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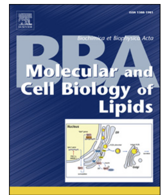

# Micromolar changes in lysophosphatidylcholine concentration cause minor effects on mitochondrial permeability but major alterations in function

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ABSTRACT

Mice deficient in group 1b phospholipase $A_2$ have decreased plasma lysophosphatidylcholine and increased hepatic oxidation that is inhibited by intraperitoneal lysophosphatidylcholine injection. This study sought to identify a mechanism for lysophosphatidylcholine-mediated inhibition of hepatic oxidative function. Results showed that in vitro incubation of isolated mitochondria with 40–200 μM lysophosphatidylcholine caused cyclosporine A-resistant swelling in a concentration-dependent manner. However, when mitochondria were challenged with 220 μM $CaCl_2$, cyclosporine A protected against permeability transition induced by 40 μM, but not 80 μM lysophosphatidylcholine. Incubation with 40–120 μM lysophosphatidylcholine also increased mitochondrial permeability to 75 μM $CaCl_2$ in a concentration-dependent manner. Interestingly, despite incubation with 80 μM lysophosphatidylcholine, the mitochondrial membrane potential was steady in the presence of succinate, and oxidation rates and respiratory control indices were similar to controls in the presence of succinate, glutamate/malate, and palmitoyl-carnitine. However, mitochondrial oxidation rates were inhibited by 30–50% at 100 μM lysophosphatidylcholine. Finally, while 40 μM lysophosphatidylcholine has no effect on fatty acid oxidation and mitochondria remained impermeable in intact hepatocytes, 100 μM lysophosphatidylcholine inhibited fatty-acid stimulated oxidation and caused intracellular mitochondrial permeability. Taken together, these present data demonstrated that LPC concentration dependently modulates mitochondrial microenvironment, with low micromolar concentrations of lysophosphatidylcholine sufficient to change hepatic oxidation rate whereas higher concentrations are required to disrupt mitochondrial integrity.

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## 1. Introduction

Phospholipases $A_2$ ($PLA_2$s) are enzymes that hydrolyze phospholipids at the *sn*-2 position to release a lysophospholipid and a fatty acid [1]. Types of $PLA_2$s include secreted, cytoplasmic, lysosomal, calcium-independent ($iPLA_2$), and platelet activating factor acetylhydrolase [2]. These enzymes are important in a variety of cellular and systemic processes such as digestion, inflammation, metabolism, cell signaling, and immunity [1,2]. The group 1b enzyme (PLA2G1B) is a secreted $PLA_2$, produced mainly in the pancreas, and is responsible for the absorption of lysophospholipids, which are taken up into the bloodstream through the portal circulation subsequent to their conversion from dietary and biliary phospholipids [3–5]. Plasma concentrations of lysophospholipids in humans range from ~150 μM in normal subjects to ~200–250 μM in diabetic patients [6]. Most if not all of the lysophospholipids transported in plasma are albumin-bound [7]. Though other secreted $PLA_2$ enzymes are present in the blood (e.g. group IIA, group V, group X), mice that are deficient in Pla2g1b activity have decreased plasma levels of lysophosphatidylcholine (LPC) compared to wild type animals, a difference which is exacerbated with high fat diet challenge [4].

Mice deficient in PLA2G1B (*Pla2g1b*$^{-/-}$ mice) have increased postprandial hepatic fatty acid oxidation rates compared to *Pla2g1b*$^{+/+}$ mice after exposure to high fat diet, leading to protection against diet-induced obesity [4,8]. Systemic supplementation of LPC prior to oral lipid load decreases hepatic fatty acid oxidation to levels similar to those observed in *Pla2g1b*$^{+/+}$ mice, as well as causes stimulation of triglyceride production in fasting *Pla2g1b*$^{-/-}$ and *Pla2g1b*$^{+/+}$ mice [9,10]. These observations suggest a direct and acute effect of LPC on hepatocytes and that Pla2g1b-mediated LPC absorption may play a role in

