Altered cholesterol homeostasis in cultured and in vivo models of cystic fibrosis

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White NM, Jiang D, Burgess JD, Bederman IR, Previs SF, Kelley TJ. Altered cholesterol homeostasis in cultured and in vivo models of cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 292: L476–L486, 2007. First published November 3, 2006; doi:10.1152/ajplung.00262.2006.—Determining how the regulation of cellular processes is impacted in cystic fibrosis (CF) is fundamental to understanding disease pathology and to identifying new therapeutic targets. In this study, unesterified cholesterol accumulation is observed in lung and trachea sections obtained from CF patients compared with non-CF tissues, suggesting an inherent flaw in cholesterol processing. An alternate staining method utilizing a fluorescent cholesterol probe also indicates improper lysosomal storage of cholesterol in CF cells. Excess cholesterol is also manifested by a significant increase in plasma membrane cholesterol content in both cultured CF cells and in nasal tissue excised from cftr−/− mice. Impaired intracellular cholesterol movement is predicted to stimulate cholesterol synthesis, a hypothesis supported by the observation of increased de novo cholesterol synthesis in lung and liver of cftr−/− mice compared with controls. Furthermore, pharmacological inhibition of cholesterol transport is sufficient to cause CF-like elevation in cytokine production in wild-type cells in response to bacterial challenge but has no effect in CF cells. These data demonstrate via multiple methods in both cultured and in vivo models that cellular cholesterol homeostasis is inherently altered in CF. This perturbation of cholesterol homeostasis represents a potentially important process in CF pathogenesis.

Cystic fibrosis (CF) is caused by the loss of function of the cAMP-dependent chloride channel cystic fibrosis transmembrane conductance regulator (CFTR). However, it is not clearly understood what mechanisms lead to aggressive inflammatory signaling in CF. Previous work demonstrated that cultured models of CF epithelial cells exhibited intracellular accumulation of unesterified cholesterol in a manner similar to what is observed in cells from patients with Niemann-Pick type C (NPC) disease (43). NPC is a disease of impaired intracellular cholesterol transport resulting in free cholesterol accumulation in late endosomes and lysosomes (15, 18). It was determined that NPC fibroblasts share a number of cell signaling alterations previously identified in CF cells, including reduced inducible nitric oxide synthase (NOS2) expression, increased RhoA and signal transducer and activator of transcription-1 (STAT1) protein expression, and reduced SMAD3 protein expression (43). These data suggest that improper cholesterol processing is a trigger for altered inflammatory signaling responses in CF cells. Further support for the importance of cholesterol-related pathways in CF cell signaling regulation in CF cells has been demonstrated previously by the impact of isoprenoid/cholesterol synthesis on these signaling cascades. Treatment with the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor mevastatin resulted in the correction of STAT1 and NOS2 signaling in both cultured cell models and mouse models of CF (20, 22, 23). Similarly, inhibition of isoprenoid transferase activity resulted in correction of SMAD3 expression and transforming growth factor-β1 (TGF-β1) signaling in CF cells (26).

The above findings suggest a relationship between cholesterol accumulation and the isoprenoid/cholesterol synthesis pathway in CF. NPC cells have been shown to exhibit increased cholesterol synthesis despite increased intracellular storage, likely due to a lack of cholesterol transport to the endoplasmic reticulum (ER) (31). The goal of this study is to further investigate cholesterol processing in CF models as a potential mechanism for alterations in CF inflammatory pathways. The hypothesis of this study is that a loss of CFTR function leads to altered cholesterol trafficking, resulting in increased cholesterol synthesis. This perturbation in cholesterol regulation is proposed to contribute to the inflammatory response present in CF.

The importance of lipid regulation in CF inflammatory responses has been explored previously. Kowalski and Pier (21) have reported that plasma membrane cholesterol is essential for CFTR localization and for proper responses to Pseudomonas aeruginosa (PA). Similarly, Grassme et al. (16) have demonstrated the importance of ceramide-rich signaling platforms to internalize PA using a mouse model of Niemann-Pick type A disease. Another component of an anti-inflammatory pathway related to cholesterol homeostasis reported to be deficient in CF models is the peroxisome proliferation-activated receptor-γ (PPARγ) (33). PPARγ has been demonstrated to be deficient in NPC cells as well, suggesting a similar regulatory relationship in CF cells (39). Each of these studies supports the hypothesis that alterations in cholesterol processing and internal trafficking would have important consequences on inflammation and on bacterial response at the plasma membrane in CF.

The cholesterol pathway has also been implicated in the regulation of CFTR trafficking to the apical membrane. Shen et al. (41) demonstrated that treatment with the HMG-CoA reductase inhibitor lovastatin reduced CFTR-mediated chloride transport.

