Remodeling of Cardiolipin by Phospholipid Transacylation*

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Mitochondrial cardiolipin (CL) contains unique fatty acid patterns, but it is not known how the characteristic molecular species of CL are formed. We found a novel reaction that transfers acyl groups from phosphatidylcholine or phosphatidylethanolamine to CL in mitochondria of rat liver and human lymphoblasts. Acyl transfer was stimulated by ADP, ATP, and ATPyS, but not by other nucleotides. Coenzyme A stimulated the reaction only in the absence of adenine nucleotides. Free fatty acids were not incorporated into CL under the same incubation condition. The transacylation required addition of exogenous CL or monolysocardiolipin, whereas dilyso-CL was not a substrate. Transacylase activity decreased in lymphoblasts from patients with Barth syndrome (tafazzin deletion), and this was accompanied by drastic changes in the molecular composition of CL. In rat liver, where linoleic acid was the most abundant residue of CL, only linoleoyl groups were transferred into CL, but not oleoyl or arachidonoyl groups. We demonstrated complete remodeling of tetraoleoyl-CL to tetrarolineoyl-CL in rat liver mitochondria and identified the intermediates linoleoyl-trioleoyl-CL, dilinoleoyl-dioleoyl-CL, and trioleoyl-oleoyl-CL by high-performance liquid chromatography. The data suggest that CL is remodeled by acyl specific phospholipid transacylation and that tafazzin is an acyltransferase involved in this mechanism.

Mitochondria contain cardiolipin (CL),1 a unique phospholipid with two phosphate groups and four fatty acids. Throughout the eukaryotic kingdom, CL is the signature lipid of the cristae membrane, suggesting it may play an essential role in mitochondrial physiology (1–4). However, only recently has the availability of CL-lacking yeast strains offered a first glimpse into this role. In the absence of CL, coupling between respiration and protonmotive force is impaired, causing reduction of the mitochondrial membrane potential (ΔΨ) and inhibition of ΔΨ-dependent functions, such as oxidative phosphorylation and protein import (5–7). CL is firmly integrated into the quaternary structure of mitochondrial protein complexes (4, 8). For instance, the position of CL in the crystal structure of the bc complex suggests that it participates directly in proton conduction (9), providing a rationale for the dependence of ΔΨ on CL. CL also supports the functional membrane conformation of the ADP-ATP carrier (10–12), and it is required for the assembly of mitochondrial supercomplexes (13).

It is not known how the function of CL is related to its unique structure. CL is not only a dimeric phospholipid, it also has an unusual composition of fatty acids (2, 4, 11). In some animal and plant tissues, CL contains mostly linoleic acid (1, 2, 4). For instance, in mammalian heart, tetrarolineoyl-CL makes up 70–80% of all molecular species (14). In certain marine animals CL contains almost exclusively arachidonic acid (15), whereas in yeast the dominant acyl groups are oleic and palmitoleic acid (11). Regardless of these variations, in most cells CL maintains a distinct fatty acid pattern that is dominated by only one or two acyl species. CL is relatively resistant to dietary manipulation of its fatty acid pattern (16, 17), suggesting that the acyl composition has functional significance. This idea was supported by the discovery of abnormal CL species in patients with Barth syndrome (14, 18, 19), an X-linked disease associated with cardiomyopathy, skeletal myopathy, neutropenia, and growth retardation (20). Barth syndrome is caused by mutations of tafazzin (21). Because tafazzin is a putative acyltransferase (22), the abnormal composition of CL may be a direct result of acyltransferase deficiency, and it may play a crucial role in the pathogenesis of Barth syndrome.

