Original Article

Lpcat3 deficiency promotes palmitic acid-induced 3T3-L1 mature adipocyte inflammation through enhanced ROS generation

Jiachun Hu, Yan Deng, Tingbo Ding, Jibin Dong, Yuning Liang, and Bin Lou*

Department of Pharmacology, School of Pharmacy, Fudan University, Shanghai 201203, China
*Correspondence address. Tel: +86-21-51980032; E-mail: blou@shmu.edu.cn

Received 11 May 2022 Accepted 03 July 2022

Abstract
Phosphatidylcholines (PCs) are major phospholipids in the mammalian cell membrane. Structural remodeling of PCs is associated with many biological processes. Lysophosphatidylcholine acyltransferase 3 (Lpcat3), which catalyzes the incorporation of polyunsaturated fatty acyl chains into the sn-2 site of PCs, plays an important role in maintaining plasma membrane fluidity. Adipose tissue is one of the main distribution organs of Lpcat3, while the relationship between Lpcat3 and adipose tissue dysfunction during overexpansion remains unknown. In this study, we reveal that both polyunsaturated PC content and Lpcat3 expression are increased in abdominal adipose tissues of high-fat diet-fed mice when compared with chow-diet-fed mice, indicating that Lpcat3 is involved in adipose tissue overexpansion and dysfunction. Our experiments in 3T3-L1 adipocytes show that inhibition of Lpcat3 does not change triglyceride accumulation but increases palmitic acid-induced inflammation and lipolysis. Conversely, Lpcat3 overexpression exhibits anti-inflammatory and anti-lipolytic effects. Furthermore, mechanistic studies demonstrate that Lpcat3 deficiency promotes reactive oxygen species (ROS) generation by increasing NOX enzyme activity by facilitating the translocation of NOX4 to lipid rafts, thereby aggregating 3T3-L1 adipocyte inflammation induced by palmitic acid. Moreover, overexpression of Lpcat3 exhibits the opposite effects. These findings suggest that Lpcat3 protects adipocytes from inflammation during adipose tissue overexpansion by reducing ROS generation. In conclusion, our study demonstrates that Lpcat3 deficiency promotes palmitic acid-induced inflammation in 3T3-L1 adipocytes by enhancing ROS generation.

Key words adipocyte, inflammation, lysophosphatidylcholine acyltransferase 3 (Lpcat3), obesity

Introduction
Phosphatidylcholines (PCs) are the major phospholipids in the mammalian plasma membrane. In addition to their role as biomass components, PCs also play regulatory roles as signaling molecules that engage specific receptors and transcription factors [1–3]. It has also become clear that the saturation of PCs can impact plasma membrane fluidity and alter membrane biophysical properties, thus regulating a variety of cell processes [4–6]. The saturation of PCs is determined by different fatty acyl chains. Saturated fatty acids are usually esterified at the sn-1 position of PC and decrease membrane fluidity, while polyunsaturated fatty acids are usually esterified at the sn-2 position and enhance membrane fluidity by preventing the molecules from packing together [7]. Although PCs are mainly synthesized de novo in the CDP-choline pathway called the “Kennedy pathway” [8], its saturation is maintained in part by a deacylation-reacylation process known as the “Lands’ cycle” [9,10]. The deacylation step of the Lands’ cycle is catalyzed by calcium-independent phospholipase A2, and the reacylation step is catalyzed by lysophosphatidylcholine acyltransferase (Lpcat). The Lpcat family consists of 4 isoforms called Lpcat1, Lpcat2, Lpcat3, and Lpcat4 [11]. Compared to the other 3 isoforms, Lpcat3 has distinct substrate preferences. Lpcat3 prefers polyunsaturated fatty acid CoA, such as linoleoyl-CoA and arachidonoyl-CoA, as the acyl donor to synthesize polyunsaturated PCs, thereby greatly increasing their unsaturation [12,13]. Previous studies have shown that polyunsaturated PCs synthesized by Lpcat3 are involved in the process of lipogenesis and inflammation in the liver, which indicates that Lpcat3 plays a pivotal role in protecting the liver...
from metabolic stress in obesity [3,14]. In addition, one recent study revealed that decreased polyunsaturated PC content caused by Lpcat3 deficiency could promote insulin sensitivity in mouse skeletal muscles, suggesting that Lpcat3 could aggravate insulin resistance and worsen diabetes in obesity [15]. These studies revealed the complicated roles of Lpcat3 in obesity-associated disorders.

Obesity is characterized by hypertrophy and hyperplasia of adipose tissue. Excessive adipose tissue expansion can be accompanied by local low-grade chronic inflammation, which leads to adipose tissue dysfunction, such as elevated fatty acid release and abnormal adipokine secretion [16,17]. These severe malfunctions may ultimately result in serious metabolic syndrome, including fatty liver, glucose intolerance, dyslipidemia, and hypertension [18]. Currently, several types of stressors have been reported to be involved in adipose tissue inflammation and dysfunctions, such as oxidative stress, endoplasmic reticulum stress, inflammatory cytokines, and elevated concentrations of free fatty acids [18,19]. Although the leading factors remain controversial, attenuating inflammation in adipose tissues may be an effective strategy to relieve metabolic syndromes [20–22].

