Accumulation of cardiolipin and lysocardiolipin in fibroblasts from Tangier disease subjects

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Abstract Tangier disease (TD) is an inherited disorder of lipid metabolism characterized by very low high density lipoprotein (HDL) plasma levels, cellular cholesteryl ester accumulation and reduced cholesterol excretion in response to HDL apolipoproteins. Molecular defects in the ATP binding cassette transporter 1 (ABCA1) have recently been identified as the cause of TD. ABCA1 plays a key role in the translocation of cholesterol across the plasma membrane, and defective ABCA1 causes cholesterol storage in TD cells. Not only cholesterol efflux, but also phospholipid efflux was shown to be impaired in TD cells. By use of thin layer chromatography, high performance liquid chromatography and time-of-flight secondary ion mass spectrometry, we characterized the cellular phospholipid content in fibroblasts from three homozygous TD patients. The cellular content of the major phospholipids was not found to be significantly altered in TD fibroblasts. However, the two phospholipids cardiolipin and lysocardiolipin, which make up minute amounts in normal cells, were at least 3–5-fold enriched in fibroblasts from TD subjects. A structurally closely related phospholipid (lysobisphosphatidic acid) has recently been shown to be enriched in Niemann–Pick type C, another lipid storage disorder. Altogether these data may indicate that the role of these phospholipids is a regulatory one rather than that of a bulk mediator of cholesterol solubilization in sterol trafficking and efflux. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Tangier disease; High density lipoprotein; Familial high density lipoprotein deficiency; Apolipoprotein A-I; ATP binding cassette transporter 1; Cardiolipin; Lysocardiolipin; Niemann–Pick type C disease; Sterol trafficking

1. Introduction

High density lipoprotein (HDL) plays an important role in reverse cholesterol transport, the process by which the cholesterol is transported from peripheral cells to the liver, where it is excreted in the form of bile salts. This process is thought to protect against the development of atherosclerosis. The cholesterol efflux process involves specific and unspecific mechanisms [1,2]. Specific cholesterol efflux from cells is mediated by HDL apolipoproteins; it involves the activation of cellular phospholipases and kinases and an active brefeldin-sensitive transport pathway [3–6]. The specific apolipoprotein-inducible cholesterol efflux is severely impaired in subjects with homozygous Tangier disease (TD) [7–10], resulting in almost complete absence of circulating HDL, massive accumulation of cholesteryl esters in many tissues and an increased cardiovascular risk [11–13].

The ATP binding cassette transporter 1 (ABCA1) was recently identified as the molecular defect in TD by genetic mapping [14] and subsequent sequencing of candidate genes on DNA from Tangier patients [15–18]. The ATP binding cassette superfamily is comprised of a large group of evolutionarily conserved proteins present in prokaryotes and eukaryotes [19]. These proteins function by coupling the hydrolysis of ATP to the transmembrane flux of a wide variety of substrates across organellar and plasma membranes. ABCA1 seems to be an essential cofactor for the translocation of cholesterol from intra- to extracellular sites [20], and defective ABCA1 in TD thus impairs apolipoprotein-mediated cholesterol translocation to and across the plasma membrane. However, the exact mechanism of ABCA1-dependent and alternative cholesterol excretion pathways is not clear.

In addition to this defect phospholipid efflux was shown to be impaired in radiolabeled TD fibroblasts [8]. Remaley et al. [21] hypothesized that phospholipid and cholesterol efflux are closely related. They proposed the following model: apolipoprotein A-I (apo A-I) first removes phospholipids by direct interaction with the plasma membrane; in the second step the resulting apo A-I phospholipid complex is able to accept cholesterol. We have used thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) to characterize the cellular phospholipid content in fibroblasts from homozygous TD patients. The cellular concentration of the major phospholipids was not altered in TD cells. By contrast,

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Abbreviations: ABCA1, ATP binding cassette transporter 1; apo A-I, apolipoprotein A-I; CL, cardiolipin; DMEM, Dulbecco's modified Eagle's medium; HPLC, high performance liquid chromatography; LC, lysocardiolipin; NPC disease, Niemann–Pick type C disease; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLD, phospholipase D; SM, sphingomyelin; TOF SIMS, time-of-flight secondary ion mass spectrometry; TD, Tangier disease; TLC, thin layer chromatography

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two minor phospholipids, cardiolipin (CL) and lysocardiolipin (LC), were enriched at least 3–5-fold.