CL species are thought to emerge from remodeling of acyl groups subsequent to de novo formation (23, 24). The remodeling idea was suggested by (i) lack of acyl specificity in the de novo pathway (25, 26), (ii) independent turnover of acyl and glycerol moieties of CL (27), and (iii) presence of lyso-CLs in mitochondria (23). The classical mechanism of phospholipid remodeling is the Lands cycle in which endogenous fatty acids are removed from phospholipids by phospholipase A2 and new fatty acids are re-attached by acyl-CoA:lyso phospholipid acyltransferase. This process requires activation of fatty acids by MgATP and CoA. Acyl-CoA:MLCL acyltransferase activity has been identified in liver and heart, but it failed to show the anticipated linoleoyl specificity (24, 28). In this paper we report that acyl groups can be transferred directly from phosphatidylcholine (PC) or phosphatidylethanolamine (PE) to CL. This transacylation is linoleoyl-specific in liver and it is deficient in patients with Barth syndrome.

EXPERIMENTAL PROCEDURES

Materials—CL from bovine heart, CoA, linoleoyl-CoA, phospholipase A2 from Naja naja venom, and all nucleotides were purchased from Sigma. Tetraoleoyl-CL (1,3-di[1-14C]dioleoyl-glycerol-3'-phosphoryl-glycerol) was purified from bovine heart CL as described previously (29). Tetraoleoyl-CL (1,3-di[1-14C]dioleoyl-glycerol-3-phosphoryl-glycerol), tetraestersoyl-CL (1,3-di[1-14C]dimyristoyl-glycerol-3-phosphoryl-glycerol), tetraestersoyl-CL (1,3-di[1-14C]1,2-dimyristoyl-glycerol-3-phosphoryl-glycerol), and DCL (1,3-dii[1-14C]acetyl-glycerol-3'-phosphoryl-glycerol) were obtained from Avanti Polar Lipids (Alabaster, AL). Methyl arachidonoyl fluorophosphonate was supplied by

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3 The abbreviations used are: CL, cardiolipin (1,3-diphosphatidylglycerol); MLCL, monolysocardiolipin; DCL, dilyso-cardiolipin; Lα, tetralinoleoyl-cardiolipin; LαO, trilinoleoyl-oleoyl-cardiolipin; Lα2O2, dilinoleoyl-dioleoyl-cardiolipin; Lα3O, linoleoyl-trioleoyl-cardiolipin; O4, tetraoleoyl-cardiolipin; ATPyS, adenosine-5′-ytrithrophosphate; CoA, coenzyme A; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine.
Cardiopin Transacylation

Cardiopin (Cardiolipin) Transacylation—Mitochondria were prepared from liver of Sprague-Dawley rats (100–200 g). Animals were housed in the Central Animal Care Facility of New York University Medical Center. Treatment was approved by the institutional animal care and use committee. Rats were anesthetized by pentobarbital injection, and the liver was placed in ice-cold saline. The liver was minced, washed, and homogenized in 15 ml of 0.25 M sucrose, 1 mM EGTA, 0.5% fatty-acid free bovine serum albumin, and 5 mM Heps, pH 7.2. Cells were treated with digitonin until more than 90% of them became permeable to trypan blue (31). Digitonin-treated cells were disrupted in a teflon/glass homogenizer. The homogenate was spun at 17,000 g for 10 min. The resulting mitochondrial pellet was suspended in 50 ml isolation buffer followed by centrifugation at 625 g for 5 min. Finally mitochondria were spun at 17,000 g for 90 min. Protein concentrations of mitochondria and microsomes were determined by the method of Lowry (30).

Mitochondria—Mitochondrial cardiolipin cell lines were established by Epstein-Barr virus transformation of leukocytes isolated from whole blood of three patients with Barth syndrome and three control subjects. All patients with Barth syndrome had a mutation in the tafazzin gene (G4.5) that resulted in total deletion of the protein. In addition these patients met clinical criteria of Barth syndrome, i.e. they had cardiomyopathy plus two or more of the following non-cardiac symptoms: skeletal myopathy, neutropenia, growth retardation, and increased urinary excretion of 3-methylglutaconic acid. Patients or their guardians gave written informed consent. The protocol was approved by the Institutional Review Board of Johns Hopkins University. Lymphoblasts were grown in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum. Cultures were expanded and 10 6 cells were harvested in mid-log growth for isolation of mitochondria (31). To this end cells were suspended in 4 ml isolation buffer, containing 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5% (w/v) fatty-acid free bovine serum albumin, and 5 mM Heps, pH 7.2. Cells were treated with digitonin until more than 90% of them became permeable to trypan blue (31). Digitonin-treated cells were disrupted in a teflon/glass Dounce homogenizer and unbroken cells were removed by repeated centrifugation at 625 g for 5 min. Finally mitochondria were spun at 10,000 g for 20 min and they were re-suspended in isolation medium (protein: 20–40 mg/ml).