*Abbreviations:* AM, acetoxymethylester; CsA, cyclosporine A; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenyhydrazone; $iPLA_2$, calcium independent phospholipase $A_2$; LPC, lysophosphatidylcholine; MOPS, 3-(N-Morpholino)propanesulfonic acid; MPT, membrane permeability transition; OCR, oxygen consumption rate; $PLA_2$, phospholipase $A_2$; PPAR, peroxisome proliferator-activated receptor; RCI, respiratory control index

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postprandial partitioning of dietary fatty acids to triglyceride (TG) production instead of β-oxidation.

While the cellular mechanisms responsible for LPC-stimulated very low density lipoprotein (VLDL) production have been investigated in several reports [11–14], the potential effects of LPC on fatty acid oxidation have been studied less extensively. Whether the reduced fatty acid oxidation observed in *Pla2g1b*$^{-/-}$ mice and increased TG production in both *Pla2g1b*$^{-/-}$ and *Pla2g1b*$^{+/+}$ mice subsequent to LPC injection are due to direct or indirect inhibition of hepatic oxidative mechanisms has not been established. This study aims to further characterize the effect of LPC on oxidation rates by interrogating murine hepatocytes directly. We isolated mitochondria in order to determine the effects of exogenous LPC on mitochondrial permeability and oxidative function. The data showed that levels of LPC need to be delicately balanced in order to control mitochondrial and cellular respiration.

## 2. Methods

### 2.1. Mice

Wild type C57BL/6J mice were originally purchased from Jackson Laboratories and a breeding colony was established in our institutional facility. Mice were maintained in accordance to protocols approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. Access to food and water was ad libitum, except as indicated. Mice had a 12 hour light/dark cycle. Male mice of at least 10 weeks of age were used in each experiment for isolation of primary hepatocytes and liver mitochondria.

### 2.2. Mitochondria isolation

Hepatic mitochondria were isolated from mice using a method based on a published protocol [15]. Briefly, freshly isolated mouse livers were kept at 4 °C, diced into <1 mm pieces, homogenized in Isolation Media (220 mM D-mannitol (Sigma), 70 mM sucrose (Fisher), 1 mM EDTA (Fisher), 10 mM 3-(N-Morpholino)propanesulfonic acid (MOPS, Sigma), 0.5% BSA (Sigma), pH 7.2 with KOH (Fisher)), and centrifuged at 500 ×g for 15 min. The supernatant was strained through gauze and then centrifuged again at 10,000 ×g for 15 min to produce a mitochondrial pellet. This pellet was resuspended two times in Wash Media (250 mM sucrose, 10 mM MOPS, pH 7.2 with KOH) and recovered by centrifugation at 10,000 ×g. The final pellet was suspended in 250–500 μL of wash medium and stored on ice until used for experiments.

### 2.3. Mitochondrial swelling and permeability

Mitochondrial protein concentration was determined by bicinchoninic acid assay (BCA, Thermo Scientific). Isolated mitochondria (0.7 mg protein/mL) were incubated in Assay Media (250 mM sucrose, 2.5 mM $MgCl_2$ (Fisher), 0.5 mM EDTA (Fisher), 10 mM MOPS, 0.72 mM $K_2HPO_4$ (Fisher), 0.28 mM $KH_2PO_4$ (Sigma), to pH 7.2 with KOH) supplemented with 5 mM glutamate/5 mM malate as respiratory substrates and egg LPC (Sigma) with or without 2 μM cyclosporine A (CsA, Sigma) at room temperature (~22 °C). Since LPC has detergent properties and is amphipathic, 1.7 mM SDS was used as a negative control to achieve total mitochondrial membrane solubilization [16]. Mitochondria were incubated in the indicated concentrations of LPC for 2 min prior to the addition of 75 μM or 220 μM $CaCl_2$ (Fisher). Absorbance at 530 nm was recorded initially after exposure to LPC and after 10 min of incubation with $CaCl_2$. In other experiments, mitochondria were incubated with LPC for 3 min and centrifuged at 4 °C at 12,000 ×g. Cytochrome c released into the media was determined by enzyme-linked immunoabsorbent assay (Abcam).