2. Materials and methods

2.1. Probands

The experiments were performed with fibroblasts from three normolipidemic healthy probands and the three homozygous Tangier patients, TD1 (66 years, male), TD2 (64 years, male) and TD3 (63 years, female, sister of TD1), whose clinical, biochemical and genetic characteristics have been described in detail in previous reports [4,7,11,12,22–25]. Patients TD1 and TD3 are characterized by an asparagine to serine mutation at position 935 of ABCA1 [25]. The unrelated patient TD2, who suffers from severe premature atherosclerosis, has a stop codon mutation at position 575 of the ABCA1 gene, predicting truncation of the encoded ABCA1 protein and deletion of most of the protein sequence, including both ATP binding cassettes [15].

2.2. Reagents

L-Lyso-3-phosphatidylcholine was obtained from Amersham Corp. (Braunschweig, Germany). All lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Autoradiography was performed by using Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). Medium components were obtained from Flow Laboratories (Meckenheim, Germany). Plastic culture dishes were purchased from Falcon Labware (Oxnard, CA, USA), scintillation counting mixture (Ultima-Gold) was obtained from Canberra-Packard (Frankfurt, Germany). Aluminum-backed silica gel 60 TLC plates and the solvents for TLC, HPLC and MS were purchased from Merck (Darmstadt, Germany). All other reagents were obtained from Sigma Chemical Co. (Deisenhofen, Germany) and were of the highest purity available.

2.3. Cell culture

Human skin fibroblasts cultured from biopsies of adult human hip skin were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM L-glutamine, and 1% antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO, USA), as previously described [7]. Once separated, the dermis was cut into small pieces (0.5 mm on each side) and placed in a flask in DMEM. When these primary cultures were confluent they were expanded by passage. Experiments were routinely performed with cells between passage levels three and six. Under these conditions, the TD cells display their typical characteristics such as cholesterol accumulation and markedly reduced cholesterol efflux [7]; however, the growth rate (which was reported to be reduced at higher cell passages) was not significantly reduced. The cells were seeded in 60-mm culture dishes at a density of $\sim 7.5 \times 10^4$/dish. The state of 70–80% confluence was reached after 3–4 days. The cell layers were rinsed twice with phosphate-buffered saline (PBS, pH 7.4) and used for measurements.

2.4. Radiolabeling and separation of radiolabeled phospholipids by TLC

At the state of near-confluence the cell layers were rinsed twice with PBS (pH 7.4) and incubated for 48 h at 37°C in DMEM. After 48 h, the medium was removed, the cells were washed three times with PBS and incubated for 2 h in 2 ml of DMEM–HEPES containing 0.05 μCi/ml [1-^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine (56 mCi/mmol) as previously described [3,4]. Thereafter, cells were washed five times in PBS, and incubated for an additional 3 h in 2 ml of DMEM–HEPES. The medium was removed and the cell dishes were placed in a liquid nitrogen bath. The cells were scraped from the dishes with a rubber policeman, once with 2 ml of ice-cold methanol and once with 2 ml of ice-cold distilled water. Radioactive lipids were extracted by the method of Folch et al. [26]. The cellular protein content was measured according to Bradford [27].