Adipose tissues play a major role in obesity-associated metabolic disorders. However, the role of Lpcat3 in adipose tissue in obesity is still unknown. Our previous study reported that Lpcat3 modulates the differentiation of 3T3-L1 preadipocytes, which indicates that Lpcat3 is involved in adipogenesis [23]. As is known, only approximately 8% of adipocytes are turned over annually, and mature adipocytes are the major cells in adipose tissues [20,24]. Meanwhile, mature adipocytes rather than preadipocytes are responsible for endocrine and energy metabolism functions. Thus, we continued to investigate the roles of Lpcat3 in mature adipocytes in obesity. Since primary adipocytes are difficult to obtain and culture in vitro directly, the preadipocyte cells and mesenchymal stem cells have been widely used for elucidating the mechanisms involved in adipogenesis and the function of mature adipocytes, such as inflammation, insulin sensitivity, and lipolysis [25–27]. The mouse preadipocyte cell line 3T3-L1 was used to obtain mature adipocytes via differentiation in this study. We used normal and palmitic acid (PA)-induced lipid overload 3T3-L1 mature adipocytes to explore the effect of Lpcat3 on triglyceride storage, inflammation, and lipolysis. Our study revealed the protective effects of Lpcat3 against adipose tissue inflammation during adipose tissue over-expansion in obesity.

Materials and Methods

Establishment of the high-fat diet mouse model

Six-week-old male C57BL/6 mice were purchased from Charles River Laboratory (Beijing, China) and housed in an approved animal care facility at Fudan University. The mice were randomly assigned to two groups. These mice were fed a chow diet (10% calories from fat; Trophic Animal Feed High-Tech Co., Ltd, Nantong, China) or a high-fat diet (60% calories from fat; Trophic Animal Feed High-tech Co., Ltd) for 6 months. Then, the mice were sacrificed. Abdominal subcutaneous adipose tissues were isolated for further analysis. Animal experiments were approved by the Animal Research Committee of Fudan University.

Lipid measurement by LC-MS/MS

Liquid chromatography was performed using an Eksigent LC100 (Eksigent Technologies, Dublin, USA) coupled to an AB SCIEX Triple TOF 5600+ mass spectrometer (AB SCIEX, Framingham, USA). Lipids extracted from adipose tissues were separated by chromatography using a precolumn of Phenomenex C18 (4 mm × 2.0 mm) and a column of Waters XBridge Peptide BEH C18 (3.5 μm, 2.1 mm × 100 mm) eluted with a gradient of solution A (10 mM ammonium acetate +0.1% formic acid water) and solution B (10 mM ammonium acetate +0.1% formic acid +49.95% acetonitrile +49.95% isopropanol). Lipids were identified by the mass spectrometer in the negative ion mode. Four lipids of 19.0 PC, 17.0 PE, and 12.0 SM were loaded into cell samples before extraction as the internal control. By comparison with the identical internal control, subspecies of the lipids were identified with the PeakView 1.2 software and further quantified with the MultiQuant 2.1 software to obtain the peak area data. The amount of certain lipid subspecies was calculated by the peak area normalized to that of the identical internal loading control and then normalized to the protein concentration.

Cell culture and differentiation

3T3-L1 cells were purchased from the China National Collection of Authenticated Cell Cultures (Shanghai, China). All media and reagents for cell culture were purchased from Gibco (Carlsbad, USA). 3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with glucose, nonessential amino acids (NEAAs), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% new calf serum (NCS). 3T3-L1 preadipocytes were never allowed to reach 100% confluence for subculture. For differentiation, 3T3-L1 cells were seeded in a 12-well plate. When the cells reached 100% confluence (day 0), they were exposed to differentiation medium I containing 0.5 mM 3-isobutyl-1-methyl-xanthine, 1 μM dexamethasone, 5 μg/mL insulin, 2 μM rosiglitazone and 10% fetal bovine serum (FBS) for two days (day 2). Then, the medium was changed to differentiation medium II containing 5 μg/mL insulin, 2 μM rosiglitazone, and 10% fetal bovine serum for another two days (day 4). From day 4, the culture medium was refreshed with 10% FBS DMEM every two days. The 3T3-L1 cells acquire the adipocyte phenotype on day 6 and become mature adipocytes on day 8. All the compounds used for the differentiation of 3T3-L1 cells were from Solarbio (Shanghai, China). Mature adipocytes were serum-starved overnight before the subsequent experiments. All the cells were cultured at 37°C in a humidified 5% CO2 atmosphere.

Construction of adenovirus (Adv)-shLpcat3

For the recombinant construction of Adv-shLpcat3, the plasmid pDKD-CMV-eGFP-U6-shRNA (Lpcat3) targeting the mouse Lpcat3 sequence GGCTTAAGGTGTACAGTC and the plasmid pDKD-CMV-eGFP-U6-shRNA targeting the scrambled sequence TTTCCTCGAACGTTCATCGT were constructed first. For the recombinant construction of Lpcat3 overexpression adenovirus, the plasmid pADV-EF1-mNeonGreen-CMV-Lpcat3-3xflag and pADV-EF1-mNeonGreen-CMV-MCS-3xflag were constructed equally. The mouse Lpcat3 (NM_145130.2) sequence was acquired from NCBI. Then, the pHBlGloxAE1,3Cre plasmid and target gene plasmid (1:1) were cotransfected into HEK293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). All plasmids were purchased from OBIO Technology (Shanghai, China). Then, the culture medium of HEK293 cells was collected as a recombinant adenovirus source,
which could continue to be used for amplification. The adenovirus in the culture medium was purified and enriched by CsCl density gradient centrifugation, followed by titer determination. The adenovirus was aliquoted and stored at −80°C.

