Abnormal intracellular lipid processing contributes to fat malabsorption in cystic fibrosis patients

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Peretti, NoëI, Claude C. Roy, Eric Drouin, Ernest Seidman, Pierre Brochu, Georges Casimir, and Emile Levy. Abnormal intracellular lipid processing contributes to fat malabsorption in cystic fibrosis patients. Am J Physiol Gastrointest Liver Physiol 290: G609–G615, 2006. First published October 13, 2005; doi:10.1152/ajpgi.00332.2005.—A common feature of cystic fibrosis (CF) is the functional derangement of the exocrine pancreas, which affects output of pancreatic lipase. This condition results in severe dietary malabsorption due to the poor hydrolysis of triacylglycerol (TG) in the lumen of the small intestine. Despite the benefits of pancreatic enzyme supplements, patients with CF present with persistent intestinal fat malabsorption. The aim of the present investigation was to determine whether defects in the intracellular phase of lipid transport occur in this pathophysiology in addition to the known disturbed digestive processes. Our hypothesis was tested by incubating intestinal biopsies from six CF and six healthy subjects with radiolabeled lipid and protein precursors. Lipid esterification and secretion were markedly decreased by 22–31% and 38–42%, respectively, in CF samples, as noted by the low incorporation of [14C]palmitic acid into TGs, phospholipids, and cholesteryl esters in patients’ duodenal explants and culture media compared with controls (100%). Accordingly, the output of TG-rich lipoproteins was substantially reduced (P < 0.05), and a similar trend was observed for high-density lipoproteins. Because intestinal lipoprotein assembly/secretion shows an absolute requirement for apolipoprotein (apo) B-48, radioactive labeling experiments were performed; these experiments demonstrated a significantly (P < 0.05) diminished synthesis of apoB-48 (40%) and apoA-I (30%). Given the critical role of microsomal triglyceride transfer protein in the formation of apoB-containing lipoproteins, its activity was determined and not found to be altered in CF intestinal tissue. Together, these results suggest that CF malabsorption may also be caused by defects in mucosal mechanisms leading to abnormal lipoprotein delivery into the blood circulation.

Lipoproteins; fatty acids; apolipoprotein B-48; microsomal triglyceride transfer protein; cystic fibrosis transmembrane conductance regulator

Cystic fibrosis (CF) is the most common life-threatening recessive genetic disease in the Caucasian population (24). It arises from a reduced density and/or activity of the CF transmembrane conductance regulator (CFTR) in the apical membranes of secretory epithelial cells, such as the airways, exocrine pancreas, salivary glands, intestine, and reproductive tissue (1). CF is described as a general exocrine disease, with mortality primarily attributable to the pathology that occurs in the lung and gastrointestinal tract (6). In fact, a major characteristic shared by CF patients is exocrine pancreatic insufficiency, which results in intestinal fat malabsorption and malnutrition as well as essential fatty acid (EFA) and fat-soluble vitamin deficiencies (37). The deficits in salt (chloride) and water transport across duct cells produce a diminution in fluid flow by the gland, which leads to duct blockage and the inspissations and precipitations of proteins in the ducts (9). Over time, cellular damage and atrophy occur, causing a shortage in the secretion of digestive enzymes. Together with conspicuous pancreatic insufficiency, various disorders of the intraluminal digestive phase, including bicarbonate deficiency and bile salt abnormalities, have been purported to be the main causes of fat malabsorption (24, 29, 39). However, despite the beneficial effects of exogenous pancreatic enzyme supplementation, malabsorption cannot be normalized in CF patients, as noted by persistent steatorrhea, poor growth, and malnutrition (2). Continuing fat malabsorption in CF subjects may also be due to considerations other than defective lipolytic enzyme activity and additional intraluminal factors. The aim of the present study was to investigate whether disturbances in CF lipid transport might also be associated with cause-related changes in the second step of fat absorption, i.e., the intracellular phase leading to lipid esterification, apolipoprotein (apo) biogenesis, and lipoprotein assembly.

