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. Supplementing CF cells with 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.—Al-Turkmani, M. R., C. Andersson, R. Alturkmani, W. Katrangi, J. E. Cluette-Brown, S. D. Freedman, and M. Laposata. A mechanism accounting for the low cellular level of linoleic acid in cystic fibrosis and its reversal by DHA. J. Lipid Res. 2008. 49: 1946–1954.

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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 cftr−/− 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 cftr−/− 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.

Abbreviations: AA, arachidonic acid; CE, cholesteryl ester; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DHA, docosahexaenoic acid; FAME, fatty acid methyl ester; LA, linoleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; TG, triglyceride; WT, wild type.

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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 eflux (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. Cell culture flasks were precoated with LHC basal medium (BioSource) containing a mixture of 10 μg/ml human fibronectin (Sigma), 3 μg/ml vitamin (Angiotech), and 0.1 mg/ml BSA (Sigma).

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 150,000 × 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 and analyzed by GC-MS as described above. The fatty acid methyl esters (FAMEs) were ex-tracted 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 50 μl. Fatty acids were separated by TLC as described below, and the radioactivity associated with each fatty acid was measured by liquid scintillation counting.

Fatty acid incorporation into lipid classes and individual PLs

Confluent monolayers of 16HBE cells were incubated with complete 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.

TLC

TLC separation of lipid classes was carried out using two solvent systems. Solvent 1: diethyl ether-petroleum ether-glacial acetic acid
(65:35:0.5; v/v/v); solvent 2; diethyl ether-petroleum ether (6:94; v/v). Plates were run in solvent 1, dried in a ventilated hood, and subjected to another run in the same direction in solvent 2. PL, triglyceride (TG), cholesteryl ester (CE), and FFA standards were run in parallel at the sides of the plate. After completing the run, the standard spots were detected using iodine vapor after covering the sample spots to prevent oxidation. Sample spots that corresponded to lipid classes were scraped directly into conical tubes for further analysis.

The TLC separation of the main PL classes, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), was carried out using the method described by Miwa et al. (24). This method uses one solvent system consisting of methyl acetate-1-propanol-chloroform-methanol-0.25% potassium chloride (25:35:20:10:10; v/v/v/v/v). Spots that corresponded to individual PLs were determined using standards running in parallel. Sample spots that corresponded to PLs were scraped directly into conical tubes for further analysis.

**HPLC**

Confluent monolayers of 16HBE cells were incubated with complete medium containing 3.5 μM [1-14C]LA. Lipids were extracted, and fatty acids were methylated as described above. After methylation, the samples were dried under nitrogen and dissolved in 100 μl acetonitrile. Forty microliters was injected into an HPLC instrument (Waters), and fatty acids were separated using a binary solvent system. Solvent A consisted of double-distilled H2O with 0.02% H2PO4, and solvent B was 100% acetonitrile (HPLC grade). The solvent program started with 76% solvent B and 24% solvent A for 0.5 min, followed by a linear gradient from 76% to 86% solvent B over 10 min, a hold for 20 min, another linear gradient from 86% to 100% solvent B over 2 min, and a hold for 18 min, followed by reconstitution of the original conditions. The peaks were identified by ultraviolet detection at 205 nm through comparison with retention times of unlabeled standards. Quantification of the peaks was performed by a scintillation detector coupled to the HPLC instrument.

**Statistical analysis**

Statistical differences between the test groups were evaluated 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. 4A). 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 difference 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 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). PS was the least-preferred esterification site for LA (2–3% of total PL-bound LA at 1 h and 4 h).

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). No differences were found in DHA incorporation into PS fraction between WT and CF cells. 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, PS 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).
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 cdfr−/− 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 cells.

*Fig. 6.* Effect of supplementation with either LA alone or LA and DHA on arachidonic acid (AA) levels in 16HBE cells. 16HBE cells cultured with horse serum were supplemented with either 50 μM LA alone, or with 50 μM LA plus 20 μM DHA for 1 week. AA levels were determined by GC-MS. Data represent mean ± SEM (n = 6 from two experiments). **P < 0.01.

*Fig. 7.* Effect of DHA supplementation on LA metabolism through the n-6 pathway in 16HBE cells. 16HBE cells were supplemented with 10 μM DHA for 24 h and then incubated with 3.5 μM [1-14C]LA for 4 h. Production of 18:3n-6, 20:3n-6, and AA was determined by HPLC. Data represent mean ± SEM (n = 3). **P < 0.01 vs. CF cells supplemented with DHA (for 18:3n-6) or vs. CF and WT cells supplemented with DHA (for AA).

*Fig. 8.* Effect of DHA supplementation on LA (A) and DHA (B) levels in 16HBE cells. 16HBE cells cultured with horse serum were supplemented with 0, 10, 20, or 40 μM DHA for 1 week. Levels of LA and DHA were determined by GC-MS. Data represent mean ± SEM (n = 3). *P < 0.01 vs. WT and CF cells with no DHA supplement.
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 transcriptional 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 low in CF cells despite its increased uptake and esterification, suggest that there might be an increased metabolism of DHA in CF cells. Recent studies have shown that DHA is metabolized to potent anti-inflammatory mediators that act locally and include D-series resolvins, docosatrienes, and neuroprotectins (28, 29). Because DHA is the end product of the n-3 pathway, one other mechanism of decreased DHA levels in CF cells might be related to its decreased synthesis from precursor n-3 fatty acids.

Correction of the n-6 pathway defect by DHA makes it a potential candidate to decrease the chronic inflammatory response present in CF patients by downregulating the production of AA-derived inflammatory eicosanoids. Our findings are supported by a mouse study in which oral administration of DHA to cftr−/− mice for 1 week caused a decrease in AA levels and an increase in DHA levels in membrane PLs to levels similar to those found in control mice expressing normal CFTR (19). This correction of the fatty acid defect in the CFTR-regulated organs (lung, pancreas, and ileum) was associated with amelioration of the CF pathology in these organs.

In conclusion, low cellular levels of LA and DHA in CF cells are associated with increased uptake and esterification of these fatty acids. In addition, supplementing CF cells with DHA corrects the upregulated LA metabolism. Therefore, aggressive dietary supplementation with DHA, which is what was employed in mouse model studies, is likely to be necessary to normalize cellular DHA and LA levels in humans with CF. On the other hand, aggressive supplementation with LA may normalize LA levels but lead to an increased inflammatory response as a result of its extensive metabolism to arachidonate and inflammatory eicosanoids.

REFERENCES


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