For TLC analysis we used a double one-dimensional technique to separate phospholipids of interest, as described in detail in reference [4]. In this approach, a series of samples were spotted 12 cm from the bottom of a 20×20 cm TLC plate. In order to separate neutral lipids from phospholipids that remained at the origin, the plates were twice developed in toluene/ether/ethanol/triethylamine (100:80:4:2, per vol). After the first run to 20 cm, the plates were thoroughly dried and developed a second time with the same solution to 18 cm. Plates were then cut 0.8 cm above the origin (i.e. 12.8 cm above the lower edge of the plate), rotated 180°, and developed to the top with chloroform/methanol/ammonium hydroxide (65:35:5, per vol). After they were dried thoroughly, autoradiography was performed by using Kodak X-OMAT film for 7–14 days. Radioactive bands were cut from the silica plates, placed in scintillation vials containing 10 ml Ultima-Gold scintillation fluid, and quantitated by liquid scintillation counting in a scintillation counter (model 1214; LKB, Bromma, Sweden). The identities of labeled bands were determined by comparing the R_f values with those of reference substances, visualized by iodine staining.

2.5. Preparation of radiolabeled LC standards

Mono-LC and di-LC were prepared by phospholipase hydrolysis of radiolabeled CL. To this purpose, fibroblasts were incubated with [1-^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine (0.1 μCi/dish), lipids were isolated and separated by TLC as described before. The CL band was scraped off the plate, extracted with chloroform and dried under a stream of nitrogen. The residue was redissolved in 2 ml 0.05 Tris–HCl (pH 7.4), incubated for 4 h (37°C) with *Rhizopus arrhizus* phospholipase (30 000 units, purchased from Sigma, Deisenhofen, Germany), extracted and separated by TLC as described before.

2.6. MS and HPLC

After incubation of the fibroblasts for 48 h at 37°C in DMEM, the medium was removed, the cells were washed three times with PBS and incubated for 2 h in 2 ml of DMEM–HEPES, containing 500 μg/ml lysophosphatidylcholine (egg). After extraction of lipids by the method of Folch et al. [26] phospholipids were separated by HPLC according to Nissen and Kreysel [28]. HPLC fractions were collected every 2 min and 10 μl of this fraction was analyzed by mass spectrometry using a TOF-SIMS II spectrometer (IONTOF, Münster, Germany), as previously described [29]. The fraction was applied to a silver target which had been pre-etched for 2 min with 20% HNO_3. After evaporating the solvent, the sample was bombarded with $^{40}Ar^+$, 10^{11} ions/cm^2 (11 keV). Measurements were performed with typical acquisition times of 100 s. The approximate mass resolution was $M/\Delta M = 10\,000$, where M is the target ion mass and ΔM is the resolved mass difference at half-width full peak mode. The identity of the phospholipid species was calculated based on the measured mass and confirmed by comparison with synthetic standards.

3. Results

3.1. Characterization of radiolabeled phospholipids by TLC

To investigate whether there were any abnormalities in the relative concentrations of radiolabeled phospholipids, control and TD fibroblasts were labeled with [1-^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine, for 2 h at 37°C, and the cellular lipids were separated by TLC. This procedure resulted in a quantitative incorporation of label into phospholipids, and no significant change of label incorporation was observed from 2 h up to 10 h onwards. The total amount of radioactivity incorporated into cellular lipids and separated on TLC was similar in TD and control cells (controls: 30 833 ± 2674 dpm/dish; Tangier: 39 741 ± 7723 dpm/dish, mean ± S.D.). 35–50% of the radiolabel incorporated was acylated to phosphatidylcholine (PC), 30–35% of the radioactivity was found in the triglycerides, ∼15% in phosphatidylethanolamine (PE), 2–2.5% in phosphatidic acid (PA), ∼3% in the lyso-PC (LPC)/phosphatidylinositol (PI)/sphingomyelin (SM) fraction, 1–1.5% in diacylglycerol, and 0.2–0.5% in monoacylglycerol (Fig. 1). The relative concentrations of these phospholipids were not significantly different between patients and controls. Three phospholipids of minor concentration, however, were found to be at least five-fold enriched in TD cells (arrows). Only minute amounts of these phospholipids were present in control cells. The most hydrophobic phospholipid comigrated with commercially available CL in one- and two-dimensional TLC.