**Infection of mature 3T3-L1 adipocytes**

Mature 3T3-L1 adipocytes were infected with recombinant adenovirus (MOI 100) on day 6 when the cells acquired the phenotype of adipocytes to knock down Lpcat3 or overexpression [28]. The recombinant adenovirus was suspended in opti-MEM (Gibco), and the cells were infected for 24 h. Then, the culture medium containing recombinant adenovirus was replaced by fresh 10% FBS DMEM upon 48 h of infection when the mature adipocytes could be used for the subsequent experiments.

**Lpcat3 activity determination**

After 6 days of differentiation and 2 days of adipogenesis infection, the adipocytes were collected and resuspended in enzyme reaction buffer (75 mM Tris, 1 mg/mL BSA, pH 6.0). The supernatant was collected to determine the protein concentration. Lpcat3 activity assays were performed as previously described [29]. Briefly, the reaction mixture in a total volume of 100 μL containing 20 μg protein, 11 μM NBD-Lyso-PC (Avanti® Polar Lipids), and 11 μM arachidonoyl-CoA (Avanti® Polar Lipids) was incubated for 10 min at 30°C. The reaction was stopped by the addition of 300 μL of chloroform/methanol (1:1; v/v) and vigorous mixing. Then, the lower organic phase was collected after centrifugation (6000 g) and dried under nitrogen gas. Lipids were redissolved and then separated with an TLC plate. The development solvent for TLC was chloroform/methanol/water (65:25:4; v/v). Bands were detected under UV, and the product content was determined using ImageJ software.

**Palmitic acid (PA)-bovine serum albumin complex preparation**

Palmitic acid (Sigma-Aldrich, St Louis, USA) was dissolved in ethanol and dried using nitrogen to obtain a certain weight of palmitic acid powder. Fatty acid-free bovine serum albumin (BSA; Beyotime Biotechnology, Shanghai, China) was dissolved in DMEM ethanol and dried using nitrogen to obtain a certain weight of buffer (75 mM Tris, 1 mg/mL BSA, pH 6.0). The cell membrane was disrupted by ultrasound and then centrifuged at 10,000 g. The supernatant was collected to determine the protein concentration. Lpcat3 activity assays were performed as previously described [29]. Briefly, the reaction mixture in a total volume of 100 μL containing 20 μg protein, 11 μM NBD-Lyso-PC (Avanti® Polar Lipids), and 11 μM arachidonoyl-CoA (Avanti® Polar Lipids) was incubated for 10 min at 30°C. The reaction was stopped by the addition of 300 μL of chloroform/methanol (1:1; v/v) and vigorous mixing. Then, the lower organic phase was collected after centrifugation (6000 g) and dried under nitrogen gas. Lipids were redissolved and then separated with a TLC plate. The development solvent for TLC was chloroform/methanol/water (65:25:4; v/v). Bands were detected under UV, and the product content was determined using ImageJ software.

**Lipid droplet oil red O staining**

Oil Red O staining was performed according to the method described by Katja Zebisch and coworkers [30]. Briefly, the Oil Red O stock solution was prepared by stirring 0.5% Oil Red O (Sinopharm Chemical Reagent, Shanghai, China) in isopropanol overnight. The solution was filtered through Whatman (Florham Park, USA) filter paper and stored at 4°C. Fresh Oil Red O working solutions were prepared by mixing the stock solution with distilled water (6:4; v/v). Adipocytes were washed twice with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 1 h at room temperature. Subsequently, the cells were washed with PBS, followed by 60% isopropanol, and dried. Then, Oil Red O working solution was added to stain the cells for 1 h. The cells were washed extensively with distilled water and photographed under a microscope.

**Reverse transcription and real-time PCR**

Abdominal adipose tissues were isolated from C57BL/6 mice. 3T3-L1 mature adipocytes and abdominal adipose tissues were dissolved in Trizol reagent (Invitrogen, Carlsbad, USA), and total RNA was extracted according to the manufacturer’s instructions. RNA was then reverse transcribed to cDNA and subjected to real-time quantitative PCR on a Bio-Rad CFX manager version 3.1 (Bio-Rad, Hercules, USA). Reverse transcription and real-time PCR reagents were purchased from Yeasen (Shanghai, China). Primer sequences for various genes are listed in Supplementary Table S1. Relative gene mRNA expression was calculated by the delta-delta CT method. 18S rRNA was used as an endogenous control.

**Xbox-binding protein 1 (XBP1) mRNA splicing measurement**

cDNA was reverse transcribed from 3T3-L1 mature adipocyte RNA as above. Then, the cDNA representing the unspliced and spliced forms of XBP1 was amplified using premix Taq (TaKaRa, Dalian, China). PCR products were resolved on a 3% agarose gel and visualized using ethidium bromide.

**Triglyceride, glycerol, and IL-6/MCP-1 measurement**

After treatment with 1 mM PA for 24 h, mature 3T3-L1 adipocytes were lysed in 1% Triton X-100 for triglyceride measurement using a kit from Nanjing JianCheng Bioengineering Institute (Nanjing, China) according to the manufacturer’s instructions. The cell supernatant was centrifuged at 10,000 g. The IL-6 and MCP-1 contents in the supernatant were analyzed using the IL-6/MCP-1 ELISA kit (Excell Bio, Taicang, China). For lipolysis measurement, mature 3T3-L1 adipocytes were treated with 1 mM PA for 21 h, and then the medium was refreshed with fresh medium containing 2% BSA for 3 h. The glycerol in the supernatant was determined using a kit (Nanjing JianCheng Bioengineering Institute) according to the manufacturer’s instructions.