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transport and CFTR trafficking to the apical membrane. Cheng et al. (6) have also recently shown that lovastatin inhibits CFTR trafficking by inhibition of the Rho family small GTPase TC10. These studies indirectly raise the possibility that observed alterations in cholesterol processing in CF cells may be an adaptive response by cells to increase CFTR content at the plasma membrane.

The current study details multiple anomalies in cholesterol-related regulation in both cultured cell models and in primary tissue of CF origin, including intracellular cholesterol accumulation, increased de novo cholesterol synthesis, and elevated plasma membrane cholesterol content. It is concluded that CF epithelial cells possess an inherent flaw in cholesterol regulation due to the loss of CFTR activity or expression. These data demonstrate in primary tissue and in multiple model systems that aberrations in cholesterol homeostasis are a CF-related phenotype that potentially influences a number of relevant cell signaling events. The control of both cholesterol synthesis and processing represents new avenues for therapeutic development for CF.

METHODS

Cell culture. IB3–1 cells, human epithelial with the delta F508 mutation (CF phenotype), and S9 cells, IB3–1 cells stably transfected with the full-length wild-type (WT) CFTR (control), were a generous gift from Pamela L. Zeitlin (Johns Hopkins University, Baltimore, MD). These cells were grown at 37°C in 95% O2-5% CO2 on Falcon 10-cm-diameter tissue culture dishes in LHC-8 Basal Medium (Biofluids, Cambridge, CA) with 5% FBS. The human alveolar type II epithelial adenocarcinoma cell line (A549) was grown under the above conditions in Ham's F-12 Kagn's Modification (Biofluids, Rockville, MD) with 10% FBS. Human epithelium 9/HTeO– cells overexpressing the CFTR R domain (pCEPR) and mock-transfected 9/HTeO– cells (pECP2), the WT phenotype, were a generous gift from the lab of Dr. Pamela B. Davis (Case Western Reserve University). Cells were cared for as previously described (34).

Mice. Mice lacking CFTR expression (CFTRtm1Unc) were obtained from Jackson Laboratories (Bar Harbor, MA). CFTR WT mice were siblings of cftr–/– mice. All mice were used between 6 and 8 wk of age and were backcrossed over 10 generations onto a C57Bl/6 background. CF mice were fed a liquid diet as described by Eckman et al. (10). Mice were cared for in accordance with the Case Western Reserve University Institutional Animal Care and Use Committee guidelines by the CF Animal Core Facility. Nasal scrapings of mouse epithelium were obtained from both WT and CF animals. The Animal Care and Use Committee of Case Western Reserve University approved all animal procedures.

NBD-cholesterol staining. Cells were seated at a density of 150–200,000 cells/well on Fisherbrand coverslips. Fifty micrograms per milliliter of NBD-cholesterol or 25-N-[7-nitrobenz-2-oxa-1,3-diazol-4-y]-27-norcholesterol (Molecular Probes, Eugene, OR) was added for ~24 h. Cells were then incubated in fresh media for another 4 h. Cells were fixed in 2% paraformaldehyde for 30 min and then rinsed in phosphate-buffered saline (PBS) three times before being mounted using SlowFade Light antifade (Molecular Probes). Confocal images were taken using a Leica DMIRE2 confocal microscope (Leica Imaging Systems, Mannheim, Germany) using the HCX PL AP × 63 1.4 oil objective. Images are representatives of average pictures taken of the z-stacks. For experiments with gly-phe-

Flow cytometry. Approximately 300,000 cells were plated in six-well dishes and treated as described with NBD-cholesterol; the cells were allowed to sit in fresh media overnight. Cells were trypsinized, rinsed once in PBS, and placed in fresh PBS for analysis. The EPICS-XL-MCL (Beckman Coulter, Miami, FL) has a 488 air-cooled argon ion laser at 15 mW. A 525-nm band pass filter was used to collect 200,000 events per sample.

Filipin staining. Cells were treated as previously described by Kruth et al. (24). Briefly, cells were grown to 75–90% confluency on Fisherbrand coverslips. Cells were rinsed three times with PBS and then fixed with 2% paraformaldehyde for ~30 min. Cells were rinsed once more with PBS and then incubated with 0.05 mg/ml filipin (Sigma-Aldrich) in PBS for 1 h on a shaker in the dark. Filipin dissolved fresh in dimethylformamide before each experiment. Cells were rinsed in PBS before mounting using SlowFade Light antifade (Molecular Probes) on slides. Cells were visualized in the ultraviolet range using a wide-field microscope on a Zeiss Axiosvert 200 and Metamorph software. A ×63 objective was used for all images. Cells were treated for 1 h with GPN at a final concentration of 50 μM or DHA for 24 h at a final concentration of 10 μM.