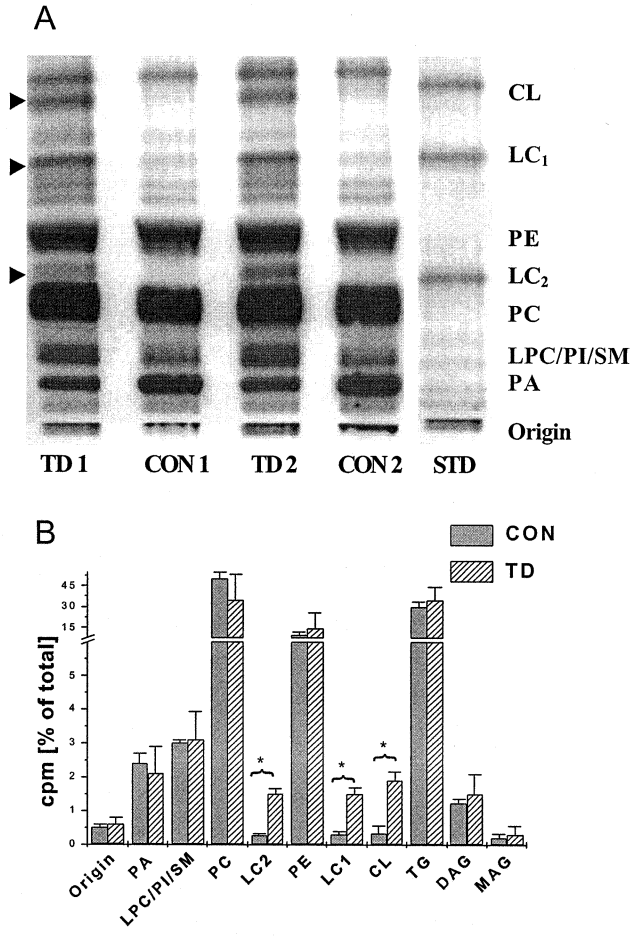

Fig. 1. A: TLC analysis of cellular lipid extracts from two control (CON1, CON2) and two Tangier (TD1, TD2) fibroblast lines labeled with 0.05 μCi/ml [1-^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine. The identities of CL, PE, PC, LPC, PI, PA and SM were determined with one- and two-dimensional TLC, by comparing the R_f values with those of commercially available reference substances, visualized by iodine staining (not shown). Mono-LC (LC1) and di-LC (LC2) were identified by comparison with radioactive standards, which were prepared by phospholipase hydrolysis of radiolabeled CL, as described in Section 2. The standard shown in the figure (STD) represents a mixture of radioactive CL, mono-LC (LC1) and di-LC (LC2), visualized by autoradiography. B: Radioactive phospholipids were cut from the plate and quantified by liquid scintillation counting as described in Section 2. Each value represents the mean (±S.E.M.) of $n=12$ repetitive experiments from a single fibroblast culture. *$P<0.01$ compared to the appropriate control value.

The other two phospholipids (lower two arrows in Fig. 1) were indistinguishable by TLC from the two deacylated forms of CL obtained from CL by incubation with *Rhizopus arrhizus* lipase (a lipase with phospholipase A1-like activity; LC1 and LC2 in Fig. 1). A cochromatographic analysis, in which radioactive standards and samples were mixed, further confirmed this identification (data not shown).