**NADPH oxidase (NOX) activity measurement**

To determine the activity of NOX, the plasma membrane of mature 3T3-L1 adipocytes was isolated with the method reported by Chang Yeop Han and coworkers [31]. Briefly, mature 3T3-L1 adipocytes were collected and incubated for 30 min with hypotonic lysis buffer (25 mM Tris-HCl, pH 7.4) containing 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail (NCM Biotech, Suzhou, China), followed by homogenization with a Dounce homogenizer and centrifugation at 1000 g at 4°C for 10 min. Then, the supernatant was ultracentrifuged at 100,000 g for 1 h at 4°C. The pellet, which contained the plasma membrane, was dissolved in suspension buffer (50 mM triethanolamine, 150 mM NaCl, 2 mM MgCl2, 0.1 mM EGTA, protease inhibitor cocktail). After protein concentration determination, equal amounts of protein were used to measure NOX activity. Then, lucigenin (5 μM; Cayman Chemical Company, Inc., Ann Arbor, USA) and NADPH (100 μM; Beyotime Biotechnology) were added and incubated for 10 min at 37°C. The chemiluminescence was recorded with a luminometer (Biotek, Winooski, USA).

**ROS staining and H2O2 measurement**

Mature 3T3-L1 adipocytes were treated with 1 mM PA-BSA complex or BSA for 3 h. For the rescue group cells, mature adipocytes were pretreated for 1 h with 20 mM N-acetyl-L-cysteine (NAC; Beyotime...
Biotechnology) or 20 mM Methyl-β-cyclodextrin (MβCD; Meilunbio, Dalian, China), followed by treatment with 1 mM PA-BSA complex supplemented with 20 mM NAC or 20 mM MβCD for another 3 h. For total ROS staining, a cellvix orange probe (Invitrogen) was added at a final concentration of 5 μM in the last 30 min. For mitochondrial ROS staining, a MitoSOX mitochondrial ROS probe (Yeasen) was added at a final concentration of 5 μM in the last 30 min of treatment. After three times wash with PBS, 3T3-L1 adipocytes were photographed with a fluorescence microscope (Olympus, Tokyo, Japan). The fluorescence intensity of these photos was quantified with ImageJ software. For H<sub>2</sub>O<sub>2</sub> measurement, 3T3-L1 adipocytes were lysed and the concentration of H<sub>2</sub>O<sub>2</sub> in the lysate was measured using a kit (Beyotime Biotechnology) according to the manufacturer’s instructions. The H<sub>2</sub>O<sub>2</sub> concentration was normalized to the protein concentration.

Isolation of lipid rafts
Lipid rafts were separated by sucrose density gradient ultracentrifugation. Briefly, cells or minced adipose tissues were lysed in ice-cold TNEU buffer (10 mM Tris-HCl) containing 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1% Triton X-100 and protease inhibitors (NCM Biotech). After protein concentration measurement, 1 mL of 85% sucrose TNEU was mixed with 1 mL lysates and laid at the bottom of the tube, followed by 6 mL of 35% sucrose TNEU buffer and 4 mL 5% sucrose TNEU buffer. Samples were ultracentrifuged at 200,000 g for 18 h and fractioned into 12 subfractions.

Western blot analysis
Mature 3T3-L1 adipocytes were collected after treatment and lysed with RIPA lysis buffer (Beyotime Biotechnology). The extracts were then centrifuged at 10,000 g for 10 min at 4°C to remove insoluble material. The total protein concentration was measured using the Bradford protein assay kit (Beyotime Institute of Biotechnology). Protein samples (20 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Billerica, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline for 1 h and incubated at 4°C overnight with the primary antibodies. After three times wash with TBST, membranes were incubated with secondary antibodies for 1 h. Target protein bands were revealed with enhanced chemiluminescence (ECL) system (Bio-Rad). Band density was analysed by ImageJ software. Primary and secondary antibodies are listed as follows: anti-CD36 antibody (ab133625; Abcam, Cambridge, UK); anti-Perilipin-1 antibody (9349; CST, Danvers, USA); anti-PPARγ antibody (16643-1-AP; Proteintech, Chicago, USA); anti-Fatty acid synthase antibody (3180; CST); anti-FABP4 antibody (ab92501; Abcam); anti-Adiponectin antibody (2789; CST); anti-GAPDH antibody (ab8245; Abcam); anti-iNOS antibody (ab178945; Abcam); anti-COX 2 antibody (12282; CST); anti-Beclin1 antibody (ab210498; Abcam); anti-LC3B antibody (NB100-2220; NOVUS, Littleton, USA); anti-β-actin antibody (ab8226; Abcam); anti-Phospho-p38 MAPK (Thr180/Tyr182) antibody (4511; CST); anti-Phospho-NF-κB p65 (Ser546) antibody (3033; CST); anti-P38 MAPK antibody (9212; CST); anti-NF-κB p65 (8242; CST); anti-CHOP antibody (2895; CST); anti-GRP78 antibody (3177; CST); anti-NOX4 antibody (14347-1-AP; Proteintech); anti-caveolin1 antibody (3267; CST); and HRP-conjugated goat anti-rabbit IgG (ab6721; Abcam).