Tissue stains were fixed in 2% paraformaldehyde and placed in paraffin blocks. Tissue was sectioned at 5 μm. These sections were stained with filipin as described above. Confocal images were taken using a Leica DMIRE2 confocal microscope (Leica Imaging Systems) using the HCX PL AP × 100 oil objective. Staining was done with one CF trachea and lung and one control tissue.

Measuring cholesterol synthesis in vivo. CFTRtm1Unc mice and the matched controls were given an intraperitoneal injection (ip) (~24 μl/g body wt) of deuterated saline (9 g NaCl in 1,000 ml of 99% 2H2O; Sigma-Aldrich). After 8 h, mice were killed using carbon dioxide. Blood was taken from the heart and plasma collected. Whole lungs and ~1.0 g of liver and small intestine were collected. Tissue samples were hydrolyzed in 1 N KOH-70% ethanol (vol/vol) for 2 h at 70°C, vortexing occasionally. Samples were then evaporated to dryness, redissolved in 2 ml of water, and acidified using 12 N HCl. Cholesterol was extracted twice by addition of ethyl ether (3 ml). The pooled ether extracts were evaporated to dryness under nitrogen and then converted to the trimethylsilyl cholesterol derivatives by reacting with 60 μl of bis(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (Regis, Morton Grove, IL) (TMS) at 60°C for 20 min. The 2H-labeling of cholesterol was determined using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system. The cholesterol was run on a DB17-MS capillary column (30 m × 0.25 mm × 0.25 μm). The oven temperature was initially held for 1 min at 150°C and then increased by 20°C/min to 310°C and maintained for 8 min. The split ratio was 20:1 with a helium flow of 1 ml/min. The inlet temperature was set at 270°C and MS transfer line was set at 310°C. Under these conditions, cholesterol elutes at ~11.1 min. Electron impact ionization was used in all analyses with selected ion monitoring of m/z 368–372 (M0-M4, cholesterol), dwell time of 10 ms/scan.

The 2H-labeling of mice plasma water was determined by exchange with water as described by McCabe et al. (30). Briefly, plasma was diluted twofold with distilled water and reacted with 2 μl of 10 N NaOH and 4 μl of a 5% (vol/vol) solution of acetone in acetonitrile for 24 h. Acetone was extracted by addition of 600 μl of chlorofom followed by addition of 0.5 g of Na2SO4. Samples were vigorously mixed, and a small aliquot of the chlorofrom was transferred to a GC-MS vial. Acetone was analyzed using the Agilent equipment described above. The oven temperature program was as follows: 60°C initial, increase by 20°C/min to 100°C, increase by 5°C/min to 220°C and maintain for 1 min. The split ratio was 40:1 with a helium flow of 1 ml/min. The inlet temperature was set at 230°C, and the mass spectrometer transfer line was set at 245°C. Acetone eluted at ~1.5 min. The mass spectrometer was operated in the electron impact mode (70 eV). Selective ion monitoring of m/z 58 and 59 was performed using a dwell time of 10 ms/scan.
Calculation of cholesterol synthesis. Following correction for natural enrichment (11), rates of de novo cholesterol synthesis were calculated using the following formula: total labeling \[\frac{M_1}{H_0H_0} + \frac{(M_2 \times 2)}{H_0H_0} + \frac{(M_3 \times 3)}{H_0H_0} + \frac{(M_4 \times 4)}{nH}\] labeling of plasma water \(n\) time, where \(M_i\) represents isotope-labeled isomeric species of cholesterol (\(M_1\) being singly labeled, \(M_2\) doubly labeled, etc.) (5) and “\(n\)” represents the number of exchangeable hydrogens, assumed to be 25 for cholesterol (27).

Electrochemical measurements of cholesterol. Platinum microelectrodes were fabricated in-house (11.5- and 100-μm-diameter wire, Goodfellow) as described (9). Platinum wire was inserted into glass capillaries (Kimax-51, Kimble Products) and placed inside a heated platinum coil. The glass was pulled to create a thin insulating layer on the platinum wire. The capillary microelectrodes were polished using a beveling machine (WPI) to produce a disk electrode. The microelectrodes were immediately immersed in a 5 mM hexane solution of 11-mercaptoundecanoic acid (95%, Aldrich) for 2 h to form a carboxylic acid-terminated monolayer on the electrode surface. Then, the microelectrodes were treated with 2 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma) in 100 mM PBS solution (pH 7.4) for 30 min to activate the carboxyl groups to an acylisourea intermediate. The modified electrode was immersed in 1 mg/ml recombinant cholesterol oxidase (Oriental Yeast, 42.0 units/mg) solution for 3 h, allowing this intermediate to react with amine, immobilizing the enzyme on the electrode surface.