MATERIALS AND METHODS

Patients. Six CF patients (13.6 ± 1.4 yr) underwent duodenal endoscopy for diagnostic purposes in view of their digestive symptoms and clinical condition, which did not improve despite pancreatic supplementation. Pancreatic insufficiency in these subjects was defined by the requirement for exogenous pancreatic enzymes for the treatment of clinically diagnosed steatorrhea: 8.4, 11.7, 15.9, 17.2, 20.3, and 21.8 g/day (normal range: 3.5–5.0 g/day). To further the investigation on malabsorption, biopsies were taken from the duodenum for pathohistological evaluation and for lipid transport assessment, which are presented in detail in the present study. The CF patients were compared with six age-matched healthy subjects who were previously suspected of having gastrointestinal diseases such as disaccharidase deficiency, celiac disease, cow’s milk-sensitive enteropathy, and failure to thrive with chronic nonspecific diarrhea. Informed consent was obtained from the parents, and the project was approved by the CHU Sainte-Justine Ethics Committee.

Explant cultures of duodenal biopsies. Fasting intestinal biopsy specimens were obtained from the region of the ligament of Treitz and immediately placed in RPMI-1640 culture medium containing 10% inactivated and dialyzed human lipoprotein-deficient serum. This medium was saturated with 95% O2-5% CO2, and cultures were set up within 20 min of completion of biopsies by methods described by 10.220.33.3 on August 27, 2017 http://ajpgi.physiology.org/ Downloaded from

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previously (17). The tissue culture medium (0.8 ml) consisted of leucine-free RPMI-1640 with lipoprotein-deficient serum, including gentamycin (100 μg/ml) and soybean trypsin inhibitor (60 μg/ml). Lipid synthesis and secretion by explants were examined by addition of 10 μCi of [14C]palmitic acid (specific activity of 40–60 mCi/mmole; New England Nuclear, Montreal, Quebec, Canada) to a micellar mixture (8 mM sodium taurocholate, 20 mM palmitic acid, and 10 mM Sn-2-mono-oleylglycerol) to stimulate intracellular lipid esterification. The final ratio of radioactivity to concentration of palmitic acid in each dish was 1 μCi/400 μM. The Petri dishes were thereafter placed in anaerobic jars, sealed, gassed with 5% CO2-95% O2, and incubated for 18 h at 37°C. The pH of the medium was maintained between 7.2 and 7.4 as indicated by phenol red. After this incubation period, tissue integrity was confirmed by morphological and biochemical studies.

**Lipid synthesis.** After an 18-h incubation, the explants were sonicated and homogenates were lipid extracted with chloroform-methanol (2:1, vol/vol). Lipid separation was carried out by TLC (silica gel; Eastman Kodak, Rochester, NY). The developing solvent system was cated and homogenates were lipid extracted with chloroform-methanol (2:1, vol/vol). The radioactivity of the separated fractions was measured in a Beckman liquid scintillation spectrometer. We corrected quenching using computerized curves generated with external standards. An aliquot of the tissue homogenate was used for protein determination.

**De novo apolipoprotein synthesis.** Duodenal biopsies were cultured with [3H]leucine (500 μCi/ml) to assess apo biogenesis. At the end of the 45-min incubation at 37°C, explants were washed (3×) for 5 min by the addition of media supplemented with 10 mM leucine and then homogenized in 1 ml of PBS (20 mM sodium phosphate, 145 mM NaCl, pH 7.4) containing 1% (wt/vol) Triton X-100, leucine (2 mM), PMFS (1 mM), and benzamid (1 mM). The homogenate was centrifuged (4°C) at 105,000 g for 60 min in a 50-Ti rotor (Beckman) and subsequently reacted with excess polyclonal apoB and apoA-I antibodies (Boehringer Mannheim) for 18 h at 4°C. Pansorbin (Calbiochem) was then added, and the immunoprecipitate was washed extensively and analyzed by linear 3–20% acrylamide gradient and a 2% stacking gel. Bands corresponding to apoA-I and apoB-48 were sectioned and counted after an overnight incubation at 20°C with 1 ml BPS-450 (Beckman) and 10 ml of liquid scintillation fluid (Ready Solv NA, Beckman). Results for each apo studied were expressed as percentage of apo/total TCA-precipitable protein.