Statistical analysis
Data are shown as the mean ± SD. Statistical analysis was performed by one-way ANOVA for independent or correlated values followed by Student’s t test. P < 0.05 was considered statistically significant. All statistical calculations were performed using Microsoft Excel 2019 (Microsoft, Redmond, USA) or GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, USA).

Results
Lpcat3 is highly expressed in over-expanded abdominal subcutaneous adipose tissues
Since polyunsaturated PC metabolism disorder is not rare in many tissues in obesity, we first investigated the polyunsaturated PC content and Lpcat3 expression in expanded adipose tissues. After six months of high-fat feeding, the body weight (Figure 1A) and abdominal subcutaneous adipocyte tissue weight of high-fat-fed mice were significantly increased compared with those in chow-diet-fed mice (Figure 1B), suggesting the successful establishment of an obesity mouse model. Moreover, elevated inflammatory gene expression was observed in these over-expanded adipose tissues from obese mice, such as IL-6 and MCP-1, indicating low-grade chronic inflammation (Figure 1C). Subsequently, we analyzed the PC composition in these adipose tissues. The results showed that polyunsaturated PC contents were significantly increased in over-expanded adipose tissues (Figure 1D). Consistently, we found that Lpcat3, the major Lpcat isoform in adipose tissue, was also the most obviously upregulated isoform of Lpcat (Figure 1E). These data suggested that Lpcat3 might participate in the overexpansion and dysfunction of adipose tissues in obesity.

It has been reported that there is a high concentration of PA in the serum of obese patients, thereby aggravating inflammatory responses in many organs [32]. In addition, PA is also used as a stimulator for lipid accumulation in certain cells [33]. To establish a hypertrophic adipocyte model in vitro and explore the functions of Lpcat3, we treated 3T3-L1 adipocytes with 1 mM PA for 24 h to induce lipid overload. As a result, we observed that PA-treated 3T3-L1 adipocytes were remarkably hypertrophic and accumulated more triglycerides (Figure 1F). Similarly, Lpcat3 expression was also upregulated in these PA-treated cells (Figure 1G). These data suggested that this cell model can partly mimic the features of hypertrophic adipocytes in overexpanded adipose tissues.

Lpcat3 deficiency does not affect lipogenesis in mature 3T3-L1 adipocytes
We have previously reported that Lpcat3 deficiency inhibits the differentiation of 3T3-L1 preadipocytes [23]. However, it is still unknown whether Lpcat3 can modulate lipogenesis in mature adipocytes. For this reason, we silenced Lpcat3 expression in mature 3T3-L1 adipocytes by using adenovirus (Adv-shLpcat3). After treatment, Lpcat3 mRNA and activity levels were reduced by 80% and 60%, respectively (shLpcat3), compared with the control (shCon) (Figure 2A). Then, Oil Red O staining was used to assess lipid droplet formation, and we found no difference between the knockdown cells and the controls (Figure 2B). Meanwhile, there was no change in cellular triglyceride content after Lpcat3 knockdown (Figure 2C). Moreover, our results showed that Lpcat3 deficiency results in no change in the expressions of major lipogenic and adipocyte-specific genes at either the mRNA or protein level (Figure 2D–F). Together, these data showed that Lpcat3 deficiency

Hu et al. Acta Biochim Biophys Sin 2022
does not affect lipogenesis in mature 3T3-L1 adipocytes.

**Lpcat3 regulates PA-induced inflammation and lipolysis**

Previous studies reported that persisting low-grade chronic inflammation in over-expanded adipose tissues could stimulate lipolysis and cause more severe metabolic syndrome [34]. In addition, PA treatment could mimic these phenomena in adipocytes [32]. Thus, we chose PA to investigate the effects of Lpcat3 deficiency on adipocytes. As shown in Figure 3A, Lpcat3-deficient 3T3-L1 adipocytes showed more obvious elevated expressions of inflammatory genes after PA treatment, including IL-6, MCP-1, iNOS, and COX2. Next, we examined IL-6 and MCP-1 secretion in Lpcat3-
knockdown 3T3-L1 adipocytes and controls, respectively. The results showed that these two cytokines were increased in PA-treated Lpcat3-deficient 3T3-L1 adipocytes compared with control adipocytes (Figure 3B). Consistent with the gene expression results, we also found that Lpcat3 deficiency increased iNOS and COX2 protein levels in PA-treated 3T3-L1 adipocytes (Figure 3C,D). In
addition, Lpcat3 deficiency was shown to promote PA-induced inflammatory gene expression, such as IL-6, in a time-dependent manner (Figure 3E) and promote PA-induced MAPK p38 phosphorylation and NF-κB p65 phosphorylation, which can activate the