Data acquisitions. Amperometric measurements were conducted using a two-electrode cell and a voltammeter-amperometer (ChemClamp, Dagan). The three-pole bessel filter in the voltammeter-amperometer was set to 100 Hz. The output was further processed using a noise-rejecting voltmeter (model 7310 DSP, Signal Recovery) to digitally filter 60-Hz noise. An Ag/AgCl (1 M KCl) reference electrode was used for all experiments, and the applied potential was 780 mV vs. Na+/H+ exchanger (NHE) for all experiments. All experiments were performed in 100 mM phosphate buffer (pH 7.4) at 36°C.

Single cells and excised tissue were captured by a capillary prepared in-house using an IM-6 microinjector (Narishige International). The electrode was initially positioned ~50 mm from the cell surface or tissue inner edge for acquisition of baseline data. The electrode was repositioned for contacting the biological sample and acquisition of electrode response.

Cytokine measurements. HE-9 pCEP and pCEPR were plated in 24-well plates at a density of 500,000 cells/well. These cells were treated for 24 h with 5 μg/ml U18666a, a cholesterol transport inhibitor. After being placed in serum-free media overnight, some of the cells were challenged with 1 × 10⁹ colony-forming units (CFU) of PA in the presence and absence of U18666a or DHA. After 1 h, cells were sterilized with 50 μg/ml gentamicin. The supernatants were collected 18 h later and assayed for IL-6 and IL-8 production. Cytokine levels were assayed by the Cell Mediator core facility using immunoreagents obtained from R&D Systems.

Western immunoblotting. Antibodies against NOS2 (mouse) were obtained from BD Transduction Laboratories (Billerica, MA). Antibody against actin (rabbit) was obtained from Sigma-Aldrich. Antibody against SMAD3 was obtained from Santa Crux Biotechnologies (Santa Cruz, CA). Protein samples were prepared with 60-mm dishes of cultured cells in ice-cold lysis buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 200 μM Na₂VO₄, and 10 μg/ml pepstatin and leupeptin) for 30 min at 4°C while shaking. Cell lysates were microcentrifuged at 4°C at 14,000 rpm for 10 min.

Fig. 1. Unesterified cholesterol accumulation in cystic fibrosis (CF) tissue. A: filipin staining of non-CF and CF trachea epithelium tissue. B: filipin staining of non-CF and CF of epithelium from upper airway tissue. At right of each filipin stain is a transmitted image to indicate tissue structure. Images are representative of multiple sections of each sample. Trachea and lung tissue is from separate individuals. Bar = 30 μm.
Proteins were separated using SDS-PAGE containing 20–40 μg of protein in 6–12% acrylamide gel. The samples were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) at 15 V for 30 min. The blots were blocked overnight in PBS (138 mM NaCl, 15 mM Na₂HPO₄, 1.5 mM KCl, and 2.5 mM KH₂PO₄) containing 5% nonfat dehydrated milk and 0.1% Tween-20 at 4°C. Blots were incubated overnight for NOS2 (1:500 dilution) in PBS containing 5% nonfat dehydrated milk and 0.1% Tween-20. Blots were washed three times in PBS and incubated in secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature (dilution 1:4,000, Sigma). Blots were washed again in PBS before visualizing, using Super Signal chemiluminescent substrate (Pierce, Rockford, IL), and exposing the membrane to Kodak scientific imaging film (Kodak, Rochester, NY). Quantification of protein expression was accomplished by densitometry software on the VersaDoc (Quality One; Bio-Rad, Hercules, CA).

Transfections. The human sterol response element (SRE)-luciferase reporter construct (SRE-luc) was provided by Dr. Timothy Osborne (University of California at Irvine). The SRE-luc construct consists of the SRE region of the HMG-CoA reductase promoter. Cells were seeded at a density of 50,000 cells/well in 24-well tissue culture dishes 24 h before transfection. For each transfection, 0.6 μl of FuGene 6 (Roche, Indianapolis, IN) was incubated for 5 min in 100 μl of OptiMEM (Gibco BRL, Gaithersburg, MD). Then, 0.03 μg of DNA and 0.008 μg of pRL-TK were added to the FuGene-OptiMEM mix and incubated for another 15 min. One hundred microliters of diluted transfection mix were added to each well, and cells were incubated at 37°C in 95% O₂-5% CO₂ for 24 h. To address the role of de novo cholesterol synthesis, cells were also examined under serum-free conditions. Cells were cultured without serum for 24 h to eliminate exogenous cholesterol influences on SRE regulation. Cells were also treated with 10 μM DHA for 24 h. Cells were then lysed in 1 × Passive Lysis Buffer (Promega, Madison, WI) at room temperature for 15 min and assayed for luciferase activity according to manufacturer’s instructions (Promega). Results are expressed in relative light units (RLU) and normalized to Renilla luciferase activity.

