A mechanism accounting for the low cellular level of linoleic acid in cystic fibrosis and its reversal by DHA

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Abstract Specific fatty acid alterations have been described in the blood and tissues of cystic fibrosis (CF) patients. The principal alterations include decreased levels of linoleic acid (LA) and docosahexaenoic acid (DHA). We investigated the potential mechanisms of these alterations by studying the cellular uptake of LA and DHA, their distribution among lipid classes, and the metabolism of LA in a human bronchial epithelial cell model of CF. CF (antisense) cells demonstrated decreased levels of LA and DHA compared with wild type (WT, sense) cells expressing normal CFTR. Cellular uptake of LA and DHA was higher in CF cells compared with WT cells at 1 h and 4 h. Subsequent incorporation of LA and DHA into most lipid classes and individual phospholipids was also increased in CF cells. The metabolic conversion of LA to n-6 metabolites, including 18:3n-6 and arachidonic acid, was upregulated in CF cells, indicating increased flux through the n-6 pathway. Supplementation of LA and DHA into most lipid classes and its reversal by DHA inhibited the production of LA metabolites and corrected the n-6 fatty acid defect.

In conclusion, the evidence suggests that low LA level in cultured CF cells is due to its increased metabolism, and this increased LA metabolism is corrected by DHA supplementation.

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Supplementary key words arachidonic acid • docosahexaenoic acid • fatty acid alterations • lipid classes • n-6 pathway • phospholipids • supplementation • uptake

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (1). CF is characterized by accumulation of viscous secretions in the respiratory, gastrointestinal, and genitourinary tracts of CF patients, resulting in pancreatic insufficiency, malabsorption, and recurrent pulmonary infections (2).

Specific fatty acid alterations have been described in CF patients. Decreased levels of linoleic acid (LA) and docosahexaenoic acid (DHA) have been the two most consistent alterations. Decreased LA levels were among the earliest fatty acid abnormalities described in CF (3). Several studies reported the presence of low LA levels in different blood components of CF patients (4–7). In addition, decreased LA levels were also found in CFTR-expressing tissues of CF patients, such as nasal tissue (8). Increased LA metabolism through the n-6 pathway was described in CF pancreatic epithelial cells expressing the ΔF508 CFTR and in cultures of pancreatic acini from Δftr−/− mice (9, 10). The other consistent fatty acid alteration (decreased DHA levels) was described in the blood and tissues of CF patients (11–14). Other reported fatty acid abnormalities in CF include increased palmitoleic acid (16:1n-7) and eicosatrienoic acid (20:3n-9) in the plasma of CF patients (6, 13, 15, 16). In addition to these fatty acid alterations, increased arachidonic acid (AA) release from CF lymphocytes and from epithelial cell lines carrying the ΔF508 CFTR mutation has been described (17, 18).

Freedman et al. (19) reported increased levels of phospholipid (PL)-bound AA and decreased levels of PL-bound DHA in the lung, pancreas, and ileum of Δftr−/− mice. Oral administration of DHA to these mice corrected the lipid imbalance and reversed certain pathological manifestations of CF in the pancreas and ileum.

Although the association between specific fatty acid alterations and CF is well established in the scientific literature, the mechanisms of these alterations are poorly understood.
The aims of this study were 1) to study the cellular uptake of LA and DHA and their distribution among lipid classes in a cell culture model expressing the CF phenotype, 2) to investigate the potential mechanisms of decreased LA levels in CF, and 3) to study the effect of DHA supplementation on LA metabolism.

**METHODS**

**Cell culture**

Human bronchial epithelial cells (16HBE cells) were a kind gift from Dr. Pamela Davis (Case Western University, Cleveland, OH). These cells have been treated with a plasmid containing the first 131 nucleotides of CFTR in the sense (S) or antisense (AS) orientations, resulting in a wild type (WT, expresses normal CFTR) or CF (does not express CFTR) phenotype (20). This has been confirmed by measurement of chloride secretion, which demonstrates that 16HBE-AS cells lack AMP-stimulated chloride secretion, whereas 16HBE-S cells show normal chloride secretion (20). 16HBE-AS cells also demonstrate an increased inflammatory response, both at a basal level and following *Pseudomonas aeruginosa* exposure (21).

Cells were grown in minimal essential medium with glutamax (Invitrogen) supplemented with a final concentration of 10% horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO2 and 95% air. Cell culture medium was changed three times/week. Cells were harvested by scraping on ice with a rubber policeman. Lipids were extracted, and fatty acids for 1 week. The medium was changed three times during the week. Cells were then washed with PBS and scraped on ice with a rubber policeman. Lipids were extracted, and fatty acids were methylated and analyzed by GC-MS as described above.