Figure 3. Lpcat3 deficiency intensifies the inflammation and lipolysis induced by palmitic acid Mature 3T3-L1 adipocytes were treated with 1 mM PA for 24 h after Adv-shLpcat3/Adv-shControl adenovirus infection for 72 h. (A) Then, the relative mRNA expression levels of inflammatory genes in Lpcat3-deficient 3T3-L1 adipocytes were determined by RT-qPCR. (B) The concentrations of IL-6 and MCP-1 in the Lpcat3-deficient 3T3-L1 adipocyte culture medium were analyzed by ELISA. (C) INOS and COX2 proteins were analyzed by Western blot analysis and quantified (D). (E) Relative IL-6 mRNA expression at various time points was measured by RT-qPCR. (F,G) Western blot analysis of p-p65 NF-κB and p-p38 MAPK was performed and quantified. (H) The concentration of glycerol in the supernatant of Lpcat3-deficient 3T3-L1 adipocytes. (I) The ATGL and HSL mRNA expression levels in Lpcat3-deficient 3T3-L1 adipocytes were determined by RT-qPCR. Data are presented as the mean ± SD, n = 3. *P < 0.05, **P < 0.01.
Persistent inflammation has been reported to cause adipocyte dysfunction, such as elevated lipolysis [35]. Our results showed that Lpcat3 deficiency also promotes lipolysis in 3T3-L1 adipocytes after PA treatment, as indicated by higher concentrations of glycerol in the culture medium of Lpcat3-deficient adipocytes (Figure 3H). To further validate this phenomenon, we examined the expressions of two important lipase genes, ATGL and HSL, which are responsible for lipolysis in adipocytes [36]. We found that both of these lipases were upregulated in Lpcat3-deficient 3T3-L1 adipocytes after PA treatment (Figure 3I).

IRE1 kinase was reported to promote inflammation-induced lipolysis in adipocytes [27]. However, we found that LPCAT3 deficiency did not influence the gene expression of IRE1 in PA-treated 3T3-L1 adipocytes (Supplementary Figure S1). This result indicated that enhanced lipolysis caused by Lpcat3 deficiency in PA-treated 3T3-L1 adipocytes could not be attributed to the activation of IRE1 kinase. In summary, these results showed the proinflammatory effect of Lpcat3 deficiency and indicated the involvement of the inflammation-lipolysis axis in Lpcat3-deficient 3T3-L1 adipocytes.

To confirm the phenomenon observed in Lpcat3-deficient adipocytes, we infected mature 3T3-L1 adipocytes with adenovirus Adv-Lpcat3 to overexpress Lpcat3 and found that Lpcat3 expression and its enzyme activity were significantly increased (Figure 4A–C). Consistently, PA-induced inflammatory gene expression levels were significantly decreased after Adv-Lpcat3 infection (Figure 4D). Meanwhile, decreased concentrations of IL-6, MCP-1, and glycerol were also detected in the culture medium of these cells (Figure 4E,F). Furthermore, phosphorylated p38 MAPK and phosphorylated NF-kB p65 induced by PA were also reduced after Lpcat3 overexpression (Figure 4G,H). Together, these data directly indicated that Lpcat3 reduced PA-induced inflammation in 3T3-L1 adipocytes.

ROS but not ER stress intensifies PA-induced inflammation in Lpcat3-deficient adipocytes

To explore the mechanism of the Lpcat3 deficiency-associated proinflammatory effect, we first checked the expressions of two classical unfolded protein response (URP) markers, CHOP and GRP78, in ER stress. Although both were slightly elevated in Lpcat3-deficient adipocytes after PA treatment, they were negligible when compared with thapsigargin (Tg), a well-known ER stress inducer [37] (Figure 5A and Supplementary Figure S2A). Furthermore, we found that XBP1, an important protein involved in the UPR, was not spliced in any groups except the positive control (Supplementary Figure S2B). These data suggested that ER stress is not involved in this inflammatory process.

Notably, some antioxidation genes, such as Nrf2, GPx1, and SOD2, were upregulated in Lpcat3-deficient adipocytes after PA treatment (Figure 5B), suggesting that these cells were under serious oxidative stress. To explore the reason, we used a cellrox orange probe to detect cellular ROS levels. Consequently, we found more obvious ROS and hydrogen peroxide generation in PA-treated Lpcat3-deficient 3T3-L1 adipocytes when compared with the PA-treated controls, while their overgeneration was attenuated once Lpcat3 was overexpressed (Figure 5C,D). To directly test whether it is the increased ROS generation that promotes inflammation, we pretreated cells with N-acetyl cysteine (NAC), a well-known antioxidant [38]. As expected, adipocytes treated with NAC could quench most of the ROS induced by PA (Figure 5C). More importantly, the expression and secretion of IL-6 from those adipocytes were notably inhibited by NAC (Figure 5E,F). Furthermore, the phosphorylation of MAPK p38 MAPK and NF-κB p65 was also completely abolished (Figure 5G,H). Collectively, Lpcat3 deficiency-stimulated ROS overproduction can exacerbate PA-induced inflammation in 3T3-L1 adipocytes.

Lpcat3 regulates NOX4 translocation to lipid rafts, thus modulating ROS generation

We next sought to identify two major sources of ROS generation. First, we used a mitochondrion-specific superoxide indicator to detect mitochondrial ROS generation. A bright fluorescence signal was observed in the positive control 3T3-L1 adipocytes, which were treated with IBMX, a phosphodiesterase inhibitor that stimulates mitochondrial oxidative metabolism. In contrast, we could barely detect any fluorescence signal in any other cells (Supplementary Figure S3). Thus, we rule out the possibility of mitochondria as the source of excessive ROS. Next, we detected the enzyme activity of another source of ROS generation, the NOX family. After isolating cell membrane fractions by ultracentrifugation, we found that there was increased NOX activity in PA-treated adipocytes compared to untreated adipocytes. In addition, the increased NOX activity was more pronounced after Lpcat3 gene knockdown and more weakened after Lpcat3 gene overexpression (Figure 6A). These results indicated that Lpcat3 plays a role in regulating NOX enzyme activity. Since the NOX family has seven isoforms, we then examined the whole NOX family gene expression in 3T3-L1 adipocytes and found that NOX4 is the major NOX isoform in 3T3-L1 adipocytes regardless of PA treatment, which is consistent with a previously reported study (Figure 6B) [31]. Therefore, we believe that NOX4 is responsible for the change in NOX activity in adipocytes. However, the mRNA and protein levels of NOX4 were not affected either by Lpcat3 gene knockdown (Figure 6C and Supplementary Figure S4) or by Lpcat3 gene overexpression (data not shown). These data suggested that the change in NOX4 activity cannot be attributed to protein changes in these adipocytes.