RESULTS

In vivo accumulation of unesterified cholesterol. Previous observations of unesterified cholesterol accumulation in CF were limited to two cultured cell models (43). To determine whether this phenotype is conserved in vivo and relevant to human disease, cholesterol content in sections from CF and non-CF trachea and upper lung airways was determined by filipin staining. Filipin is a protein from the bacteria Streptomyces filipinensis that binds to unesterified cholesterol, becoming detectable in the ultraviolet range. Compared with control tissue, CF trachea exhibited increased intracellular cholesterol content and more intense staining (Fig. 1A). The same pattern of accumulation observed in the CF trachea was
also present in the epithelium of the upper airway of the lung (Fig. 1B). These data support previous findings of increased intracellular unesterified cholesterol content in cultured CF models and demonstrate that primary tissue of CF origin exhibits the aberrant cholesterol transport phenotype.

**NBD-cholesterol accumulation in CF cell models.** Filipin staining demonstrates accumulation of unesterified cholesterol in cell and tissue models of CF. To determine whether these findings were due to a disruption of transport or simply due to deficient reesterification, the transport of a fluorescently labeled cholesterol analog was examined. With the use of a fluorescent cholesterol probe, 25-[N-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino]-27-norcholesterol (NBD-cholesterol), cholesterol trafficking in cultured cell models was measured. After ~24 h of incubation with NBD-cholesterol, cells were placed in fresh media for 4 h before being fixed. Confocal images demonstrate a clear accumulation of NBD-cholesterol in two different cultured CF cell models (9/HTEo−/pCEPR and IB3) compared with respective controls (Fig. 2A). Quantification of NBD-cholesterol accumulation was accomplished using flow cytometry analysis. Cells were treated similarly as before but allowed to process cholesterol in fresh media for longer periods of time (approximately overnight). There was a significant increase in mean fluorescence present in both CF-like cells; 9/HTEo− pCEPR had a 1.4 ± 0.2-fold (P = 0.003) increase compared with 9/HTEo−/pCEP controls, and IB3 cells had a 2.0 ± 0.2-fold (P = 0.005) increase compared with S9 controls (Fig. 2B). These data confirm by separate technique the observation of cholesterol accumulation in CF cells initially determined by filipin staining and suggest that the observation is due to a flaw in lipid transport mechanisms.

On the basis of the increased content of free cholesterol in CF cells and tissues, it is postulated that cholesterol is accumulating in late endosomes and lysosomes, similar to NPC. To determine whether the cholesterol accumulation phenotype could be reversed, 9/HTEo− pCEPR (CF phenotype) cells were treated with GPN, a cathepsin C substrate that causes lysosomal disruption (3). The pattern of cholesterol accumulation was examined by visualization of NBD-cholesterol. 9/HTEo− pCEPR cells were treated with 50 μM GPN and 5 μg/ml NBD-cholesterol for ~24 h. GPN treatment reduces cholesterol accumulation in pCEPR to near control (pCEP) levels (Fig. 3A). NBD-cholesterol content was objectively determined by flow cytometry analysis. Flow cytometry analysis reveals a significant decrease in NBD-cholesterol fluorescence in GPN-treated cells compared with untreated 9/HTEo− pCEPR cells (Fig. 3B). GPN treatment significantly reduces mean fluorescence in pCEPR (CF phenotype) cells ~34% to control (pCEP) levels. These data suggest that cholesterol is at least in part being accumulated in lysosomal compartments. The use of NBD-cholesterol for trafficking studies must be viewed with some caution, as the hydrophobic NBD moiety can potentially interact with the membrane itself and interfere with processing. However, consistency and quantification balanced these concerns for this study.
Increased cholesterol content in the plasma membrane of CF model systems. Intracellular cholesterol accumulation in cultured and in in vivo CF models suggests a direct flaw in cholesterol transport mechanisms. NPC cells have been reported to exhibit reduced plasma membrane cholesterol as a result of impaired NPC1 function (28). Given the potential importance of membrane cholesterol content in immune responses to bacterial challenge (21) and in cell signaling through lipid raft formation, plasma membrane cholesterol content was measured in 9/HTEo− pCEP and pCEP cells and in excised nasal epithelium from WT and cfr−/− mice. Plasma membrane cholesterol content was measured utilizing a micro-electrode containing cholesterol oxidase, as described in METHODS (9). In both cultured cell models and nasal epithelial tissue, CF samples exhibited an approximate twofold (P < 0.01) increase in detectable current, indicating increased membrane cholesterol content (Fig. 4). These results are consistent with earlier work demonstrating increased cholesterol content in plasma membranes of CF lymphoblasts (29). Whether this increase in plasma membrane cholesterol content in CF cells and tissues is due to elevated NPC1 expression (43) or to passive diffusion of cholesterol-rich lipid droplets is unclear. These data do demonstrate an inherent alteration in cholesterol homeostasis leading to increased de novo cholesterol synthesis in an in vivo model of CF compared with WT increase of cholesterol synthesis compared with matched controls in an 8-h time period (Fig. 5). Cholesterol synthesis in the liver was also increased 1.8 ± 0.2-fold (P < 0.001) in cfr−/− mice compared with control. No difference in cholesterol synthesis between WT and cfr−/− mouse small intestine was observed. These data demonstrate an inherent alteration in cholesterol homeostasis leading to increased de novo cholesterol synthesis in an in vivo model of CF compared with WT.