In Vitro Experiments—Mitochondria (1.0–1.5 mg protein) were incubated in 0.3 ml buffer, containing 50 mM Tris, pH 7.5, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 4 mM ATP, 33 μM acetyl acceptor (CL, MLCL, or DLCL), and 0.1 μM 6.0–6.9 μM) radioactive acyl donor (14C)PC or (14C)PE at 37 °C shaking water bath. First, organic stock solutions of phospholipids were added and organic solvents were evaporated. Then, aqueous buffer was added and phospholipids were dispersed by sonication in a Branson 8210 sonicator bath for 45 s. The mitochondrial pellet was suspended in 50 ml isolation buffer followed by centrifugation at 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.5% fatty-acid free bovine serum albumin, and 5 mM Heps, pH 7.2. Mitochondria were incubated with 14C-linoleoyl-PC and MLCL. Addition of phospholipase A2 by 10 μM methyl arachidonyl fluorophosphonate had no effect on the reaction (activity in the presence of [14C]linoleoyl-PC, MLCL, ADP, and inhibitor was 110 ± 20% of activity without inhibitor), suggesting that neither cytosolic nor Ca2+-independent phospholipases A2 were involved in transacylation. The specific transacylation activity was 3–4 times higher in the mitochondrial preparation than in the microsomal preparation, suggesting a specific localization on mitochondria or mitochondria-associated membranes.

We compared the effect of various nucleotides on [14C]Cardiolipin transfer. ADP, ATP, and ATPγS stimulated [14C]Cardiolipin transfer from PC to CL and from PC to PE, whereas other nucleotides had little or no effect (Fig. 3). Half-maximal stimulation occurred at a concentration of 1.5 ± 1.1 μM ADP. CoA

RESULTS

Transacylation in Rat Liver Mitochondria—Mitochondria were prepared from rat liver mitochondria with 14C-linoleoyl-PC and MLCL. Within 40 min, radioactivity of PC decreased by 7.5 ± 0.2% (n = 3). Of this portion, 50 ± 3% was recovered as free linoleic acid, 31 ± 4% was recovered in CL, 10 ± 1% was recovered in lyso-PC, 6 ± 1% was recovered in PE, and 3 ± 1% was recovered in other phospholipids (n = 3). Incorporation of [14C]Chlorophylin to CL and PE occurred at a steady rate that was increased by ADP (Fig. 1). To investigate whether liberated 14C-linoleic acid was the source of radioactivity in CL, we added free 14C-linoleic acid to the incubation instead of [14C]linoleoyl-PC. The amount of added [14C]linoleic acid was identical to the amount of [14C]linoleic acid released during incubation with [14C]PC. In the presence of [14C]linoleic acid, incorporation of radioactivity into CL was negligible. In contrast, [14C]linoleoyl-PE was an effective donor of radioactivity to CL (Fig. 2). Acyl transfer from [14C]linoleoyl-PE showed similar dependence on ADP and incubation time as the transfer from [14C]linoleoyl-PC (data not shown). These results suggest that [14C]Chlorophylin groups were transferred from either PC or PE to CL by transacylation.
increased $[^{14}C]$linoleoyl transfer from PC into CL 2.2 ± 0.2-fold ($n = 5$), and the CoA concentration required for half-maximal effect was 5.8 ± 2.7 μM. However, the effect of CoA disappeared in the presence of ADP, i.e. $[^{14}C]$linoleoyl transfer with ADP + CoA was 99 ± 9% of the transfer with ADP alone ($n = 4$). $[^{14}C]$Linoleoyl transfer from PC to CL was dependent on exogenous MLCL (Fig. 1). When exogenous CL was added instead of MLCL, the same rate of $[^{14}C]$linoleoyl transfer was measured.