3.2. Characterization of phospholipids by HPLC and TOF-SIMS

Non-radioactive phospholipid content of control and TD cells was examined by HPLC analysis of cellular lipid extracts. As shown in Fig. 2, the cellular phospholipid content of control and TD cells was very similar. We did not find a significant quantitative difference of the major phospholipid classes. Mass spectroscopy analysis of the HPLC lipid fractions and comparison of the masses with a computerized search program identified the major HPLC fractions as PC and PE, as indicated in Fig. 2. The relatively hydrophobic CL-containing HPLC fraction was 3–5-fold increased (Fig. 2). Fig. 3 represents mass spectra of this HPLC fraction in the mass range 1100–1600 amu. In the positive secondary ion mass spectra, lipid species were detectable in protonated $[M+H]^+$, cationized $[M+Na]^+$ or intact $[M]^+$ forms. The theoretical masses of the molecular ions of CL (based on tetra-linoleoyl CL) were as follows: $[M]^+$, 1447 amu; $[M-C_6H_{12}]^+$, 1363 amu; $[M-C_{16}H_{29}+Na]^+$, 1249 amu. Using a computerized lipid library (composed of 2780 different masses of phospholipids and fragments) these molecular masses corresponded exactly to CL. The identity of this compounds was further confirmed by comparison with mass spectra and HPLC retention times of commercially available CL (Figs. 2 and 3). Moreover, the most intense signal in the respective negative spectrum revealed the molecular mass of the fatty acid linoleic acid ($[M]^-$, 280 amu), which is the most abundant fatty acid in CL. On the basis of the HPLC results for lipid fractions

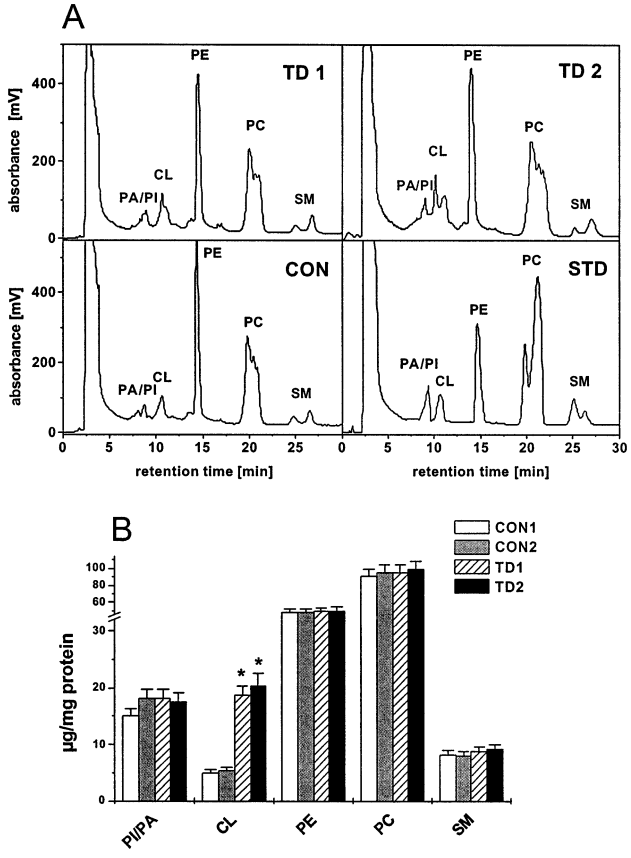

Fig. 2. HPLC analysis of phospholipid extracts from one representative control (CON1) and two Tangier (TD1, TD2) fibroblast lines. A: HPLC chromatograms of CON1, TD1, TD2 and the standard mixture (STD): CL (25 μg/ml), PE (50 μg/ml), PC (100 μg/ml), SM (50 μg/ml), PA (25 μg/ml) and PI (25 μg/ml). B: Content of phospholipids, estimated by integration of the respective HPLC peaks. Each value represents the mean (±S.E.M.) of $n=6$ repetitive experiments from a single fibroblast culture. *$P<0.01$, TD1 vs. CON2, and TD2 vs. CON2.

