Ursodeoxycholyl lysophosphatidylethanolamide protects against hepatic ischemia/reperfusion injury via phospholipid metabolism-mediated mitochondrial quality control

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Abstract
Mitochondrial dysfunction is the leading cause of reactive oxygen species (ROS) burst and apoptosis in hepatic ischemia/reperfusion (I/R) injury. Ursodeoxycholyl lysophosphatidylethanolamide (UDCA-LPE) is a hepatotargeted agent that exerts hepatoprotective roles by regulating lipid metabolism. Our previous studies have shown that UDCA-LPE improves hepatic I/R injury by inhibiting apoptosis and inflammation. However, the role of UDCA-LPE in lipid metabolism and mitochondrial function in hepatic I/R remains unknown. In the present study, we investigated the role of UDCA-LPE in hepatic I/R by focusing on the interface of phospholipid metabolism and mitochondrial homeostasis. Livers from 28-week-old mice, primary hepatocytes and HepG2 cells were subjected to in vivo and in vitro I/R, respectively. Analyses of oxidative stress, imaging, ATP generation, genetics, and lipidomics showed that I/R was associated with mitochondrial dysfunction and a reduction in phospholipids. UDCA-LPE alleviated mitochondria-dependent oxidative stress and apoptosis and prevented the decrease of phospholipid levels. Our study found that cytosolic phospholipase A2 (cPLA2), a phospholipase that is activated during I/R, hydrolyzed mitochondrial membrane phospholipids and led to mitochondria-mediated oxidative stress and apoptosis. UDCA-LPE inhibited the interaction between cPLA2 and mitochondria and reduced phospholipid hydrolysis-mediated injury. UDCA-LPE might regulate the crosstalk between the phospholipid metabolism and the mitochondria, restore mitochondrial function and ameliorate I/R injury.

Keywords
cPLA2, defect mitochondria, mitochondrial membrane, oxidative stress, phospholipid metabolism disorders, reperfusion injury

Abbreviations: CL, cardiolipin; cPLA2, cytosolic phospholipase A2; GSH, Glutathione; I/R, ischemia/reperfusion; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MDA, Malondialdehyde; MPTP, mitochondrial permeability transition pore; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PETM, phosphatidylethanolamine methyltransferase; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; t-BHP, tert-butyl hydroperoxide; UDCA-LPE, ursodeoxycholyl lysophosphatidylethanolamide.
1 INTRODUCTION

Ischemia/reperfusion (I/R) is a pathological phenomenon that aggravates cell and tissue damage previously initiated during hypoxia or anoxia after blood reperfusion. Hepatic I/R injury is a severe complication commonly associated with liver surgery, such as hepatectomy and liver transplantation. Hepatic I/R is the main cause of postoperative complications and seriously affects postoperative recovery, morbidity, and mortality. Due to its complex dynamic pathogenesis, no effective treatment strategy exists to prevent hepatic I/R injury. Although the mechanism of hepatic I/R remains uncertain, mitochondrial defects have been identified as critical factors underlying the pathogenesis of hepatic I/R.

Mitochondria are widely distributed intracellular organelles surrounded by double membranes, and they produce 90% of the cellular ATP and participate in cellular signaling pathways such as cell differentiation, signal transduction, and apoptosis. The main functions of mitochondria include energy conversion (from carbohydrates, lipids, and proteins), the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), and the storage of Ca\(^{2+}\). Disruptions in mitochondrial bioenergy and metabolic function can lead to many diseases such as Alzheimer’s disease (AD) and cancer.

Studies have shown that mitochondria are regulators of reactive oxygen species (ROS), apoptosis, and Ca\(^{2+}\) levels in the development of hepatic I/R. During the ischemic phase, hypoxia stimulates ROS production though complex III of the mitochondrial electron transport chain (ETC). During the reperfusion phase, oxygen is rapidly reintroduced into cells and reacts with reduced electron carrier molecules, which are caused by the interruption of OXPHOS during the ischemic phase, and ROS production rises sharply. During hepatic I/R, the decreases in the Na\(^{+}\) pump and Na\(^{+}\)-Ca\(^{2+}\) exchange pump activity and the organelle membrane damage lead to an increase in the Ca\(^{2+}\) concentration. Ca\(^{2+}\) and ROS can subsequently initiate mitochondrial permeability transition pores (mPTPs). mPTPs cause ATP depletion, the loss of mitochondrial membrane potential and the expression of many proapoptotic proteins in the cytoplasm, inducing cytochrome c (cyto c).

Lipids are essential for three physiological processes, which include maintaining membrane integrity, providing a source of energy and acting as signaling molecules to regulate cell proliferation, metabolism, inflammation, and apoptosis. Phospholipids are the main components of lipids. These molecules are generally composed of two fatty acids linked via ester bonds to a glycerol backbone containing a polar head-group. The main phospholipids in the mitochondrial membrane are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL), which account for approximately 40%, 30%, and 15% of the total lipids, respectively. Changes in mitochondrial membrane phospholipid levels may have profound physiological and physical effects on the activity of key transmembrane proteins, such as respiratory chain proteins, and can cause mitochondrial dysfunction, resulting in cell apoptosis. Therefore, mitochondrial quality control mediated by regulating mitochondrial lipid metabolism may have great therapeutic potential in mitochondria-related diseases.

