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Accumulation of 3-Hydroxy-Fatty Acids in the Culture Medium of Long-Chain L-3-Hydroxyacyl CoA Dehydrogenase (LCHAD) and Mitochondrial Trifunctional Protein-Deficient Skin Fibroblasts: Implications for Medium Chain Triglyceride Dietary Treatment of LCHAD Deficiency

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ABSTRACT

Dietary management of long-chain L-3-hydroxyacyl CoA dehydrogenase (LCHAD) deficiency involves a regimen that contains adequate carbohydrate, protein, and essential lipids, and supplementation with medium-chain fatty acids in the form of medium-chain triglycerides, providing energy from fats that bypasses the long-chain metabolic block. This study analyzes the effects of dietary treatment of LCHAD deficiency in an *in vitro* model. Cultured skin fibroblasts from LCHAD-deficient and normal individuals were grown in media supplemented with physiologic combinations of medium-chain fatty acids octanoate and decanoate, and the long-chain palmitate. Medium was removed from the cells after various incubation times, and assayed for 3-hydroxy-intermediates of fatty acid oxidation. The 3-hydroxy-fatty acids were measured by stable-isotope dilution gas chromatography/mass spectrometry. We found that the addition of medium-chain fatty acids caused a decrease in the accumulation of long-chain fatty acid oxidation intermediates in LCHAD-deficient cells when the cells were incubated in untreated medium, and also when they were incubated in this medium with palmitate added. Medium with decanoate alone was better at achieving this effect than medium with only octanoate added. A 1:3 ratio of octanoate to decanoate worked best over an extended time period in LCHAD-deficient cells in untreated medium, whereas a 1:1 ratio of octanoate to decanoate worked best in the same cells incubated in medium containing palmitate. In all dietary medium-chain triglyceride preparations, the ratio of octanoate was greater than that of decanoate. Our results suggest that a medium-chain triglyceride preparation that is higher in decanoate may be more effective in reducing the accumulation of potentially toxic long-chain 3-hydroxy-fatty acids in LCHAD deficiency. **(*Pediatr Res* 53: 783–787, 2003)**

Abbreviations

LCHAD, long-chain L-3-hydroxyacyl CoA dehydrogenase
MTP, mitochondrial trifunctional protein
LCFA, long-chain fatty acid
MCT, medium-chain triglyceride
FAO, fatty acid oxidation
3OHFA, 3-hydroxy-fatty acid
DMEM, Dulbecco's modified Eagles medium
FBS, fetal bovine serum
FA, fatty acid

Mitochondrial FAO is a complex pathway of energy metabolism in which more than 17 inborn errors of metabolism have currently been elucidated (1–4). Management of these disorders by prevention of fasting is extremely efficacious for some defects such as medium-chain acyl-CoA dehydrogenase deficiency, but less successful in other defects. The outcome of treatment depends on the specific enzymatic step involved and also on the degree of enzyme deficiency.

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LCHAD is the third enzyme responsible for the β-oxidation of LCFA and is found as part of the MTP. Isolated LCHAD deficiency has been reported in over 200 cases and is generally regarded as one of the more severe FAO defects (1). MTP deficiency seems to be rarer than LCHAD deficiency but has similar biochemical findings. Treatment of LCHAD deficiency has generally involved the approach that applies to all long-chain FAO defects that includes provision of adequate carbohydrate caloric intake, and preventing circumstances that may result in low glucose levels and thus induce increased FAO flux. An example would be fasting or intercurrent infections that may cause metabolic decompensation. Along with this

carbohydrate maintenance, LCHAD-deficient individuals are usually provided with adequate protein and treated with dietary MCT to bypass the long-chain metabolic blockage while still providing energy from fats (5, 6). Like many inborn errors that present in infancy, diet regimens involve a balancing act between providing essential nutrients for growth and normal development, and restricting dietary elements that will result in the build-up of toxic metabolites. In the case of LCHAD deficiency, although LCFA are often restricted, linoleic and α-linolenic acids are essential and must be supplied in the diet. Along with being important for maintaining the integrity of biologic membranes, these unsaturated C18 FA are required for the synthesis of arachidonic acid and docosahexanoic acid, two FA that are essential for the proper development of the eyes and nervous system. LCHAD-deficient individuals are susceptible to neuropathy and degenerative retinopathy (7), which may be related to the build-up of toxic LCFA intermediates that interfere with the proper synthesis of these necessary LCFA (8, 9).