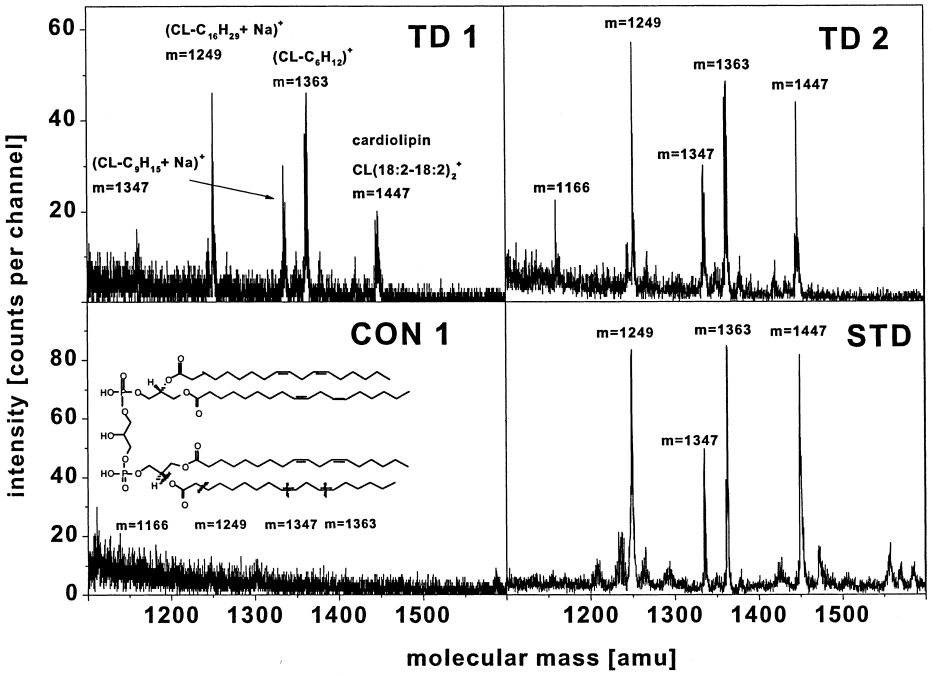

Fig. 3. TOF-SIMS spectrum of the CL fraction from control and TD cells. The characteristic CL fragments are presented in the insert of CON1.

obtained from three separate cellular lipid preparations, the concentration of CL in TD cells was estimated to be 18.7 ± 1.68 (TD1) and 24.3 ± 2.19 (TD2) μmol/mg cell protein in two Tangier cell lines and 5.1 ± 0.46 (CON1) and 5.5 ± 0.49 (CON2) in two control cell lines. Since the CL-containing HPLC fraction contained minor amounts of additional lipids, the CL concentration especially in controls tended to be overestimated. On the basis of MS, the CL concentration in controls was close to the lower detection limit of TOF-SIMS both in the positive (Fig. 3) and in the negative spectrum (data not shown). Also, CL was not detectable at higher concentrations in any other HPLC fraction in controls.

4. Discussion

The cellular cholesterol content is markedly increased in TD fibroblasts [8], probably due to impaired translocation of cholesterol across the plasma membrane [7–10]. It has previously been shown that not only cholesterol efflux but also phospholipid efflux is defective in TD fibroblasts [8,21]. Since ATP binding cassette transporters are involved in the transmembrane flux of a wide variety of substrates, we hypothesized that cholesterol and phospholipid mass efflux might be coordinately regulated, and thus that both processes might be impaired leading to significant cellular accumulation of phospholipids.

The novel finding provided by this investigation is that in TD fibroblasts the content of CL and LC is severalfold increased as compared to normal fibroblasts. However, we could not detect significant differences in the cellular content of major cellular phospholipids of normal and TD fibroblasts. Of course, these data do not exclude the possibility that a quantitatively minor fraction of cellular PC is effluxed together with cholesterol and is required for the solubilization of