Since a previous study reported that NOX4 activity is associated with its distribution in lipid rafts [31], we isolated lipid rafts from these adipocytes. A widely used anti-caveolin-1 (CAV1) antibody was used to identify the location of lipid rafts (Figure 6E,F). Our results showed that most NOX4 was distributed in nonlipid rafts of adipocytes without PA treatment (Figure 6D,E, left). However, after PA treatment, NOX4 was translocated to the lipid rafts (Figure 6D,E, right). In addition, we also found that the distribution of NOX4 in lipid rafts was increased in abdominal subcutaneous adipose tissue of HFD-fed mice compared with that in chow-fed mice (Supplementary Figure S5). This result suggests that the translocation of NOX4 to lipid rafts in adipose tissues is associated with obesity and adipose tissue overexpansion. Furthermore, our results demonstrated that Lpcat3 deficiency in adipocytes could significantly promote this translocation (Figure 6D, right) and vice versa when the gene is overexpressed (Figure 6E, right). The quantification of NOX4 translocation in different cases is shown in Figure 6F,G. According to a previous study, we speculate that Lpcat3 modulates NOX4 activity via regulating the distribution of NOX4 in lipid rafts [31]. To verify whether the increased distribution of NOX4 in lipid rafts causes enhanced ROS generation in Lpcat3-deficient 3T3-L1 adipocytes, we used 20 mM methyl-β-cyclodextrin (MβCD) to
disrupt lipid rafts [39]. Indeed, the incubation of cells with MβCD decreased the \( \text{H}_2 \text{O}_2 \) content in Lpcat3-deficient 3T3-L1 adipocytes (Figure 6H). Furthermore, MβCD treatment normalized PA-induced IL-6 mRNA expression with Lpcat3 deficiency to control levels (Figure 6I). These findings indicate that PA-induced ROS generation and inflammation in Lpcat3-deficient 3T3-L1 adipocytes could be...
Figure 5. Lpcat3 deficiency promotes inflammation after PA treatment via excessive ROS production but not ER stress. Mature 3T3-L1 adipocytes were treated with 1 mM PA for 24 h after Adv-shLpcat3/Adv-Lpcat3 adenovirus infection for 72 h. (A) The relative mRNA expression levels of CHOP and GRP78 in Lpcat3-deficient 3T3-L1 adipocytes were determined by RT-qPCR. Thapsigargin treatment (10 μM) was used as a positive control for ER stress. (B) Relative antioxidative gene expression in Lpcat3-deficient 3T3-L1 adipocytes was determined by RT-qPCR. After three hours of PA treatment, the total ROS in Lpcat3-deficient or Lpcat3-overexpressing 3T3-L1 adipocytes were labelled with the ROS probe cellrox (C), and the cellular H₂O₂ content was determined (D). Then, 20 mM N-acetylcysteine was used to quench the ROS. The relative mRNA expression (E) and secretion (F) of IL-6 in Lpcat3-deficient 3T3-L1 adipocytes were determined by RT-qPCR and ELISA. (G,H) Western blot analysis of p-p65 NF-κB and p-p38 MAPK was performed and quantified. Data are presented as the mean ± SD, n = 3. *P < 0.05, **P < 0.01.
**Figure 6.** Lpcat3 modulates ROS production via regulating NOX4 distribution in lipid rafts

Mature 3T3-L1 adipocytes were treated with 1 mM PA for 24 h after adenovirus infection for 72 h. (A) NOX activity was measured after isolating the cell membrane of Lpcat3-deficient 3T3-L1 adipocytes. Relative mRNA expressions of NOX family (B) and NOX4 (C) in Lpcat3-deficient 3T3-L1 adipocytes were determined by RT-qPCR. (D,E) After 15 min of treatment with 1 mM PA, the lipid rafts of Lpcat3-deficient or Lpcat3-overexpressing 3T3-L1 adipocytes were isolated by sucrose density gradient ultracentrifugation. Different fractions were subject to western blot analysis. Lipid rafts were indicated by caveolin-1 (CAV1). NOX4 distribution was quantified in (F) and (G). Lpcat3-deficient 3T3-L1 adipocytes were pretreated with 20 mM MβCD for 30 min, and the cellular H₂O₂ content (H) and relative IL-6 mRNA expression (I) were measured after 3 h of treatment with 1 mM PA. Data are presented as the mean ± SD, n = 3. *P < 0.05, **P < 0.01.
attributed to the distribution of NOX4 in lipid rafts.

Discussion

The fatty acyl chain of phosphatidylcholine determines cell membrane biological properties and influences various metabolisms in cells. Lpcat3, as an important phospholipid remodelling enzyme, can enhance membrane fluidity and is involved in many metabolic disorders [14,40–42]. Here, we demonstrated that Lpcat3 impacts inflammation in mature 3T3-L1 adipocytes by regulating ROS generation. We showed that after treatment with PA, Lpcat3-deficient adipocytes exhibited increased NOX4 translocation to lipid rafts on the cell membrane and enhanced NOX4 enzyme activity, thus accelerating ROS generation. As a result, excessive ROS induced intensive inflammation followed by enhanced lipolysis in these mature adipocytes. These findings suggest a new link between the saturation of PCs and ROS generation that regulates inflammatory responses of adipocytes in obesity.