The concentration required for half-maximal stimulation was 1.4 ± 0.6 nmol/mg protein for CL and 1.0 ± 0.5 nmol/mg protein for MLCL. In contrast, DLCL was not a substrate of the transacylation (Fig. 4).

Remodeling of CL in Rat Liver Mitochondria—To measure acyl specificity of the transacylation between PC and CL, we incubated rat liver mitochondria with different species of $[^{14}C]$acyl-PC and different species of CL. $[^{14}C]$Linoleoyl-PC was
an effective acyl donor in the presence of either tetralinoleoyl-CL, tetraoleoyl-CL, or tetramyristoyl-CL. In contrast, neither $[^{14}\text{C}]\text{oleoyl-PC}$ nor $[^{14}\text{C}]\text{arachidonoyl-PC}$ participated in the transacylation regardless of the acyl acceptor (Fig. 5). Acyl transfer from PE to CL also favored linoleoyl groups, albeit the specificity was lower; i.e. transacylation from $[^{14}\text{C}]\text{linoleoyl-PE}$
to CL was only 4.6 ± 1.3-fold faster than from [14C]arachidonoyl-PE to CL (n = 3).

Incubation of liver mitochondria with [14C]linoleoyl-PC and tetraoleoyl-CL (O4) led to complete remodeling, generating the end product tetralinoleoyl-CL (L4), as well as the intermediates LO3, L2O2, and L3O, all of which were identified by high-performance liquid chromatography (Fig. 6). Although ultimately all acyl positions of CL were accessible to transacylation, sn-2 positions turned over faster than sn-1 positions. This was suggested by treatment of isolated [14C]CL with phospholipase A2, releasing 76 ± 4% of radioactivity as linoleic acid (representing sn-2 bound residues), 15 ± 3% as DLCL (representing sn-1 bound residues), and 9 ± 1% as MLCL (n = 3). The data are consistent with the following remodeling pathway: O4 → 2-L-1,1′,2′-O3 → 2,2′-L0-1,1′-O2 → 1,2,2′-L0-1′-O → L4. Likewise, phospholipase A2 released 88 ± 2% of radioactivity as linoleic acid from the sn-2 position of [14C]PE (n = 3).

Transacylation in Human Lymphoblast Mitochondria—We measured phospholipid transacylation in mitochondria from human lymphoblast cell lines. Transacylation was measured with [14C]oleoyl-PC because oleic acid was the most abundant residue in PC, CL, and PE of lymphoblasts. Like in liver, transacylase activity was stimulated by ADP and it was independent of CoA in the presence of ADP (data not shown). In lymphoblasts with tafazzin deficiency, derived from patients with Barth syndrome, [14C]oleoyl transfer from PC to CL and to PE was significantly reduced (Fig. 7, lower panel). At the same time there were drastic changes in the molecular composition of CL in tafazzin-deficient lymphoblasts; i.e., the four characteristic CL species of normal lymphoblasts, containing primarily oleic and palmitoleic acid, were replaced by multiple molecular species representing a more diverse fatty acid pattern (Fig. 7, upper panel).

**DISCUSSION**

We demonstrated formation of tetralinoleoyl-CL by linoleoyl-specific phospholipid transacylation in rat liver mitochondria. Linoleoyl groups were transferred directly from PC or PE, because (i) free linoleic acid was not incorporated into CL under conditions that allowed incorporation of phospholipid-bound linoleoyl residues (Fig. 2), and (ii) linoleoyl transfer did not require MgATP/CoA (Figs. 1 and 3). The notion of a transacylation contradicts our previous conclusion that CL is remodeled with CoA activated fatty acids (23). On the one hand the present data confirmed that ATP and CoA stimulated [14C]CL formation, on the other hand we found that neither component was absolutely necessary for [14C]linoleoyl transfer from PC to CL. ATP had no greater effect than ADP or ATPγS, and CoA did not stimulate linoleoyl transfer in the presence of adenine nucleotides. However, ATP may be the physiological effector of the transacylation because it required adenine nucleotide concentrations above 2 μM for full activity. Transacylation also occurred between PC and PE, suggesting that all three major mitochondrial phospholipids can exchange acyl groups by this mechanism.