**Isolation of lipoproteins.** The separation of lipoproteins from the medium was carried out by sequential ultracentrifugation using a TL-100 ultracentrifuge as described previously (10, 15, 22). Briefly, after the removal of triacylglycerol (TG)-rich lipoproteins [chylomicrons and very-low density lipoproteins (1.006 g/ml)] at 100,000 g for ~2.5 h at 5°C, the low-density lipoproteins and high-density lipoproteins were obtained together (1.21 g/ml) by centrifuging for 6.5 h at 100,000 g. Each lipoprotein fraction was exhaustively dialyzed against 0.15 M NaCl and 0.0001 M EDTA, pH 7.0, at 4°C for 24 h.

**Microsomal triglyceride transfer protein activity assay.** Microsomal triglyceride transfer protein (MTP) activity was determined by evaluating the transfer of radioactivity from TG to TG-rich lipoproteins (Table 1). The levels of lactase, maltase and sucrase were not significantly different between the control and the CF patients' intestinal explants.

**RESULTS**

Concomitantly with the evaluation of mucosal fat transport, studies were conducted to assess the integrity of duodenal tissue obtained from CF patients and control subjects. Figure 1 illustrates the general morphology of intestinal tissue at the light microscopy level. Both patient and control duodenal specimens exhibited well-shaped villi and crypts. The epithelial cells lining the villi were tall, and the epithelium of the crypts was well preserved in CF and control subjects. Furthermore, no damaged cells were noted in the CF epithelium compared with normal mucosa.

Experiments were also carried out to examine the enzymatic activities of duodenal biopsies (Table 1). The levels of lactase, maltase, and sucrase were not significantly different between CF patients and controls.

In a subsequent step, we used the model of intestinal organ culture, a reliable and useful technique, to explore the intracellular processes of lipid absorption. The excellent viability of CF and control tissues was reflected by similar levels of alkaline phosphatase activity and thymidine incorporation, as well as by measuring the leakage of lactate dehydrogenase into the culture medium after an 18-h incubation period (Table 2). Overall, these data suggested that marked undesired gastrointestinal effects occurred as a result of the CF condition or after organ culture of CF patients’ intestinal explants.

**Lipid synthesis and secretion were assessed by following [14C]palmitic acid incorporation into different lipid classes.** As noted in Fig. 2, the formation and release of lipids were decreased in CF duodenal explants compared with controls. Importantly, the incorporation of [14C]palmitic acid into TG, phospholipids (PL), and cholesteryl ester (CE) was moderately reduced in CF duodenal explants (22, 31, and 30%, respectively) and contrasted with the markedly impaired output of these lipids in the culture medium (40, 38, and 42%, respectively) when compared with controls. The discrepancy between the synthesis of newly formed lipids and their secretion became particularly evident when expressed as a medium-to-tissue...
ratio. Accordingly, the discharges of TG-rich lipoproteins and high-density lipoprotein were much lower \((P < 0.05)\) in CF samples than in controls (Fig. 3A). In keeping with these biosynthetic lipid abnormalities, the radioactivity incorporated into apoB-48 and apoA-I in CF specimens amounted to 60 and 70\%, respectively, of controls \((P < 0.05)\) (Fig. 3B). However, no significant correlation \((r = 0.39, P < 0.41)\) was found between fecal fat excretion and the lipoprotein production rates in the CF biopsies.