### 2.4. Membrane potential

Mitochondria were incubated in Assay Media containing 25 μM safranin O (Sigma) and LPC for 2 min at room temperature. Safranin was used as a spectrophotometric indicator of mitochondrial voltage since there is a tight positive correlation between absorbance and membrane potential. Thus, absorbance values were measured at its peak absorption at wavelength of 530 nm [17]. Baseline absorbance at 530 nm was recorded prior to the addition of 5 mM succinate. Negative control for total mitochondrial membrane solubilization was achieved by incubation with 1.7 mM SDS. After a 3 min equilibration period, 5 mM succinate was then added by multichannel pipette to each well. Absorbance at 530 nm was measured every 30 s for 10 min after succinate addition.

### 2.5. Mitochondrial calcium uptake

Mitochondria were incubated in Assay Media containing 5 mM glutamate, 5 mM malate, 1 μM Calcium Green-5N (Invitrogen), which is a fluorescent probe that binds $Ca^{2+}$ and is impermeable to membranes, and varying concentrations of LPC. Fluorescence intensity was determined at 538 nm after excitation at 485 nm at baseline and every 2.5 s for 10 min after the addition of 75 μM $CaCl_2$. In negative controls, the mitochondrial membrane was solubilized by incubation with SDS.

### 2.6. Mitochondrial oxygen consumption

In order to determine the effect of intracellular LPC on mitochondrial fatty acid oxidation, $O_2$ consumption was measured at 37 °C with a Gilson oxymeter. Isolated mitochondria (1 mg protein/mL) were added to Assay Media containing one of the following sets of substrates: 5 mM succinate, 5 mM glutamate/5 mM malate, or 10 μM palmitoylcarnitine/1 mM malate. After 2 min, 441 nmol ADP was added and state 3 and 4 respiration rates, ADP/O ratio, and respiratory control index (RCI) were determined.

### 2.7. Cellular oxygen consumption

Chow-fed C57BL/6J mice were anesthetized with inhaled isofluorane and primary hepatocytes were isolated as previously described by perfusion with 100 U/mL collagenase [18]. Primary mouse hepatocytes were then plated on a collagen-coated 96-well Seahorse (Seahorse Bioscience) plate at a density of 5000 cells/well. The cells were allowed to adhere overnight and then treated with 50 μM oleate and indicated levels of LPC, 10 μg/mL oligomycin, 3 μM carbonyl cyanide 4-(trifluoromethoxy) phenyhydrazone (FCCP) and 4 μM antimycin A/1 μM rotenone to measure oxidation changes and construct mitochondrial bioenergetics profiles [19–21]. Basal oxygen consumption rate (OCR) was established prior to injection of oleate and LPC.

### 2.8. Hepatocyte viability and mitochondrial permeability transition

Primary hepatocytes were plated in dark-walled microtiter plates for overnight incubation. Cells were then washed with phosphate-buffered saline (PBS), acclimated in Hepatozyme-SFM (Invitrogen) for 1 h, treated with 100 μM oleate complexed to BSA at a ratio of 5:1 and indicated concentrations of LPC for 10 min, washed three times with PBS, and then incubated in 2 μM calcein-acetoxymethylester (AM, BD Biosciences) for 30 min at 37 °C in the dark [22,23]. Fluorescence intensity after excitation at 485 nm and emission at 538 nm was measured in a microplate fluorimeter to determine hepatocyte viability. Viability was also determined by measuring oxygen consumption during experiments using the XF96 Analyzer. In order to measure mitochondrial permeability, 8 mM $CoCl_2$ (Sigma), which quenches cytosolic fluorescence

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