**Fatty acid analysis**

Cells were grown in 6-well plates until confluence, and monolayers were washed three times with cold Ca2+-, Mg2+-free PBS (Invitrogen). Cells were harvested by scraping on ice with a rubber policeman and pelleted by centrifugation at 150 g at 4°C for 8 min. The cell pellet was resuspended in 0.5 ml of cold buffer, and cellular lipids were extracted using the modified method of Folch, Lees, and Sloane Stanley (22) for lipid extraction. In this method, 6 vols of chloroform-methanol (2:1) were used to extract total cellular lipids. The mixture was then incubated for 10 min on ice, vortexed, and centrifuged (600 g for 10 min), and the resulting lower organic phase was transferred to a new tube. An aliquot of 30 μl of 1 mg/ml solution of heptadecanoic acid (17:0, NuCheck Prep) in chloroform-methanol (2:1) was added as an internal standard for fatty acid analysis by GC-MS. Fatty acids in lipid extracts were methylated using boron trifluoride (BF3)-methanol and methanolic-base reagent (23). Lipid extracts were dried under a stream of nitrogen and incubated with 0.5 N-methanolic NaOH (Acros Organics) for 3 min at 100°C, followed by incubation with BF3-methanol reagent (Sigma) for 1 min at 100°C. The resulting fatty acid methyl esters (FAMEs) were extracted with hexane, and the extract was washed by adding 6.5 ml of saturated NaCl solution. FAMEs in the hexane layer were analyzed by GC-MS after concentrating the samples to a final volume of 100 μl under a gentle stream of nitrogen. FAMEs were identified and quantified by GC-MS using a Hewlett-Packard Series II 5890 gas chromatograph equipped with a Supelcowax SP-10 capillary column coupled to an HP-5971 mass spectrometer. The oven temperature was maintained at 150°C for 2 min, ramped at 10°C/min to 200°C and held for 4 min, ramped again at 5°C/min to 240°C and held for 3 min, and then finally ramped to 270°C at 10°C/min and maintained for 5 min. The injector and detector were maintained at 260°C and 280°C, respectively. Carrier gas flow rate was maintained at a constant 0.8 ml/min throughout. Total ion monitoring was performed, encompassing mass ranges from 50–550 amu. Peak identification was based upon comparison of both retention time and mass spectra of the unknown peak to that of known standards within the GC-MS database library. FAME mass was determined by comparing areas of unknown FAMEs to that of the 17:0 internal standard. The response of the GC-MS in terms of assignment of area units to each fatty acid is variable and is influenced by the degree of unsaturation of the fatty acid, the properties of the column selected, and the inherent ability of the GC-MS instrumentation itself. To correct for this variability, response factors were determined for each individual FAME of the GC-MS response based on known mass ratios of the reference standard mixture.

**Fatty acid supplementation**

Aliquots of free LA or free DHA dissolved in chloroform-methanol (2:1) were transferred to 15 ml conical tubes and dried under a stream of nitrogen. After complete drying, culture medium containing 10% horse serum was added to the conical tubes on ice to extract the dried fatty acid from the walls of conical tubes. Tubes were vortexed, and the resulting extract was transferred to a new tube. This step was repeated five times to completely extract the dried fatty acid, and extracts were pooled. Complete culture medium was added to final concentrations of 50 μM of supplemental LA or 10, 20, and 40 μM of supplemental DHA. After filtering the culture media using 0.2 μm filter, 16HBE cells were incubated with the media containing supplemental fatty acids for 1 week. The medium was changed three times during the week. Cells were then washed with PBS and scraped on ice with a rubber policeman. Lipids were extracted, and fatty acids were methylated and analyzed by GC-MS as described above.

**Fatty acid uptake**

Confluent monolayers of 16HBE cells were incubated with complete culture medium containing 0.9 μM [1-14C]LA (PerkinElmer) or 0.9 μM [1-14C]DHA (PerkinElmer) for 1 h and 4 h. After incubation, aliquots of supernatant were removed and centrifuged to remove cell debris. Cells were washed three times with PBS and lysed using 1 N NaOH. Radioactivity of supernatant and cell lysate was quantified using liquid scintillation counting.