To determine whether the defect of apoB-48 biogenesis and TG-rich lipoprotein secretion might be related to MTP, we studied protein transfer activity using the transport of \[^{14}C\]tri-oleylglycerol from donor to acceptor small unilamellar vesicles. No significant changes in MTP activity \((%\)transfer) were detected in CF biopsy homogenates \((16 \pm 5)\) compared with controls \((18 \pm 4)\).

FA compositions of CF and control plasma were determined. The CF profile displayed both a significant reduction in the relative content of polyunsaturated fatty acids and a considerable increase in the proportion of saturated FAs (Fig. 4). These alterations in CF patients led to an elevation of the ratio 20:3 \((n-9)\) to 20:4 \((n-6)\), a very sensitive index of essential fatty acid deficiency (EFAD), and to a decrease in the polyunsaturated fatty acid-to-saturated FA ratio.

Overall, these findings underline the CF patient’s limited ability to esterify lipids, elaborate apoproteins, and assemble lipoproteins. Thus our studies show, for the first time, that the intracellular phase of fat absorption is impaired in the enterocytes of CF patients.

**DISCUSSION**

In the present study, we used intestinal tissue in culture to further our understanding of the complex biosynthetic molecular events essential for the formation and secretion of lipoproteins relevant to CF patients. This efficient and reliable intestinal model affords the opportunity to investigate lipid transport while minimizing the influence of the many confounding digestive intraluminal factors seen in the in vivo situation, thus facilitating the interpretation of data. We can, therefore, conclude that these intraluminal factors are unlikely to be responsible for the diminished capacity of CF patients’ biopsies to absorb lipids, since the intraluminal lipolysis of TG by lipases and the solubilization of lipolytic products by bile components are circumvented.

Results from our biochemical analyses provide novel information regarding lipid processing in the intestine of CF patients. First, cultured CF duodenal explants were incapable of the normal biosynthesis of neutral (TG, CE) and polar (PL) lipids. Second, they were inefficient in delivering lipids into the medium, which was confirmed by defective lipoprotein secretion. Third, they displayed less efficiency in elaborating apo (B-48 and A-I) biogenesis.

At present, our findings cannot indicate whether the impaired capacity of the small intestine to produce TG in CF is due to the limited activity of the monoacylglycerol (MG) pathway. As reported previously \((14, 35)\), MG and fatty acyl
CoA are covalently joined to form diacylglycerol in a reaction catalyzed by monoacylglycerol acyltransferase. Diacylglycerol and fatty acyltransferase are then used to synthesize TG in a reaction catalyzed by diacylglycerol acyltransferase (14, 35). In our study, the measurement of monoacylglycerol acyltransferase and diacylglycerol acyltransferase could not be carried out given the limited tissue availability, nor were we able to define the status of the enzymes responsible for cholesterol esterification and PL formation.

Importantly, the analysis of free FAs and MG in the mucosa of CF patients did not disclose quantitatively significant changes compared with controls. The absence of FA accumulation in CF mucosa may suggest a reduced uptake of exogenous FAs in view of the limited lipid esterification and secretion. However, more evidence is necessary to confirm this mechanism since FAs may have alternative metabolic fates, including mitochondrial β-oxidation, peroxysomal degradation, elongation and conversion to prostaglandins, protein binding and acylation, intracellular signalization, and membrane formation. It is important to mention that biopsies from control and CF patients were obtained after fasting, thus excluding a possible dilution of labeled palmitic acid substrate with residual FAs in the mucosa.

It is generally accepted that the intracellular mechanism of TG-rich lipoprotein assembly requires apoB synthesis and association. An early step in this process is the cotranslational lipidation of apoB that is transiently bound to the endoplasmic reticulum (ER) membrane where it is folded. The addition of lipids stabilizes apoB-48 and prevents its proteolytic degradation via the ubiquitin-dependent proteasomal pathway (4, 38). It is therefore reasonable to suggest that the defective synthesis of TG, CE, and PL in the small intestine of CF patients reduced apoB-48 protection from misfolding and degradation, resulting in the lessened secretion of apoB-48-containing lipoproteins. Additional studies are required to determine the role of COP II machinery (8, 31) and apoB-48 glycosylation (13) in the defective transport of TG-rich lipoproteins in CF patients.