The phospholipase A\(_2\) (PLA\(_2\)) protein family is a diverse group of enzymes that hydrolyze the acyl group attached to the 2-position of phospholipids. The PLA\(_2\) protein family mainly includes six major types: secreted phospholipase A\(_2\) (sPLA\(_2\)), cytosolic phospholipase A\(_2\) (cPLA\(_2\)), calcium-independent phospholipase A\(_2\) (iPLA\(_2\)), lipoprotein-associated phospholipase A\(_2\) (Lp-PLA\(_2\)), lysosomal phospholipase A\(_2\) (LPLA\(_2\)), and adipose-specific phospholipase A\(_2\) (AdPLA\(_2\)). PLA\(_2\) is mainly regulated by calcium ions and phosphorylation. Activated PLA\(_2\) hydrolyzes phospholipids and releases lysophospholipids and arachidonic acid. Lysophospholipids have strong surface activity, can damage cell membranes, and can cause hemolysis or cell necrosis. Arachidonic acid is the source of prostaglandins, leukotrienes, and thromboxanes, which promote inflammation. The PLA\(_2\) superfamily of enzymes plays a significant role in regulating lipid storage and liver diseases such as nonalcoholic fatty liver disease (NAFLD) and liver fibrosis. Several studies have shown that PLA\(_2\) acts in I/R as a regulator of inflammation through phospholipid hydrolyzation. However, little is known about the role of cPLA\(_2\) in mitochondrial dysfunction.

Ursodeoxycholyl lysophosphatidylethanolamide (UDCA-LPE) is a new targeted hepatoprotective drug that achieves liver-targeted drug delivery using bile acid as a targeting carrier, thus increasing the local drug concentration in the liver and reducing toxic adverse effects. Previous studies found that UDCA-LPE stimulates hepatocyte growth in an in vitro starvation model by activating the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways. UDCA-LPE inhibits TNF- \(\alpha\)-induced apoptosis by upregulating the protein expression of cFLIPL. UDCA-LPE exerted strong protective properties against high-fat diet-induced liver steatosis by inhibiting apoptosis and inflammation, and modifying the expression of genes involved in lipid metabolism such as acetyl-CoA carboxylase 1 (ACC1), fatty acid synthetase (FASN), and sterol regulatory element binding protein 1c (SREBP1c). UDCA-LPE protects against hepatic fibrogenesis by blocking TGF- \(\beta\)-Smad2/3 signaling.

Our previous studies found that UDCA-LPE protects against hepatic I/R-induced apoptosis, inflammation, and portal fibrogenesis by regulating apoptosis-related signals (cleaved caspase 3), inflammation-related signals...
(IL-1, CD11b, chemokine ligands 2 and 3, chemokine receptor 2), and fibrogenesis-related signals (α-smooth muscle actin, plasminogen activator inhibitor 1), respectively. The aim of the present study was to investigate the protective efficacy of the novel conjugate UDCA-LPE as a phospholipid-based approach for the treatment of I/R, focusing on the crosstalk of lipid metabolism and the mitochondria.

2 | MATERIALS AND METHODS

2.1 | Materials

All chemicals were from Sigma (Munich, Germany) unless stated otherwise.

2.2 | Animals

Six- to eight-week-old male C57BL/6 mice were obtained from Beijing Vital River Laboratory Animal Technology Co. The animals were housed in the Animal Care Facility of Tongji Medical College. All experiments were approved by The Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, China.

2.3 | Surgical procedures

At 28 weeks, the mice were divided into three groups: sham, I/R, and I/R + UDCA-LPE with 10 mice per group. The surgical procedures were performed as previously described to induce warm hepatic I/R injury in 70% of the liver. Fasted mice were anesthetized with pentobarbital sodium and chloral hydrate. An atraumatic clamp was used to interrupt the blood supply of the portal triad to the cephalad liver lobes to induce ischemia for 90 minutes. After unclamping the liver, hepatic reperfusion was allowed for 2 hours. UDCA-LPE stock was prepared at 5 mg/mL in 5% of carboxymethylcellulose (CMC). In the sham and I/R groups, the vehicle CMC was injected intraperitoneally. In the I/R + UDCA-LPE group, 100 mg/kg of UDCA-LPE was intraperitoneally injected 30 minutes prior to clamping.

2.4 | Primary hepatocyte isolation and culture

Primary hepatocytes were isolated from 6- to 8-week-old mice by the collagenase perfusion method as previously described. In vitro hypoxia-reoxygenation injury was induced by paraffin oil submersion for 90 minutes. Reoxygenation was induced by replacement of the oil layer by culture medium for 2 hours.

2.5 | Cell culture

HepG2 cells (American Type Culture Collection [ATCC], Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; GE Healthcare Life Sciences), with 10% of fetal bovine serum (FBS; GE Healthcare Life Sciences) and penicillin/streptomycin (WelGene, Inc, Daegu, Republic of Korea).

2.6 | Cell viability

Cell viability was determined by the MTT assay. Briefly, cells were washed with phosphate buffer saline (PBS). MTT (5 mg/mL) in PBS solution was added to medium and incubated for 4 hours. Then, the medium was carefully removed and 150 µL of dimethyl sulfoxide (DMSO) was added in each well to solubilize the crystals. Finally, the optical density (OD) was measured by a microplate reader at 490 nm. All viability assays were performed in duplicate; the percentage growth inhibition was calculated using the following formula: Cell viability (%) = (A490 treatment − A490 blank)/(A490 control − A490 blank) ×100 (Control: serum-free DMEM with cells; Blank: serum-free DMEM without cells).

2.7 | MDA, GSH, and superoxide dismutase (SOD) level measurement

The levels of GSH, MDA, and SOD, which are indicators of oxidative stress, were determined using commercially available kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Briefly, for MDA and SOD measurements, the liver tissues and hepatocytes were rinsed with PBS, and then homogenized and sonicated in lysis buffer on ice. After sonication, the samples were centrifuged at 4000 g for 10 minutes to remove debris. For GSH measurements, liver tissues and hepatocytes were homogenized, and then the samples were centrifuged at 3500 rpm for 5 minutes at 4°C. The MDA, GSH, and SOD levels in the supernatant were measured. MDA was measured based on the level of thiobarbituric acid-reactive substances in samples at 530 nm using a microplate reader. GSH was measured based on the level of 5,5'-dithiobis (2-nitrobenzoic acid)-reactive substances in samples at 405 nm using a microplate reader. GSH was measured based on the level of WST1-reactive substances in samples at 450 nm using a microplate reader. The protein concentration of each sample was determined by a BCA protein assay kit. In addition, MDA, GSH, and SOD levels were normalized according to the protein concentrations.
2.8 | Immunoblotting

Lysates of cells and liver tissues were centrifuged at 13,000 g, 4°C for 15 minutes. The proteins in liver homogenates and lysates were determined by using the Bio-Rad protein kit (Bio-Rad Laboratories, Munich, Germany). Proteins were separated by gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% of milk, and were probed sequentially with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The antibodies are shown in Table S1.