**Fatty acid incorporation into lipid classes and individual PLs**

Confluent monolayers of 16HBE cells were incubated with complete medium containing 9.1 μM [1-14C]LA or 9.1 μM [1-14C]DHA for 1 h and 4 h. After incubation, cell monolayers were washed three times with cold PBS and then scraped off the dish with a rubber policeman on ice. Cellular lipids were extracted as described above. Lipid extracts were concentrated to final volumes of 50 μl and spotted on silica gel plates under a gentle stream of nitrogen to prevent oxidation. Lipid classes and individual PLs were separated by TLC as described below, and the radioactivity associated with these fractions was quantified using liquid scintillation counting.

The concentrations of radiolabeled LA and DHA in these extraction studies were 10 times higher than those in the uptake experiment. We chose a much lower fatty acid concentration in the uptake experiment to increase the likelihood of observing a difference in fatty acid uptake that might have been obscured at higher fatty acid concentrations.

**TLC**

TLC separation of lipid classes was carried out using two solvent systems. Solvent 1: diethyl ether-petroleum ether-glacial acetic acid
using Student's t test. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

CF-specific fatty acid alterations in LA and DHA are present in cultured CF cells

WT and CF 16HBE cells were subjected to fatty acid analysis after culturing with medium containing 10% horse serum, which has much higher LA levels than PBS. CF cells demonstrated decreased LA and DHA levels compared with WT cells (Fig. 1).

LA and DHA levels are decreased in major lipid classes of CF cells

One hypothesis is that the low LA and DHA levels in CF cells are due to a selective decrease in one specific lipid compartment. To address this, we studied the fatty acid composition of the main cellular lipid classes, including total PLs, TGs, CEs, and the FFA fraction in 16HBE cells after separating these classes by TLC. LA levels were significantly decreased in the PL (P < 0.05) and TG (P < 0.01) fractions of CF cells (80% and 35% of WT levels, respectively), with no changes seen in the FFA fraction (Fig. 2A). DHA levels were significantly decreased (P < 0.01) in the PL fraction of CF cells (21% of the WT levels) (Fig. 2B). DHA was not detectable in other lipid fractions.

CF cells demonstrate increased uptake of LA and DHA

To investigate whether the decreased levels of LA and DHA in CF cells were due to reduced uptake, the cellular uptake of [1-14C]LA and [1-14C]DHA was studied after incubating 16HBE cells with the corresponding radiolabeled fatty acid for 1 h and 4 h. LA uptake by CF cells was significantly higher (P < 0.01) than the uptake by WT cells at 1 h (4-fold) and 4 h (2.8-fold) (Fig. 3A). Similarly, the uptake of DHA by CF cells was significantly higher (P < 0.01) than the uptake by WT cells at 1 h (2.8-fold) and 4 h (2-fold) (Fig. 3B).

Incorporation of LA and DHA into lipid classes is increased in CF cells

To determine whether the increased uptake of radiolabeled LA and DHA results in a parallel increase in the incorporation of LA and DHA into the main lipid classes in CF cells, the radiolabeled cells described for the uptake studies were subjected to TLC separation of lipid classes, and the radioactivity associated with these classes was quantified. The incorporation of LA and DHA into all lipid fractions was significantly higher (P < 0.01) at 4 h than their incorporation at 1 h (Fig. 4). LA incorporation into the PL, FFA, and CE fractions was significantly increased (P < 0.05) in CF cells compared with WT cells at both 1 h and 4 h (Fig. 4A). Unlike the case with other lipid fractions, LA incorporation into the TG fraction was significantly decreased (P < 0.01) in CF cells compared with WT cells at 1 h, with no change found after 4 h of incubation.

Similar to LA, the incorporation of DHA into the PL and FFA fractions at 4 h and into the CE fraction at both time points was significantly increased (P < 0.05) in CF cells.
cells compared with WT cells (Fig. 4B). Unlike LA, DHA incorporation into the TG fraction of CF cells was increased at the 4 h time point.

The percentage of LA and DHA incorporation within each lipid class was calculated. Because PUFAs are preferably esterified at the sn-2 position of PLs, not unexpectedly the largest portion of newly incorporated LA and DHA was associated with the PL fraction (76–91% of total incorporated LA and 66–72% of total incorporated DHA). The incorporation of LA and DHA was lowest in the CE fraction (0.5–0.9% of total incorporated LA and 0.7–1.8% of total incorporated DHA).

**Esterification of LA and DHA into individual PLs is increased in CF cells**

The increased incorporation of radiolabeled LA and DHA into total PLs in CF cells (Fig. 4) could be due to increased esterification into one specific PL class. The esterification of LA and DHA into individual PLs was analyzed by incubating 16HBE cells with [1-14C]LA or [1-14C]DHA for 1 h and 4 h, followed by separation of the main PLs (PC, PE, PI, and PS) by TLC and quantification of the radioactivity associated with each individual PL.