We found a similar transacylase reaction in human lymphoblast mitochondria. The transacylase activity was decreased in lymphoblasts with tafazzin deletion (Fig. 7), suggesting that tafazzin plays a role in the transacylation mechanism. Tafazzin deletion also resulted in altered composition of CL, suggesting that tafazzin is involved in CL remodeling (Fig. 7). Tafazzin was predicted to be an acyltransferase based on its amino acid sequence (22). Several splice variants of tafazzin exist and the splicing pattern is characteristic for cell type and tissue (21). It was suggested that tafazzin splicing may confer acyl specificity (22). Acyl specificity of the transacylation corresponded to the molecular composition of CL in liver and lymphoblasts, respectively. In liver where CL was rich in linoleic acid, we found strict linoleoyl-specificity of the transacylation. No such specificity was observed in lymphoblasts where CL contained mostly oleic and palmitoleic acid.

Overall the present data supported the notion that phospholipid transacylation is the underlying mechanism of CL remodeling. The data also suggested that CL was not released from the transacylase until remodeling was nearly complete. This is because more than 80% of the remodeling products in liver cannot be released from CL because of the presence of phosphorylase A2. The presence of phosphorylase A2 in liver is consistent with the hypothesis that CL remodeling is a mechanism of phospholipid transacylation.

**Fig. 7.** CL composition and transacylation in lymphoblast mitochondria from patients with tafazzin deletion (ΔTAZ) and controls. Upper panel, analysis of molecular species of CL by fluorescence-HPLC. Fluorescence intensity is plotted against retention time (RT). The major molecular species of CL in control lymphoblasts are tripalmitoleoyl-oleoyl-CL (P, O), dipalmitoleoyl-dioleoyl-CL (P2O2), palmitoleoyl-trioleoyl-CL (PO32), and tetraoleoyl-CL (O4). Lower panel, human lymphoblast mitochondria were incubated with [14C]oleoyl-PC, MLCL, and ADP for 30 min. Lipids were extracted and separated by two-dimensional thin-layer chromatography. Radioactivity was measured in CL and PE. Data are mean ± S.E. of three separate experiments.

**Fig. 8.** Proposed scheme of CL remodeling. The putative enzyme (E) binds either MLCL or CL. The two lipids are in equilibrium via the acyl−E−MLCL intermediate. The CL backbone remains bound until remodeling is complete. Acyl groups are transferred from PC into acyl−E−MLCL by acyl-specific transacylation. Acyl groups are transferred out of acyl−E−MLCL by nonspecific transacylation. The acyl acceptor of this transacylase (X) has not been identified. Hypothetically it may be CoA, lysophospholipid, or water.
were tetralinoleoyl-CL and trilinoleoyl-oleoyl-CL (Fig. 6), a result that cannot be achieved by single acyl exchange with exogenous tetraoleoyl-CL. CL and MLCL had equal effect on the transacylation (Fig. 4), suggesting that both substrates readily exchanged acyl groups with the putative acyl–enzyme (acyl–E) intermediate. Thus we propose the presence of an active complex acyl–E–MLCL, that is in equilibrium with E–CL via intramolecular acyl transfer (Fig. 8). This complex may exchange acyl groups with other lipids by transacylation until the desired molecular species of CL emerge. Inflow of acyl groups into the complex may show tissue-specific acyl selectivity. In contrast, outflow of acyl groups is non-selective because different species of CL are equally good substrates of the acyl transferase from liver mitochondria (28). Although this enzyme cannot provide specific incorporation of linoleoyl groups into tissues (34). In contrast, acyl-CoA-dependent acyltransferases only distinguish between saturated and unsaturated fatty acids (34, 35). This is also true for purified MLCL:acyl-CoA acyltransferase from liver mitochondria (28). Although this enzyme cannot provide specific incorporation of linoleoyl groups into CL, it may still be involved in the remodeling process. For instance it may transfer acyl groups out of CL to CoA or other acyl acceptors.

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