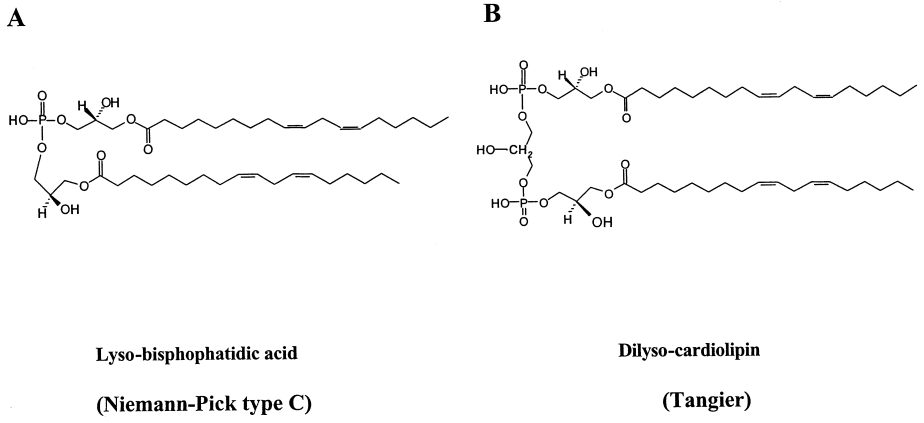

Fig. 4. Molecular structure of lyso-bis-PA and di-LC.

cholesterol. Moreover, it is possible that phospholipid accumulation, comparable to cholesterol accumulation, is prevented by decreased synthesis or enhanced catabolism of cellular phospholipids. The accumulation of two quantitatively minor phospholipids in TD fibroblasts suggests that phospholipids may play a regulatory role in cellular cholesterol sorting and trafficking rather than acting solely via solubilization of cholesterol. Two possibilities may account for the cellular accumulation of (lyso)CL in TD. First, the translocation of cellular cholesterol is closely related to translocation of (lyso)CL and both processes are coordinately regulated and impaired in TD. Second, enhanced synthesis and/or accumulation of CL may reflect enhanced mitochondrial activity, possibly as the result of an adaptive mechanism.

CL is ubiquitous in eukaryotes. It is unique in structure, subcellular localization and potential function. Because it is found predominantly in the mitochondrial inner membrane, it is an excellent marker for mitochondrial biogenesis. Its function, however, has remained unclear ever since it was discovered in the 1940s [30]. CL was proposed to be an important cofactor for cholesterol translocation from the outer to the inner mitochondrial membrane where it enhances the availability of cytochrome P-450 to inner membrane cholesterol [31,32]. In steroidogenic tissues, CL activates mitochondrial cholesterol side chain cleavage and is a potent stimulator of steroidogenesis. Recent data from Björkhem's group suggest that mitochondrial cholesterol oxidation may also occur in non-steroidal cells to enhance the cholesterol solubility and to stimulate cholesterol efflux [33,34]. Moreover, oxysterols are physiological ligands of nuclear hormone receptors (liver X receptors), which may play an important role in reverse cholesterol transport [35]. Mitochondrial cholesterol oxidation may therefore represent a defence mechanism for removal of excess cholesterol, and could be affected in TD cells.

It is striking that the structure of (lyso)CL is closely related to the structure of lyso-bis-PA (Fig. 4). Lyso-bis-PA accumulates in fibroblasts from patients with Niemann–Pick type C (NPC) disease, another cholesterol storage disease. Mutations in the NPC1 gene result in accumulation of low density lipoprotein-derived cholesterol in endosomes and lysosomes, which cholesterol cannot be translocated to the plasma membrane [36,37]. The translocation of endogenously synthesized cholesterol to and across the plasma membrane (which is defective in TD) seems to be normal in NPC1-deficient cells [38]. Defective cholesterol storage in late endosomes and lysosomes secondarily leads to a redistribution of membrane proteins and phospholipids. In this context, it was hypothesized that lyso-bis-PA (by means of its unique structure and physicochemical properties) is a key player in the regulation of sorting and trafficking of lipids [39–41]. Based upon our data it is possible that a variety of such regulatory phospholipids may exist. Thus, further characterization of phospholipids in TD and other lipid storage disorders could be helpful for elucidation of sterol trafficking, which process is poorly understood at the molecular level.

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