2.9 | Electron microscopy

The mitochondrial ultrastructure in liver tissue and HepG2 cells was evaluated via electron microscopy. The samples were fixed in 2.5% of glutaraldehyde at 4°C for 24 hours. After washing in PBS, samples were postfixed in 1% of OsO4 4°C for 3 hours, dehydrated, and embedded in Embed-812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed using a FEI Tecnai G2 20 TWIN transmission electron microscope (FEI, USA).

2.10 | ROS measurement

ROS, a characterization of oxidant status, is involved in the injury of cells in the setting of oxidation stress. DCFH-DA (Invitrogen, Germany) was used to detect the intracellular ROS and 10 μM of DCFH-DA was added to the cells culture medium, which was then incubated in the dark. After being washed with PBS, the cells were analyzed by fluorescence microscopy. Mitochondrial superoxide levels were determined by using MitoSOX. Briefly, cells were incubated with MitoSOX (5 μM) for 10 minutes at 37°C. After washing, the fluorescent images were analyzed immediately with fluorescence microscopy (Olympus Corporation, Tokyo, Japan) and flow cytometry.

2.11 | ATP levels measurement

The level of ATP in liver tissue was determined using the ATP Assay Kit (Beyotime Institute of Biotechnology, China), according to the manufacturer’s instructions. Briefly, liver tissue were lysed with a lysis buffer, and were centrifuged at 12,000 g for 10 min at 4°C. The level of ATP was determined by mixing 50 mL of the supernatant with 50 mL of luciferase reagent. Luciferase uses ATP to catalyze the oxidation of luciferin to produce light. The emitted light, which is linearly related to the ATP concentration, was measured using a microplate luminometer (Varioskan Flash, 5250040, Thermo).

2.12 | Mitochondrial potential (ΔΨm) measurement

The mitochondrial potential (ΔΨm) was analyzed using JC-1 staining (Beyotime Institute of Biotechnology). Briefly, cells were washed with ice-cold PBS, and then stained with 2.5 g/mL of JC-1 for 30 minutes at 37°C. After being washed with binding buffer, the cells were analyzed by flow cytometry.

2.13 | Mitochondrial DNA (mtDNA) level

Mitochondrial DNA (mtDNA) was extracted from liver tissue using a DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA). To amplify mtDNA, quantitative real-time RT-PCR (qRT-PCR) was used with a using Applied Biosystems StepOne Real-Time PCR system. The primers used were as follows: 5'-ACGCTTCCGTACGATCAAC-3' (sense) and 5'-ACTCCCGCTGAAAAATTTG-3' (antisense) for mitochondrial NADH dehydrogenase 1 and 5'-AGCCA TGACGTAACCATCCC-3' (sense) and 5'-GCTGTGG TGGTGAAGCTGTGA-3' (antisense) for β-actin. The mtDNA copy number was quantified relative to both β-actin and the average of all delta cycle threshold (Ct) values in each sample using the Ct method. All analyses were conducted in duplicate to ensure amplification integrity.

2.14 | Immunofluorescence staining

For immunofluorescence, samples were washed with PBS and fixed with 4% of paraformaldehyde for 20 minutes. The samples were then incubated with the primary antibody at 4°C overnight. Next, the samples were stained with a fluorescent secondary antibody for 60 minutes. 4, 6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. Cell fluorescence was imaged on an epifluorescence microscope (Olympus Corporation, Tokyo, Japan) or on a confocal microscope (Zeiss Germany, Oberkochen, Germany). The primary antibodies used in the present study are described in the supplemental materials.

2.15 | Gene expression by real-time polymerase chain reaction

Total RNA was extracted from cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc) according to the manufacturer’s protocol. Subsequently, cDNA was synthesized from 2 μg of total RNA. Real-time PCR was performed using an Applied Biosystems StepOne Real-Time PCR system, using ChamQTM SYBR qPCR Master Mix (Vazyme), containing 5 ng of cDNA and 10 pM of each primer. The expression level of targets in quadruplets was calculated using the ΔΔCt transformation
method, and determined as a ratio of the target gene normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers are shown in Table S2.

2.16  |  Isolation of mitochondria

Cells was homogenized in medium containing 250 mM of sucrose, 10 mM of trishydrochloride, and 1 mM of ethylene-diaminetetraacetic acid (EDTA) at 4°C. The homogenate was centrifuged at 600 g for 10 minutes, and the supernatant was subsequently centrifuged for 5 minutes at 15 000 g to obtain a mitochondrial pellet. The mitochondrial pellet was washed with the same medium without EDTA and centrifuged for 5 minutes at 15 000 g, producing a final pellet.

2.17  |  Cells transfection

HepG2 cells were transfected with 1.5 µg/9.5 cm² culture well of pcDNA cPLA₂ and pcDNA3.1 (EV) (ThermoFisher Scientific) using lipofectamine 3000 Reagent (Life Technologies), according to manufacturer protocol.

2.18  |  PLA₂ activity measurement

Enzymatic activity of PLA₂ was measured in liver tissue using a commercial kit (Axxora). Briefly, liver homogenates (0.5 g/mL in PBS) were centrifuged at 10 000 g at 4°C for 20 minutes. The supernatant was removed and the concentration of proteins was determined. PLA₂ activity of lysates was determined by incubating the samples with arachidonoyl thio-PC for 1 hour at 25°C in a Ca²⁺-free buffer as specified by the manufacturer. The reaction was terminated by adding 5,5-dithio-bis-2-nitrobenzoic acid for 5 minutes. A405 was measured and the specific PLA₂ activity was expressed as moles per min per mg protein in lysates.