The esterification of newly incorporated LA into the PC fraction was greater in CF cells than in WT cells at 1 h and 4 h (Fig. 5A). LA esterification was also increased in all other PLs (PE, PI, and PS) of CF cells at 4 h. LA was preferably esterified into PC (76–77% and 58–61% of total PL-bound LA at 1 h and 4 h, respectively). PE was the second-preferred esterification site for LA (14–16% and 24–25% of total PL-bound LA at 1 h and 4 h, respectively).

DHA esterification into the PE and PI fractions at 1 h and 4 h and into the PC fraction at 1 h was increased in CF cells compared with WT cells (Fig. 5B). PE was the preferred PL for DHA esterification (61–63% and 61–70% of total PL-bound DHA at 1 h and 4 h, respectively), followed by PC (24–26% and 18–27% of total PL-bound DHA at 1 h and 4 h, respectively). As with LA, PE was the least-preferred PL for DHA esterification (2–3% and 3–4% of total PL-bound DHA at 1 h and 4 h, respectively).

**DHA supplementation inhibits LA metabolism through the n-6 pathway**

Decreased LA levels in CF cells despite its increased uptake and esterification suggests that there is increased metabolism of this fatty acid. Increased LA metabolism through the n-6 pathway was previously reported in cultured CF cells and in cftr−/− mice (9, 10). In another study, supplementing cftr−/− mice with DHA corrected the fatty acid abnormalities and reversed CF pathology in certain organs (19). We investigated whether decreased LA levels in CF cells are a result of increased LA metabolism, and whether DHA supplementation has an effect on this metabolism.

16HBE cells were supplemented for 1 week with either 50 μM LA alone, or with 50 μM LA plus 20 μM DHA. LA
supplementation caused a significant increase in AA levels in CF cells compared with WT cells, indicating increased metabolic conversion of LA to AA (Fig. 6). DHA supplementation blunted this response, resulting in a significant decrease in AA levels in both CF and WT cells compared with LA supplementation alone.

To further examine the inhibitory effect of DHA on LA metabolism, 16HBE cells were supplemented with DHA for 24 h, and then incubated with [1-14C]LA for 4 h. In the absence of DHA supplementation, there was increased production of radiolabeled LA metabolites, including 18:3n-6 (2.7-fold) and AA (1.9-fold) in CF cells compared with WT cells (Fig. 7). DHA supplementation downregulated LA conversion to 18:3n-6 in CF cells, a step that is mediated by the enzyme Δ5-desaturase. In addition, DHA supplementation inhibited the formation of AA from 20:3n-6 in both WT and CF cells, with much greater effect in CF cells. This conversion is mediated by the enzyme Δ5-desaturase.

To determine the effect of DHA supplementation on endogenous steady-state levels of LA and DHA, 16HBE cells were supplemented with 10, 20, or 40 μM DHA for 1 week. This resulted in a significant increase in LA levels in CF cells at all DHA doses used, with values approaching WT levels (Fig. 8A). On the other hand, DHA supplementation did not cause significant changes in LA levels in WT cells. Although DHA supplementation resulted in a large increase in DHA levels in both CF and WT cells, there was still a persistent difference in DHA levels between CF and WT cells (Fig. 8B).

**Fig. 4.** Incorporation of LA (A) and DHA (B) into lipid fractions of 16HBE cells. 16HBE cells were incubated with 9.1 μM [1-14C]LA or 9.1 μM [1-14C]DHA for 1 h and 4 h. Lipid fractions were separated by TLC, and fatty acid incorporation was determined by quantifying the radioactivity associated with the fractions. Data represent mean ± SEM (n = 3). * P < 0.05, ** P < 0.01. CE, cholesteryl ester.
DISCUSSION

These studies address the mechanisms by which LA is decreased in CF, and the effect of DHA on LA metabolism. To this end, we examined the cellular uptake of LA and DHA, their incorporation into lipid classes, and the metabolism of LA in cultured airway cells expressing the CF phenotype. Because DHA could reverse certain CF pathological manifestations in a mouse model (19), the effect of DHA supplementation on these mechanisms was also examined. We have demonstrated that there is increased uptake and esterification of LA and DHA in cultured CF cells. We have also shown that there is increased metabolic conversion of LA to downstream fatty acids, in particular AA, and that DHA corrects this fatty acid defect by inhibiting LA metabolism to more elongated and desaturated fatty acids.