We have also noted that apoA-I biogenesis was diminished in the intestinal tissue of CF patients. Even if the values of apoA-I were normalized and expressed as %TCA-precipitable protein, they remained lower than those for controls. These data are consistent with our previous study (12), which showed decreased concentrations of plasma apoA-I in CF patients. It is possible that the decline of apoA-I synthesis by the intestine contributes to diminished apoA-I levels in the blood circulation.

MTP is a resident protein in the lumen of the ER that facilitates the transfer of lipids from their site of synthesis in the ER membrane into the lumen during the assembly of TG-rich lipoproteins (30, 36). However, the MTP requirement for the export of apoB-48 remains controversial. Various studies have stressed that liver-specific MTP knockout mice...
will still secrete apoB-48, thus excluding the obligatory role of MTP (25, 26). Our findings show that CF intestinal tissue was unable to normally release TG-rich lipoproteins despite adequate MTP activity.

EFAD is another factor capable of contributing to fat malabsorption. Indeed, our previous observations have already emphasized that EFAD in rats impaired both the extracellular and intracellular mechanisms responsible for normal fat absorption (11). Therefore, not only does the elevation of the eicosatrienoic acid (20:3n-9)-to-arachidonic acid (20:4n-6) ratio, the most sensitive index of EFAD, indicate an abnormal EFA status in CF patients with respect to controls, but it may also account for the lipid enterocyte dysfunction. Evidence has
also been reported indicating that EFA status is associated with severe CFTR mutations, suggesting a relationship between the basic protein defect, abnormal EFA metabolism, and defective intracellular processing of dietary lipids in CF patients (34). Finally, the alterations in the mucous layer reported by previous investigators (3, 28) may persist in the present intestinal model and may interfere with lipid uptake and processing. In the present investigation, the output of lipids as reflected by the increased ratio of medium to tissue lipid content shows that the secretory mechanism is operational in CF, which reasonably excludes the role of the mucous layer in intracellular abnormalities in these patients. Moreover, we could not find a correlation between EFAD measured in the plasma and the abnormalities observed in lipids, apoproteins, and lipoproteins in the intestine of CF patients. We therefore believe that the differences noted are CF related. Of particular importance is the abundance of CFTR expression in the apical regions of normal intestinal epithelial cells (27). The presence of defects in CFTR may predispose to disturbed ion transport and to other intestinal manifestations, including intracellular lipid trafficking.

It is difficult to reconcile the failure of appropriate pancreatic enzyme replacement therapy with the persistent malabsorption. It was therefore reasonable to propose that quantitative and qualitative mucosal defects can obviously contribute to aberrations in lipid transport in CF. However, in our studies, no correlation has been established between steatorrhea and the enterocyte phase abnormalities, suggesting that several digestive and cellular factors act in combination to dramatically affect fat absorption process.

Our data have highlighted the defects in the intracellular phase of lipid transport in CF. Conversely, enhanced glucose transport was demonstrated in duodenal biopsies from CF (7), and Frase et al. (5) revealed glucose absorption in the jejunum of patients with CF. Other studies noticed that the inhibition of CFTR did not modify the activity of Na+/glucose cotransporter and taurocholate uptake were increased in cfr−/− mice (33). Overall, these data emphasize that the defect in intestinal lipid absorption in the present study is not specific and that the expression of proteins involved in other metabolic pathways is not modified.

In conclusion, data from the present study indicate that continuing fat malabsorption is not due to pancreatic insufficiency alone. An additional explanation of persistent fat malabsorption in CF patients may include abnormal intraenterocyte events that lead to aberrant plasma lipid transport. The task ahead, however, is to define the relationship between defective mechanisms governing intracellular lipoprotein assembly and CFTR aberrations.

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GRANTS

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