**In vivo new cholesterol synthesis in cfr−/− mouse tissue.** Lysosomal accumulation of cholesterol is predicted to stimulate de novo cholesterol synthesis because of a lack of cholesterol transport to the ER. An increase in de novo cholesterol synthesis is reported in NPC models (31), supporting this hypothesis. To directly test this hypothesis in an in vivo model of CF, cfr−/− mice were injected (ip) with deuterium-labeled water. After 8 h, deuterium incorporation into newly made cholesterol in various tissues was determined using GC-MS analysis. cfr−/− mouse lung had a 1.7 ± 0.1-fold (P < 0.001) increase of cholesterol synthesis compared with matched controls in an 8-h time period (Fig. 5). Cholesterol synthesis in the liver was also increased 1.8 ± 0.2-fold (P < 0.001) in cfr−/− mice compared with control. No difference in cholesterol synthesis between WT and cfr−/− mouse small intestine was observed. These data demonstrate an inherent alteration in cholesterol homeostasis leading to increased de novo cholesterol synthesis in an in vivo model of CF compared with WT.
controls. D$_2$O can potentially impact ion channel function, such as some proton channels in airway epithelium (8). The potential influence of D$_2$O on ion transport must be considered when interpreting these data.

Impaired cholesterol transport is sufficient to cause CF-like cytokine release. Previous work demonstrated identical alterations in the expression of a number of signaling proteins between cultured CF and NPC cell models (43). To determine whether alterations in cholesterol homeostasis described in this manuscript are capable of influencing inflammatory signaling, 9/HTEo pCEP and pCEPR cells were treated with the cholesterol transport inhibitor described above, U18666a (5 g/ml). Cells were challenged with $10^9$ CFU/well PA for 1 h and then assayed for IL-6 and IL-8 production after 24 h as previously described (25). WT pCEP cells exhibited a 54.7 ± 13.1% (P < 0.002) increase in IL-6 and a 74.5 ± 15.6% (P < 0.0001) increase in IL-8 production in U18666a-treated cells compared with cells challenged with bacteria alone (Fig. 6, A and B). CF phenotype pCEPR cells exhibited no difference in cytokine production in response to U18666a treatment. The effect of U18666a exposure on cholesterol transport in 9/HTEo pCEP and pCEPR cells is demonstrated in Fig. 6C to confirm that cholesterol transport is being inhibited.

To further verify that U18666a treatment recapitulated CF phenotypes, the impact of U18666a on SMAD3 and NOS2 expression was examined in Fig. 7. Both NPC and CF cells exhibit reduced SMAD3 expression and fail to induce NOS2 expression. Fig. 6 and Fig. 7 demonstrate that impaired cholesterol transport is sufficient to cause CF-like cytokine release.
expression (43); therefore, U18666a treatment is predicted to lower SMAD3 protein content and impair NOS2 induction as well. 9/HTEo− pCEP cells were treated with U18666a (5 μg/ml) for 72 h and examined for SMAD3 protein content by Western blot. Duplicate experiments reveal reduced SMAD3 expression in response to U18666a treatment, a finding consistent with observations in both CF and NPC cells.

A549 cells were chosen for NOS2 studies because of their robust induction of NOS2 protein expression (22). Exposure to U18666a (5 μg/ml) for 72 h results in an inhibition of NOS2 expression (43); therefore, U18666a treatment is predicted to lower SMAD3 protein content and impair NOS2 induction as well. 9/HTEo− pCEP cells were treated with U18666a (5 μg/ml) for 72 h and examined for SMAD3 protein content by Western blot. Duplicate experiments reveal reduced SMAD3 expression in response to U18666a treatment, a finding consistent with observations in both CF and NPC cells.