2.19  |  Lipid analysis

Liver tissue sample was grounded by liquid nitrogen. Tissue samples were sonicated for 2 minutes in 400 µL ice-cold 75% to break up the cells. Next, 1 mL of methyl tert-butyl ether (MTBE) was added and the samples were shaken for 1 hour at room temperature. Next, phase separation was induced by adding 250 µL of water, letting sit for 10 minutes at room temperature and centrifuging for 15 minutes at 14 000 g, 4°C. Because of the low density and high hydrophobicity of MTBE, lipids, and lipophilic metabolites are mainly extracted to the upper MTBE-rich phase. The lipid was transferred to fresh tubes and dried with air nitrogen. The samples were separated using a UHPLC system Ultimate 3000 ultra performance liquid chromatography system (Thermo Scientific). Column temperature 45°C; flow rate 250 µL/min; injection volume 5 µL. Mobile phase A is prepared by dissolving 0.77 g of ammonium acetate to 400 mL of HPLC-grade water, followed by adding 600 mL of HPLC-grade acetonitrile. Mobile phase B is prepared by mixing 100 mL of acetonitrile with 900 mL isopropanol. Gradient is generated as shown in Table S3. Then, Lipids analysis was performed on Q Exactive Orbitrap mass spectrometer (Thermo, CA). Lipids were identified and quantified using LipidSearch 4.1.30 (Thermo, CA). Mass tolerance of 5 ppm and 10 ppm were applied for precursor and product ions.

2.20  |  Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 6.0 (GraphPad, La Jolla, CA). Significant differences between multiple groups were determined using Student’s t test or one-way ANOVA with Dunnett’s posttest. All data are presented as the mean ± SEM unless stated otherwise. For all experiments, P values < .05 were considered significant.

3  |  RESULTS

3.1  |  UDCA-LPE alleviated I/R-induced oxidative stress

Previous studies have shown that UDCA-LPE can alleviate hepatic I/R injury. To explore the protective mechanism of UDCA-LPE, we first examined whether UDCA-LPE could alleviate the oxidative stress caused by I/R in mouse liver. Mice were subjected to 90 minutes of warm ischemia followed by 2 hours of reperfusion. The MDA content was significantly enhanced in the I/R group. In contrast, the increased MDA levels were attenuated by UDCA-LPE treatment (Figure 1A). We then examined the endogenous antioxidant defenses by quantifying the SOD activity and GSH levels in liver tissues. Liver I/R significantly decreased the SOD and GSH levels. UDCA-LPE treatment significantly restored the reduced SOD and GSH levels induced by liver I/R (Figure 1B,C). Mitochondria are the main cellular sites of ROS generation during I/R. We then examined the mitochondrial specific antioxidant systems. Our results showed that hepatic I/R decreased the Thioredoxin-2 (TRX2) and Thioredoxin reductase 2 (TXNRD2) protein levels. UDCA-LPE treatment reversed the changes in these proteins (Figure 1D). Additionally, primary hepatocytes were exposed to hypoxia for 90 minutes by submersion under a layer of paraffin oil,
FIGURE 1  UDCA-LPE alleviated I/R-induced hepatic oxidative burden. A-C, (A)MDA, (B)GSH, and (C)SOD levels of liver lysates from the sham, I/R, and I/R +UDCA-LPE mice (n = 6). D, Immunoblots of liver extracts from the sham, I/R, and I/R +UDCA-LPE mice (n = 6). E-G, (E)MDA, (F)GSH, and (G)SOD levels of primary hepatocytes lysates from the normoxia, reoxygenation, and re-ox +UDCA-LPE group (n = 6). H-I, (H)MDA and (I)GSH levels of cell lysates from the control, t-BHP, and t-BHP +UDCA-LPE group (n = 4). J, ROS levels of HepG2 cells from the control, t-BHP, and t-BHP +UDCA-LPE group. K, Mitochondrial ROS levels of HepG2 cells from the control, t-BHP, and t-BHP +UDCA-LPE group.  

P values by one-way ANOVA. *P versus sham, normoxia or control, *P versus I/R, reoxygenation, or t-BHP.
followed by 2 hours of reoxygenation in medium (Figure S1A). UDCA-LPE treatment also reduced the level of oxidative stress in primary hepatocytes (Figure 1E-G). HepG2 cells were subjected to tert-butyl hydroperoxide (t-BHP, 40 μM), an organic peroxide widely used in various oxidation processes, for 3 hours to mimic the oxidative stress of the I/R process in vitro (Figure S1B). In the UDCA-LPE treatment group, different UDCA-LPE concentrations were first added to the cell culture medium for 1 hour and were then incubated with t-BHP for 3 hours. UDCA-LPE did not affect cell viability under normal conditions (Figure S1C). The effective concentration of UDCA-LPE in the t-BHP-induced oxidative stress model may be between 0.1 and 10μmol (Figure S1D). The MDA and GSH results also reflected the results in the liver and primary hepatocytes (Figure 1H,I). Intracellular ROS levels significantly increased under oxidative stress, and UDCA-LPE reduced the ROS levels in a dose-dependent manner (Figure 1J). Further, we measured mitochondrial ROS levels. The changes in mitochondrial ROS levels are consistent with intracellular ROS (Figure 1K). These findings indicated that UDCA-LPE can alleviate the hepatic I/R-induced oxidative burdens.