In CF cells, the levels of LA and DHA are decreased when cultured in horse serum. The presence of these CF-specific fatty acid alterations in cultured cells expressing the CF phenotype indicates an association between the fatty acid abnormality and the CF basic defect. Fatty acid analysis of lipid fractions demonstrates that the deficiency of LA in CF cells is related to decreased levels in the PL and TG fractions, whereas DHA deficiency is related to its decreased content in the PL fraction, which is the preferred lipid compartment for esterification of PUFAs.

The observed decrease in LA and DHA levels within CF cells might be explained by decreased cellular uptake by cells. However, the uptake of LA and DHA by CF cells was actually higher than that by WT cells. The results from the lipid compartmentation study are in agreement with the uptake data showing increased incorporation of LA.
and DHA into most lipid fractions in CF cells, the highest being in total PLs. The increased incorporation of LA and DHA into the total PL fraction is a result of increased esterification into the main individual PLs (PC, PE, and PI). The largest portion of newly incorporated LA was esterified into PC, whereas the newly incorporated DHA was mostly esterified into PE.

Bhura-Bandali et al. (25) reported decreased incorporation of radiolabeled LA into the PL fraction and increased incorporation into the triglyceride fraction of a CF-phenotypic cell line compared with cells expressing normal CFTR. The discrepancy between the results of this study and our results could be due to the use of different sera in the cell culture medium. Bhura-Bandali et al. grew the cells in medium containing FBS, which has a much lower content of LA compared with horse serum. This low LA content in the cells that occurs under these growth conditions could have affected the distribution of the newly incorporated LA among different lipid compartments.

The decreased levels of LA in most lipid fractions of CF cells despite its increased incorporation into these fractions suggests that low LA levels are the result of increased metabolism of this particular fatty acid. Increased LA metabolism in CF cells is supported by our data, which demonstrate increased conversion of LA to 18:3n-6 and AA in CF cells. The production of the intermediate fatty acid (20:3n-6) was not different between WT and CF cells. This might be related to a very rapid conversion of this fatty acid to AA through the activity of the enzyme Δ5 desaturase before it accumulates in CF cells. Increased metabolism of LA in CF was demonstrated in a previous study showing increased metabolism of radiolabeled LA to AA in a human pancreatic epithelial cell line carrying the ΔF508 mutation compared with cells expressing the normal CFTR protein (25). Another study reported the presence of enhanced flux through the n-6 pathway in primary cultures of pancreatic acini from cfr<sup>-/-</sup> mice compared with cultures from WT mice (10). Increased LA metabolism is likely to be due to increased activity of the enzymes Δ6 desaturase and Δ5 desaturase, which mediate the formation of 18:3n-6 and AA, respectively. Increased LA metabolism may partially explain the presence of low LA.
levels in CF patients. AA is the substrate for biosynthesis of inflammatory mediators, and therefore its increased production from LA may have great importance in the hyper-inflammatory process in CF.

Supplementing 16HBE cells with different doses of DHA for 1 week resulted in a significant increase in LA levels in CF cells, indicating that this is the result of inhibition of the metabolism of LA through the n-6 pathway by DHA. Because LA levels did not change in WT cells after DHA supplementation, this would suggest that DHA supplementation corrects a defect specific to the loss of CFTR function. When supplemental DHA was added in combination with LA, DHA inhibited the LA-derived increase in AA levels in CF cells and normalized them to WT values. The effect of DHA supplementation to inhibit 18:3n-6 and AA production from [1,14C]LA further supports the role of DHA to correct the defective fatty acid metabolism within the n-6 pathway. This inhibitory effect of DHA was greater on AA production, and it was stronger in CF than in WT cells. These findings are consistent with the reported downregulatory effect of DHA on Δ-5 (which mediates the formation of AA) and Δ-6 (which mediates the formation of 18:3n-6) desaturase enzymes (26). DHA inhibition of the desaturase enzymes has been shown to take place at the transcripational level (27).

Supplementing 16HBE cells with relatively high levels of DHA did not equalize DHA levels in WT and CF cells. These findings, combined with the fact that DHA level is decreased in CF cells. Recent studies have shown that DHA is the end product of the n-3 pathway, one other mechanism of decreased DHA levels in CF cells. Because DHA is the end product of the n-3 pathway. This inhibitory effect of DHA was greater on AA production, and it was stronger in CF than in WT cells. These findings are consistent with the reported downregulatory effect of DHA on Δ-5 (which mediates the formation of AA) and Δ-6 (which mediates the formation of 18:3n-6) desaturase enzymes (26). DHA inhibition of the desaturase enzymes has been shown to take place at the transcripational level (27).

Correction of the n-6 pathway defect by DHA makes it a potential candidate to correct the defective fatty acid metabolism in CF cells.