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Fig. 8. Effect of docosahexaenoic acid (DHA) on cholesterol transport and sterol response element (SRE) activation. A: filipin staining of CF phenotype pCEPR cells with and without exposure to DHA (10 μM) for 24 h. Control pCEP 9/HTEo− cells are shown as a control. No effect of DHA treatment is observed. Images are representative of triplicate experiments. B: NBD-cholesterol visualization of CF phenotype pCEPR cells with and without exposure to DHA (10 μM) for 24 h. Control 9/HTEo− pCEP cells are shown as a control. No effect of DHA treatment is observed. Images are representative of 2 coverslides with multiple fields viewed for each treatment. C: DHA reduces SRE activation in pCEP and pCEPR cells. SRE activity is elevated in pCEPR cells while having no significant influence in control pCEP cells. The apparent specificity for the effect of DHA on CF model cells may correlate with the elevated SRE activity demonstrated in these cells in Fig. 8. Whether reduced SRE activity in the presence of DHA is responsible for its anti-inflammatory properties needs further examination.
tion, but this interaction may represent a point of convergence between these pathways.

**DISCUSSION**

Previous work demonstrated an increase in filipin staining in two cultured cell models of CF, indicating an accumulation of free cholesterol compared with respective control cells (43). The pattern of free cholesterol accumulation was similar to that characteristic of NPC cells. In a comparative study, NPC cells were found to share a number of cell signaling characteristics with CF epithelial cells including increased RhoA expression and reduced NOS2 induction (43). These earlier results suggest that aberrations in cholesterol processing in CF epithelial cells could represent an important initiating step in aggressive inflammatory signaling. The goal of this manuscript is to further define cholesterol homeostasis in CF epithelial cells using both cultured models and primary samples to determine whether intracellular accumulation of free cholesterol is a potential source for other downstream signaling events.

The current study demonstrates free cholesterol accumulation by filipin staining in human CF bronchial airway and tracheal sections compared with non-CF tissues. These data are consistent with results obtained in cultured models of CF epithelial cells and suggest that free cholesterol accumulation is a CF-related phenotype relevant to the in vivo condition. The cholesterol accumulation phenotype was also confirmed in cultured cell models using a second technique by visualizing the accumulation of a fluorescent cholesterol analog, NBD-cholesterol. Correction of the cholesterol transport phenotype by expression of full-length CFTR in S9 cells, coupled with the fact that pCEPR cells are a model of functional CFTR inhibition, suggests that CFTR activity is necessary for proper cholesterol movement.

Another aspect of cholesterol processing is the regulation of cholesterol transport to the plasma membrane. Given the potential importance of membrane cholesterol in regulating cell signaling and bacterial responses (21), membrane cholesterol content was examined in cultured cells and nasal epithelial tissue from CF mice by utilizing a cholesterol-specific electrode. CF cells and tissue both exhibit an approximate twofold increase in membrane cholesterol content compared with respective controls. The existence of this cholesterol phenotype in mice null for CFTR expression further indicates that a loss of CFTR function, as opposed to a trafficking defect, is responsible for altered cholesterol movement. Whether the increase in membrane cholesterol content in CF cells is due to passive diffusion of lipid droplets to the membrane or to an increase in active transport mechanisms is unclear. Passive transport is unlikely, since CF and NPC cells have similar signaling but opposite membrane cholesterol content changes. However, how bacterial interactions are influenced by increased membrane cholesterol content is not addressed by these studies.

The functional consequences of aberrant cholesterol transport in CF cells also need to be explored. Endosomal/lysosomal accumulation of free cholesterol is expected to result in increased de novo cholesterol synthesis as described in NPC (31). To address this process in an in vivo model, de novo cholesterol synthesis was measured in lung, liver, and small intestine of WT and cftr−/− mice. Both lung and liver exhibit increased de novo cholesterol synthesis in cftr−/− mice compared with controls, although cholesterol synthesis is unchanged in the small intestine. Why cholesterol synthesis is elevated in the liver is unclear. Previous reports suggest that CFTR is not expressed in hepatocytes, with most expression centered in bile duct epithelial cells (7). These data imply a functional cross talk between cell types within the liver or the possibility that hepatocytes do have some low-level CFTR expression. Overall, these data are consistent with a disruption of cholesterol homeostasis in CF.

The impact of disrupted cholesterol transport on epithelial inflammatory signaling was also examined. Pharmacologically inducing endosomal/lysosomal accumulation of free cholesterol with the compound U18666a in WT 9/HTEo−/− pCEP cells resulted in elevated IL-8 and IL-6 production in response to PA challenge. However, U18666a treatment had no influence on cytokine production in CF phenotype 9/HTEo−/− pCEPR cells. These data establish that disruption of cholesterol transport is sufficient to mimic CF-like inflammatory signaling. The lack of effect of U18666a in pCEPR cells demonstrates that U18666a is unlikely to be having a nonspecific effect on cytokine production and that impaired cholesterol movement is already contributing to responses to PA challenge in these cells. U18666a also recapitulates the CF phenotype of impaired NOS2 induction in A549 cells, further verifying cholesterol
transport inhibition as a mechanistic source of cell signaling changes in CF cells. NOS2 expression inhibition by U18666a is restored by mevastatin, consistent with findings in CF mouse models (22). The restoration of NOS2 expression with mevastatin directly implicates isoprenoid-dependent pathways in the effects mediated by U18666a. In addition to inhibiting cholesterol transport, U18666a has been shown to inhibit cholesterol synthesis by indirect interactions while considerably stimulating isoprenoid production (42). These data are consistent with previous work demonstrating the role of the isoprenoid-modified RhoA in CF signaling cascades (22, 23).