3.2 | UDCA-LPE attenuates hepatic I/R-induced mitochondrial damage

The above results indicate that UDCA-LPE attenuates mitochondria-mediated ROS production, so we further evaluated the quality and quantity of mitochondria to explore the mechanism of protective properties. In addition, susceptibility to mitochondria-induced apoptosis plays an important role in I/R pathogenesis. Therefore, we examined the mitochondrial structure, ATP generation, mtDNA, ΔΨm, and mitochondria-mediated apoptosis. We first performed high-resolution transmission electron microscopy on liver tissue and cell sections to examine mitochondrial morphology. As shown in Figure 2A,B, I/R, or t-BPH treatment caused extensive mitochondrial cristae breakdown, mitochondrial condensation with a heavy electron-dense matrix, highly swollen mitochondria, and the rupture of the mitochondrial membrane, whereas UDCA-LPE pretreatment caused limited mitochondrial damage. Apart from the changes in mitochondrial morphology, I/R reduced cellular ATP production. UDCA-LPE maintained ATP levels (Figure 2C,D). After I/R, the mitochondrial copy number decreased significantly, and UDCA-LPE reversed this trend (Figure 2E). In addition, we detected ΔΨm using JC-1 staining. In the oxidative stress group, ΔΨm was significantly lower than in the control group. In contrast, UDCA-LPE stabilized the mitochondrial membrane potential in a dose-dependent manner (Figure 2F).

Bcl-2-associated X protein (BAX), B-cell lymphoma 2 (BCL2), and cyto c were examined as surrogate markers for the sensitivity to mitochondria-mediated apoptosis. Our results showed that I/R increased the BAX protein levels and decreased BCL2. UDCA-LPE treatment reversed the changes in these proteins (Figure 2G,H). In the oxidative stress model, BAX was initially activated in the oxidative stress group but was reduced to baseline levels after UDCA-LPE treatment. Furthermore, BCL2 was markedly decreased in the oxidative stress model and was significantly inhibited in UDCA-LPE-treated cells. The western blot analysis of cyto c levels showed that oxidative stress promoted cyto c release from the mitochondria into the cytosol. UDCA-LPE pretreatment reduced the cyto c release into the cytosol (Figure 2I). These results indicate that there is a damage to the mitochondrial structure and function during I/R and that UDCA-LPE attenuates mitochondrial damage.

3.3 | UDCA-LPE inhibited I/R-induced mitochondrial fission

Mitochondria are in a dynamic renewal process of continuous fission/fusion, which is important for the mitochondrial quality. Previous studies have shown that I/R promotes mitochondrial fission, induces mitochondrial fragmentation, and represses mitochondrial fission-attenuated I/R-induced cell death.28,29 We further observed whether UDCA-LPE controls the mitochondrial quality by regulating mitochondrial dynamics. The confocal images revealed that although normal cells had tubular and elongated mitochondria, most mitochondria in cells treated with t-BHP had a fragmented morphology. UDCA-LPE pretreatment markedly rescued mitochondrial morphology (Figure 3A). The electron microscopy of liver tissues showed that mitochondria divided into several fragments after I/R and that this configuration change was reversed by UDCA-LPE (Figure 3B). Then, we examined the expression of mitochondrial dynamics related signals in liver tissues, primary hepatocytes, and HepG2 cells. I/R resulted in a significant increase in the expression of dynamin-related protein 1 (DRP1) and mitochondrial fission factor (MFF), two key proteins in mitochondrial division, and UDCA-LPE partially reversed the increase in DRP1, and MFF. I/R significantly reduced the expression of the mitochondrial fusion-related proteins Optic atrophy protein 1 (OPA1), mitofusin 1 (MFN1), and mitofusin 2 (MFN2), UDCA-LPE increased the content of OPA1, MFN1, and MFN2 (Figure 3C-E). These data indicate that UDCA-LPE inhibits fission and promotes fusion to maintain mitochondrial function.

3.4 | UDCA-LPE alleviated liver I/R-induced phospholipid metabolism disorder

Previous studies have shown that UDCA-LPE, as a lipid molecular analog, can alleviate liver damage in NAFLD by
FIGURE 2  UDCA-LPE protected liver mitochondria against I/R-induced injury. A, Representative electron micrographs from the sham, I/R, and I/R +UDCA-LPE mice. B, Representative electron micrographs of HepG2 cells from the control, t-BHP, and t-BHP +UDCA-LPE cells. C, Analyses of ATP production from the sham, I/R, and I/R +UDCA-LPE mice (n = 8). D, Analyses of ATP production of primary hepatocytes from the normoxia, reoxygenation, and re-ox +UDCA-LPE group (n = 6). E, Analyses of mtDNA copy number from the sham, I/R, and I/R +UDCA-LPE mice (n = 8). F, Mitochondrial ΔΨm analyses of HepG2 cells from the control, t-BHP, and t-BHP +UDCA-LPE cells (n = 4). G, Immunoblots of liver extracts from the sham, I/R, and I/R +UDCA-LPE mice (n = 6). H, Immunoblots of primary hepatocytes lysates from the normoxia, reoxygenation, and re-ox +UDCA-LPE group (n = 6). I Immunoblots of cells lysates from the control, t-BHP, and t-BHP +UDCA-LPE group (n = 4). P values by one-way ANOVA, *P versus sham, normoxia, or control. *P versus I/R, reoxygenation, or t-BHP.
regulating lipid metabolism, and the dynamic changes in the lipid composition in the I/R process have not been elucidated. Therefore, we further evaluated the effect of UDCA-LPE on the dynamic changes in lipids in the I/R process. We obtained mouse liver tissues for lipid mass spectrometry. The PC content was markedly decreased (by twofold) after I/R. The PC level substantially increased in response to UDCA-LPE (Figure 4A). Similarly, PE levels showed a decreasing trend in the I/R group, and UDCA-LPE reversed this trend, returning PE levels to close to normal (Figure 4B). Compared to the normal control group, the level of phosphatidylserine (PS) was significantly decreased in the I/R group, and UDCA-LPE reversed this trend, returning the PS level to normal (Figure 4C). The phosphatidylinositol (PI) content was almost

![Figure 3](image-url)