Lpcat3 remodels the composition of PCs via Lands’ cycle, which is associated with various metabolic diseases. Previous studies have revealed that Lpcat3 is associated with metabolic disturbances in different tissues in obesity [14,15,43]. Since Lpcat3 has been demonstrated to regulate the differentiation of adipocytes [23], it would be of interest to further investigate whether Lpcat3 is also involved in the malfunction of adipose tissues. We first examined triglyceride synthesis in Lpcat3-deficient 3T3-L1 mature adipocytes. Surprisingly, we found that Lpcat3 deficiency does not influence triglyceride accumulation in mature 3T3-L1 adipocytes. This finding seems to conflict with our previous study showing that Lpcat3 deficiency has an anti-differentiation effect on 3T3-L1 preadipocytes [23]. However, both studies are reasonable because the effect of Lpcat3 deficiency on adipocytes is dependent on the stage of differentiation. The process of preadipocyte differentiation can be divided into two stages. The first stage is adipocyte phenotype formation, which is accomplished on day 6, followed by the second stage of triglyceride accumulation [28]. We observed that rosiglitazone, a PPAR-γ agonist added at day 0–2 to increase the differentiation efficiency of 3T3-L1 preadipocytes, could counteract the anti-differentiation effect of Lpcat3 deficiency (data not shown). Thus, we confirmed that downregulation of Lpcat3 could inhibit the differentiation of 3T3-L1 cells at the first stage rather than at the second. In addition, one study suggested that Lpcat3 can promote lipogenesis of liver cells by accelerating SREBP-1c maturation [14]. Therefore, it will be interesting to investigate different mechanisms of Lpcat3 in regulating the lipogenesis of hepatocytes and adipocytes.

Endocrine disruption of adipose tissue has been reported to be directly associated with obesity-related disorders. Chronic low-grade inflammation in hyperplastic adipose tissue increases inflammatory cytokine and chemokine secretion, followed by high infiltration of proinflammatory macrophages, all of which finally lead to systemic insulin resistance [44]. Our data showed that Lpcat3 knockdown can enhance inflammatory responses and lipolysis of 3T3-L1 adipocytes induced by PA. Consistently, previous studies have also reported similar proinflammatory effects of Lpcat3 on hepatocytes and macrophages [3,45]. Thus, we supposed that the elevated polyunsaturated PCs and Lpcat3 in abdominal adipose tissues of obese C57BL/6 mice were more likely a compensatory consequence for reducing inflammation in obesity. However, a very recent study demonstrated the effect of polyunsaturated PCs and Lpcat3 on decreasing insulin sensitivity in skeletal muscle cells [15]. Given the complex functions of Lpcat3 in different tissues, it will be necessary to develop effective Lpcat3 inhibitors and to further explore their efficacy in alleviating metabolic syndromes such as systemic insulin resistance.

The most surprising finding of this study is that we revealed the mechanism by which Lpcat3 regulates the inflammatory reaction of mature 3T3-L1 adipocytes by modulating ROS generation. Previous studies reported that loss of Lpcat3 enhances endoplasmic reticulum (ER) stress by increasing phospholipid saturation in the cell membrane of hepatocytes, thus exacerbating cellular inflammation [3,46]. However, we did not find any signs of ER stress in Lpcat3-deficient 3T3-L1 adipocytes, which means that ER stress is not responsible for adipocyte inflammation after Lpcat3 reduction. Instead, we revealed that excessive ROS generation is the cause of inflammation in Lpcat3-deficient adipocytes. Furthermore, our data also substantiated that Lpcat3 could regulate NOX4 enzyme activity by modulating the distribution of NOX4 in lipid rafts, thus impacting ROS generation. Lipid rafts, defined as dynamic ordered micro-domains in the cell membrane, are responsible for creating regions in the membrane to recruit other lipids and proteins that are involved in membrane trafficking and cellular signaling [47,48]. It has been reported that the composition of PCs may modulate the formation and function of lipid rafts [49,50]. It seems that more saturated PC in plasma could contribute to assembling more large and compact lipid rafts. As an important enzyme in the synthesis of unsaturated PCs, Lpcat3 deficiency may promote TLR4 [45], insulin receptors [15], and NOX4 distribution in lipid rafts by promoting lipid raft assembly, thus affecting downstream physiological processes. Nevertheless, further investigation is needed to elucidate its mechanism.

In conclusion, Lpcat3 deficiency does not affect triglyceride accumulation but intensifies PA-induced inflammation by facilitating ROS generation in 3T3-L1 adipocytes.

Supplementary Data

Supplementary data is available at Acta Biochimica et Biophysica Sinica online.

Funding

This work was supported by the grant from the National Natural Science Foundation of China (No. 31770864)

Conflict of Interest

The authors declare that they have no conflict of interest.

References

5. Thomas C, Jalil A, Magnani C, Ishibashi M, Queré R, Bourgeois T, Bergas...
Lpcat3 deficiency promotes adipocyte inflammation


36. Eto M, Shindou H, Koeberle A, Harayama T, Yanagida K, Shimizu T. Lysophosphatidylcholine acyltransferase 3 is the key enzyme for incorporating arachidonic acid into glycerophospholipids during adipo-