Although not completely analogous, these data are consistent with a report by Grassme et al. (16) demonstrating aggressive inflammatory responses in a mouse model of Niemann-Pick type A, an acid sphingomyelinase null mouse. In addition to a loss of ceramide production, this mouse model also exhibits endosomal/lysosomal cholesterol accumulation. Grassme et al. show that challenge with PA elicits excessive production of IL-1 from these mice (16), suggesting that lipid transport is essential in modulating inflammatory responses.

The mechanism by which cholesterol accumulation triggers increased cytokine production is currently unclear. In addition to the associated cell signaling alterations previously observed (43), several studies demonstrate a relationship between lipid pathways and inflammation in CF. Oral treatment of DHA as a means to correct the DHA and arachidonic acid (AA) imbalance in cftr−/− mice corrected intestine pathology and the inflammatory response in lipopolysaccharide (LPS)-treated mice (12, 13). Similarly, treatment with the AA metabolite lipoxin (LXA4) ameliorated CF-like inflammation. Mice challenged with PA and treated with LXA4 to restore deficient endogenous levels exhibited a suppression of neutrophil inflammation and decreased bacterial burden (19). This manuscript demonstrates that DHA does not correct cholesterol transport, but its ability to inhibit SRE activation may be a source of its anti-inflammatory properties. This relationship, however, needs to be further explored.

In addition, the loss of the anti-inflammatory properties of PPARγ is likely directly related to the cholesterol transport aberration in CF cells. NPC cells have also been shown to be deficient in PPARγ activation (39), and RhoA is a known inhibitor of PPARγ (1). Inhibition of RhoA function by preventing isoprenoid/cholesterol synthesis with statins restores NOS2 expression in cftr−/− (23) mice and has been shown in other systems to increase PPARγ signaling (17). A pilot study to determine the potential efficacy of statin therapy in reducing CF-related inflammation is underway.

Determining the relationship between CFTR and intracellular cholesterol trafficking is a clear next goal. CFTR is part of the superfamily of ABC transports, and other family members such as ABCA1 are important for cholesterol trafficking. Direct cholesterol trafficking by CFTR has yet to be seen; however, there is some evidence that it can transport lipids such as sphingosine-1-phosphate (4). It has also been determined that both channels, ABCA1 and CFTR, are inhibited by glibenclamide, and inhibition of ABCA1 causes cholesterol accumulation (32). These data potentially indicate a direct role of chloride function and cholesterol regulation. Another link between cholesterol and CFTR function is proper pH of the endocytic pathway. The importance of pH has been shown in cholesterol trafficking and in CF model systems. Furuchi et al. (14) have determined that an acidic pH is needed to properly facilitate cholesterol transport from the endosomal/lysosomal vesicles. CFTR is known to be recycled within the endosomal pathway (36, 44), and the regulation of organelle pH by CFTR has been examined with mixed conclusions (2, 38, 40). The relative ambiguity of the role of CFTR in pH regulation makes conclusions regarding the role of these processes in cholesterol regulation difficult to assess. However, the building evidence regarding organelle pH and cell signaling is compelling. Recent work linking endocytic acidification and disrupted nitric oxide production directly draws these processes together (37).

This same group has also demonstrated that reversing the acidification with chloroquin corrects CF-related changes in TGF-β1 production (35). On the basis of these relationships, strong consideration of organelle pH as a mechanistic source of aberrant cholesterol transport in CF must be given.

Data presented in this manuscript demonstrate an inherent defect in cholesterol homeostasis in multiple models of CF, including primary lung and trachea tissue. Aberrations in cholesterol processing associated with CF cells and tissue include endosomal/lysosomal storage, elevated plasma membrane cholesterol content, and elevated rates of de novo cholesterol storage in lung tissue. Data also demonstrate that inhibition of cholesterol transport is sufficient to elevate cytokine production in a manner consistent with the CF phenotype. In conclusion, these data demonstrate a novel cell biological process disrupted in CF that has profound implications on the understanding of the pathobiology of CF and represents important new therapeutic targets for the treatment of CF.

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