**Figure 3** UDCA-LPE regulates mitochondrial dynamics. A, Representative confocal images of cells from the control, t-BHP, and t-BHP + UDCA-LPE group, stained labeled with Tom20 to determine mitochondrial fragmentation. B, Mitochondrial length in the electron micrographs from the sham, I/R, and I/R + UDCA-LPE mice (n = 8). C, Immunoblots of liver extracts from the sham, I/R, and I/R + UDCA-LPE mice (n = 6). D, Immunoblots of primary hepatocytes lysates from the normoxia, reoxygenation, and re-ox + UDCA-LPE group (n = 6). E, Immunoblots of cells lysates from the control, t-BHP, and t-BHP + UDCA-LPE group (n = 4). P values by one-way ANOVA. *P versus sham, normoxia, or control, *P versus I/R, reoxygenation, or t-BHP
the same across the three groups, and the difference was not statistically significant (Figure 4D). Similar to PC, the phosphatidylglycerol (PG) content was markedly reduced in the I/R group, and UDCA-LPE reversed this trend (Figure 4E). Overall, I/R led to a lower than normal phospholipid content and lipid metabolic disorders. Lysophosphatidylcholine (LPC), a metabolite of PC, is elevated during I/R, but UDCA-LPE may slightly decrease LPC levels in tissues (Figure 4F). A clear upward trend occurs during the I/R process, which can be blocked by UDCA-LPE (Figure 4G). Free fatty acids (FFAs) increase after I/R, and UDCA-LPE can block this increase (Figure 4H). These data indicate that a phospholipid hydrolyzation process occurs during I/R, in which the phospholipid component is significantly reduced, and the metabolite, lysophospholipid, is produced along with a variety of fatty acids. Phosphatidylethanolamine methyltransferase (PETM) is suppressed after I/R, and UDCA-LPE can reactivate this enzyme (Figure S2A). These data indicate that phospholipid synthesis is relatively insufficient during I/R.
The levels of CL, a phospholipid characteristic of mitochondria, decrease after I/R, and UDCA-LPE partially reversed this effect (Figure 4I). These data indicate that mitochondrial lipid metabolism is also disordered during I/R, and many mitochondrial phospholipids are hydrolyzed.

3.5 | UDCA-LPE inhibited cPLA2-induced hydrolysis of membrane phospholipids

Since the above results indicated that there was a decrease in the level of phospholipids during the I/R process, we further evaluated the phospholipid hydrolysis-related enzymes, the PLA2 family. Due to the presence of Ca2+ overload and the decrease in mitochondrial CL during I/R, we chose to examine calcium-sensitive cPLA2. In the in vivo experiments, the mRNA content of cPLA2 was elevated in the I/R model, and UDCA-LPE partially reversed this effect (Figure 5A, left). In the oxidative stress model, the mRNA content of cPLA2 increased during the early stage of oxidative stress, and then decreased, indicating that cPLA2 was activated and exerted its damaging effects during the early stage of I/R (Figure S2B). UDCA-LPE partially reversed this effect (Figure 5A, right). Western blot results showed that UDCA-LPE treatment inhibited increase in cPLA2 after I/R in the in vivo and in vitro models (Figure 5B). These data indicate that UDCA-LPE can inhibit cPLA2 expression. After I/R, mice showed an increase in the PLA2 activity, and UDCA-LPE pretreatment reduced the increased hepatic PLA2 activity to close to normal (Figure 5C). Subsequently, we analyzed the catalytic activity of cPLA2 in regard to the phospholipid composition with a focus on PC levels, the LPC/PC ratio, PE levels and the LPE/PE ratio. As mentioned earlier, PC and PE levels decreased during the I/R process (Figure 6A,B). Compared to the sham control, the LPC/PC ratio was significantly elevated upon I/R exposure. Pretreatment with UDCA-LPE normalized the LPC/PC ratio (Figure 5D). Compared with the normal controls, in the I/R group, the LPE/PE ratio significantly increased, and UDCA-LPE suppressed this effect (Figure 5E). To investigate whether UDCA-LPE can inhibit cPLA2 binding to mitochondrial membrane phospholipids, we performed a double-label immunofluorescence experiment with cPLA2 and the mitochondrial outer membrane protein, TOM20 (Figure 5F). cPLA2 was diffusely distributed inside the nonoxidative stress cells, which showed a normal mitochondrial morphology. However, in the oxidative stress cells, the cPLA2 fluorescence signal was significantly enhanced, and obvious dot-like enhancement occurred. The mitochondrial morphology changed from tubular to punctate, and cPLA2 and the mitochondria showed obvious colocalization. UDCA-LPE reduced the fluorescence intensity of cPLA2 and blocked the ability of cPLA2 to bind mitochondrial membranes (Figure 5G). These results indicate that cPLA2 expression is elevated in I/R and that the activity is significantly increased. UDCA-LPE can reduce the expression level of cPLA2 and block its binding to phospholipids, reduce its catalytic activity, and protect the mitochondria.

3.6 | cPLA2-mediated hydrolysis of membrane phospholipids leads to mitochondrial dysfunction

To establish the relative contribution of the cPLA2-mediated hydrolysis of membrane phospholipids in mediating mitochondrial dysfunction, we transiently transfected HepG2 cells with a plasmid overexpressing cPLA2. The qRT-PCR and western blots results demonstrated the efficiency of the plasmid transfection (Figure 6A,B). The transient transfection of cPLA2 significantly increased BAX expression compared with the transfection with the empty vector (EV) control. BCL2 levels significantly decreased in cells transfected with a plasmid overexpressing cPLA2 relative to the EV controls (Figure 6C). In line with our previous results, the MDA, GSH, and SOD test results showed that HepG2 cells transfected with a plasmid overexpressing cPLA2 experienced more oxidative stress than HepG2 cells transfected with the EV control (Figure 6D-F). Moreover, we observed increased ROS levels in HepG2 cells following the transient overexpression of cPLA2 relative to those in cells transfected with the EV control after UDCA-LPE and t-BHP treatment (Figure 6G). Collectively, these data indicate that the cPLA2-mediated degradation of membrane phospholipids leads to mitochondrial dysfunction.

4 | DISCUSSION

UDCA-LPE has been shown to perform well in several liver diseases by inhibiting apoptosis and inflammation, stimulating growth, and modifying lipid metabolism. In this study, UDCA-LPE also reduced oxidative stress caused by I/R. Additionally, we found UDCA-LPE protected mitochondria, the main source of ROS in I/R, through maintaining mitochondrial ATP production, the mitochondrial copy number, and stabilizing ΔΨm, keeping the balance between fission and fusion, and inhibiting mitochondria-mediated apoptotic pathways. Mass spectrometry results showed that UDCA-LPE significantly inhibited lipid metabolic disorders, especially the phospholipid degradation caused by I/R. Our results also showed that UDCA-LPE could be an atypical downstream product of cPLA2. UDCA-LPE inhibited the expression level and activity of cPLA2. UDCA-LPE prevented cPLA2 from binding to mitochondria, maintained the stability of mitochondrial membrane, and alleviated mitochondria-mediated ROS production and apoptosis. Hence, UDCA-LPE...
FIGURE 5  UDCA-LPE inhibits the cPLA₂-mediated degradation of mitochondrial membrane phospholipids. A, Relative mRNA levels of cPLA₂ in liver tissues (left) and cells (right) (n = 4-6). B, Immunoblots of cPLA₂ in liver tissues (top) and cells (bottom) (n = 4-6). C, PLA₂ activity in liver tissue lysates from the sham, I/R, and I/R + UDCA-LPE mice (n = 6). D-E, (D) The LPC/PC ratio and (E) the LPE/PE ratio in tissue lipid extracts (n = 4). F, Representative confocal images of cells from the control, t-BHP, and t-BHP + UDCA-LPE group, stained labeled with Tom20 and cPLA₂ to determine mitochondria and cPLA₂, respectively. Nuclei were labeled by DAPI. Organ immunofluorescence is the hallmark of the interaction of mitochondria and cPLA₂. G, Line scan data showing fluorescence intensities in the corresponding images demonstrating the degree of colocalization between TOMM20 and cPLA₂.  P values by one-way ANOVA. #P versus sham or control, *P versus I/R or t-BHP
FIGURE 6  cPLA₂-mediated degradation of membrane phospholipids leads to mitochondrial dysfunction. A, Relative mRNA levels of cPLA₂ in HepG2 cells transfected with either pcDNA3 or p-cPLA2. *P values by Student’s t test. *P versus pcDNA3. B, Immunoblots of cPLA₂ in HepG2 cells transfected with either pcDNA3 or p-cPLA2. C, Immunoblots of cells transfected with either pcDNA3 or p-cPLA2 from the control, t-BHP, and t-BHP + UDCA-LPE group (n = 4). D-F, (D)MDA, (E)GSH, and (F)SOD levels of cells transfected with either pcDNA3 or p-cPLA2 from the control, t-BHP, and t-BHP + UDCA-LPE group (n = 4). #P versus control, *P versus t-BHP. G, ROS levels of cells transfected with either pcDNA3 or p-cPLA2 from the control, t-BHP, and t-BHP + UDCA-LPE group.
improves mitochondrial quality by regulating the mitochondrial membrane phospholipid content, thus playing a protective role in hepatic I/R (Figure 7).

The functional status of mitochondria is determined by both the quality and quantity of mitochondria. The copy number of mitochondria is controlled by mitochondrial biogenesis and mitophagy. The previous study shows that during the I/R process, PINK1-Parkin-mediated mitophagy is activated and that there are no significant changes in the levels of mitochondrial biogenesis genes (Ppargc1a, Nrf1, Cox4, and Tomm20). Our mtDNA results indicate that there was a decrease in mitochondrial copy numbers during I/R, which may have been due to the severe impairment of mitochondria being cleared by mitophagy. In addition, during oxidative stress conditions, the production of ROS in mitochondrial ETC is a self-enhancing process, which has been termed “ROS-induced ROS release” (RIRR). The overproduction of mitochondrial ROS-induced by t-BHP administration will lead to the generation of mPTPs, which cause the collapse of \( \Delta \Psi_m \), resulting in mitochondrial swelling. Additionally, \( \Delta \Psi_m \), a main component of proton motive force across the inner mitochondrial membrane, is crucial for driving ATP production. ROS induce the collapse of \( \Delta \Psi_m \) by mPTPs and cause the decrease of ATP synthesis. Moreover, ROS directly oxidizes the mitochondrial membrane lipids and mitochondrial proteins, modifying their structural and functional properties, and resulting in mitochondrial damage. Additionally, elevated levels of ROS associated with mtDNA damage.

In mitochondria, two antioxidant systems are able to counteract increased ROS levels: the Glutathione (GHS) reductase system and the Thioredoxin reductase system. Our results show that I/R also significantly decreases the quality of mitochondria, as evidenced by decreased mitochondrial membrane potential, decreased ATP synthesis, high ROS production, antioxidant system dysfunction, and mPTP opening. Since the Thioredoxin reductase system plays an important role in mitochondrial oxidative stress levels, additional work, including measuring the activity of thioredoxin reductase, should be performed to explore the role of the Thioredoxin reductase system in I/R.

UDCA-LPE can play a protective role in three ways by using the chemical structure of UDCA, using the chemical structure of LPE, and utilizing the chemical structure of UDCA-LPE, the unique coupling molecule. UDCA-LPE may play two roles in the above manner: maintaining membrane integrity and acting as signaling molecule to regulate cell processes. UDCA has confirmed that it can stabilize mitochondrial membrane potential. Mitochondria are unique organelles with a bilayer membrane, and their function depends on the coordination of proteins and lipids. PC, PE, and CL, acting as the main lipid molecules in the mitochondrial membrane, maintain the mitochondrial membrane integrity, and regulate proteins on the mitochondrial membrane. CL, a phospholipid containing four, instead of two, FAs, can help to make the inner membrane impermeable. CL affects the formation of respiratory supercomplexes, fusion and fission events, and the activity of respiratory complexes I, III, IV, and V. A reduction in the content of CL disrupts the supercomplexes of the respiratory chain and leads to the generation of ROS. CL provides binding sites on the inner membrane for cyto c, so a decrease in its content favors the loss of cyto c, which is a process that occurs during I/R. PE affects the inner mitochondrial membrane \( \Delta \Psi_m \), regulating mitophagy by converting LC-1 to LC-2. When the PE content of mitochondria was decreased, oxygen consumption, cellular ATP levels, and the rate of ATP production were markedly reduced, which was consistent with defects in complexes I and IV of the ETC. Our results indicate that there was a decrease in PC, PE, and CL during I/R. UDCA-LPE may promote the quality of mitochondria by increasing PC, PE, CL, and UDCA.

The impairment of mitochondrial dynamic change contributes to I/R injury. In hepatic I/R, the fusion of damaged mitochondria is more conducive to maintaining mitochondrial survival functions. Inhibiting mitochondrial fission protects the liver against I/R injury. Other studies have shown that inhibiting mitochondrial fission protects the heart against I/R injury. Continuous mitochondrial division and fusion require a constant and well-regulated phospholipid supply to ensure membrane integrity. Research has shown that OPA1 and CL work together to contribute to mitochondrial fusion.

**FIGURE 7** Proposed model of the mechanism of ursodeoxycholyl lysophosphatidylethanolamide mediated protective effects in hepatic ischemia/reperfusion. Abbreviations: LysoPLs, lysophospholipids; PLs, phospholipids
Parallel phosphatidic acid interactions contribute to mitochondrial division.44 Our results showed that UDCA-LPE may promote mitochondrial fusion and inhibit mitochondrial division by regulating key phospholipids such as PC and CL.

cPLA₂, a member of the PLA₂ family, has shown high specificity toward arachidonic acid at the sn-2 fatty acyl bond. The regulation of cPLA₂ involves transcriptional and posttranslational processes, particularly increases in calcium and phosphorylation. cPLA₂ hydrolyzes phospholipids and releases lysophospholipids and arachidonic acid. Inherited human cPLA₂ deficiency is associated with impaired PLA2 activity and a reduction in prostacyclin, prostaglandin E2, prostaglandin D2, and thromboxane A2.45 A recent study showed that the knockdown of cPLA₂ leads to abnormal architecture, including of the nuclear envelope, nuclear pore, and synapses in cortical neurons. The authors conclude that cPLA₂ affects membrane fluidity and permeability by catalyzing the hydrolysis of membrane phospholipids.46 Our results indicate an increase in the content and activity of cPLA₂ during I/R. Ca²⁺ overload during I/R may increase cPLA₂ activity. Our previous studies have shown that IL-1, CD11b, chemokine ligands 2 and 3, chemokine receptor 2 inflammatory cytokine activation during I/R, and UDCA-LPE can reduce the level of inflammatory signals. UDCA-LPE may inhibit inflammation by inhibiting cPLA₂-mediated release of arachidonic acid. Our results indicate that I/R can cause mitochondrial dysfunction and UDCA-LPE can reduce its damage. UDCA-LPE may protect mitochondria by inhibiting the hydrolysis of cPLA₂-mediated mitochondrial key membrane phospholipids PC, PE, and CL. Due to the special chemical structure of UDCA-LPE, the effective range of UDCA-LPE, the elimination kinetics, and the concentration distribution inside and outside the cell need to be further evaluated to investigate the protective role of UDCA-LPE and the pathogenesis of I/R.

Three findings support the hypothesis that mitochondria can be therapeutic targets for common liver damage. First, many liver diseases are secondary mitochondrial diseases because mitochondrial dysfunction contributes to the disease process or its clinical progression. Therefore, targeting organelles can improve patient outcomes. Second, different pathologies can be simultaneously promoted through the mitochondrial pathway, and a single treatment could, therefore, be applied to various diseases.57,48 Finally, common diseases that are promising targets for mitochondrial therapies have an ever-increasing impact on health, society, and the economy in terms of the aging population.49-51 Five broad treatment strategies exist in which small molecules can be used to directly or indirectly affect secondary mitochondrial diseases: the repair or prevention of damage to organelles, the induction of mitochondrial biogenesis, the enhancement of organelles by stimulating the degradation of damaged mitochondria or organelle components (quality control), the coselection of mitochondrial functions to induce cell death, and the alteration of mitochondrial signaling pathways or metabolic processes. UDCA-LPE provides a new target for mitochondrial strategy by regulating the homeostasis of mitochondrial membrane phospholipids to prevent damage to mitochondria.

UDCA-LPE may represent a new treatment that can be used for I/R by regulating lipid metabolism and stabilizing mitochondrial membranes, thereby avoiding excessive mitochondrial damage. Therefore, UDCA-LPE can be used as a mitochondrial protective agent in liver disease, in which mitochondria may play roles in various types of liver damage.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

J.L. Wang, H.L. Li, and J. Gu conceived and designed the study; J. Gu, T. Zhang, and J.R. Guo performed experiments; J. Gu, T. Zhang, and K. Chen analyzed data; J. Gu, T. Zhang, and H.L. Li wrote the manuscript; J.L. Wang, H.L. Li, and J. Gu conceived and designed the study; J. Gu, T. Zhang, and J.R. Guo performed experiments; J. Gu, T. Zhang, and K. Chen analyzed data; J. Gu, T. Zhang, and H.L. Li wrote the manuscript; J. Gu, T. Zhang, and K. Chen analyzed data; J. Gu, T. Zhang, and J.R. Guo performed experiments; J. Gu, T. Zhang, and K. Chen analyzed data; J. Gu, T. Zhang, and H.L. Li wrote the manuscript; J. Gu, T. Zhang, and G.B. Wang supervised the study; and all authors reviewed the manuscript.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

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