This study examines the results of incubating cultured skin fibroblasts from LCHAD-deficient individuals and from normal individuals in media that has been modified by the addition of various chain-length FA. The results demonstrate that medium-chain FA in the media actually prevent accumulation of the long-chain 3-hydroxy-intermediates of FAO, a potential additional benefit of MCT therapy that has not been anticipated or demonstrated before our studies.

METHODS

Samples. Fibroblasts used in this study were from three LCHAD-deficient patients whose diagnoses were confirmed by enzymic and molecular analysis. The genotypes of the patients from which the three lines were derived are as follows: one homozygous G1528C mutation and one heterozygous G1528C/5 bp deletion crossing a splice site after exon 15, representing isolated LCHAD deficiencies, and one heterozygous C685T(β-subunit)/s mutation, as yet unknown, with enzymically confirmed MTP deficiency. Three cell lines with normal FAO flux were used as controls. This study has applicable institutional review board approval from our institution.

Materials. Low-glucose DMEM, no-glucose DMEM, and FBS were obtained from Life Technologies (Invitrogen, Carlsbad, CA, U.S.A.). Palmitate, octanoate, decanoate, fatty-acid-free BSA, sodium sulfate, and 2-phenylbutyric acid were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). 3-OHFA stable isotope standards were synthesized and used as previously described (10). *N,O*-bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (99:1) was obtained from Supleco (Bellefonte, PA, U.S.A.) and used as the derivatizing agent. HPLC-grade ethyl acetate and HCl were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Anhydrous ethanol was obtained from Electron Microscopy Services (Ft. Washington, PA, U.S.A.).

Media preparation. Nonisotopic octanoate, decanoate, or palmitate was dissolved in anhydrous ethanol to a concentration of 22 mM to make each stock FA. The stock FA were then added to 40 g/L BSA to a final concentration of 1.0 mM or 3.0 mM and incubated at 37°C for 30 min to allow binding of the FA to the BSA. The FA/BSA mixture was then added to the DMEM-10% FBS medium to the final concentration used in each experiment. For example, for an experiment run with 300 μM octanoate, after incubation at 37°C for 30 min, the 3.0 mM FA/BSA mixture would be diluted 1/10 into DMEM-10% FBS to give a final concentration of 300 μM octanoate.

Cell culture. T25 flasks of cells were cultured in DMEM-10% FBS, and studied between passages 5 and 15 when cells were confluent, usually 5–7 d after subculture. To run an experiment, medium was removed from the cells and 5 mL of FA-containing medium was added. Flasks were incubated for 24–96 h at 37°C in a 5% CO_2-95% air incubator. After incubation, media was removed and saved for analysis, and cells were returned to DMEM-10% FBS.

Extraction and analysis. Media sample extraction, derivatization, assay on the gas chromatography/mass spectrometry, and analysis of results were performed as previously described (10, 11).

RESULTS

Previously, we have shown that incubation of LCHAD-deficient fibroblasts in 100 μM palmitate results in the accumulation of diagnostic amounts of 3-OH-C16 in the culture medium (11). Because LCHAD-deficient individuals are often treated with dietary MCT, we attempted to determine what effect the addition of medium-chain FA would have on the accumulation of intermediates in cultured LCHAD-deficient fibroblasts as a potential *in vitro* model to study the effects of MCT supplementation. A review of the literature for physiologic concentrations of octanoate and decanoate in individuals treated with MCT failed to reveal definitive information. Ranges were given that varied from 0 to 972 μM for octanoate and 20 to 350 μM for decanoate, and under a variety of conditions (12–15). These values were similar to those determined in previous studies (unpublished data) at our hospital on serum MCT concentrations from patients receiving MCT in their diet. We found octanoate concentrations ranging from 0 to 900 μM and decanoate concentrations of 0 to 110 μM. The physiologic ranges are very broad because of the rapid turnover of MCT in the body and the unknown timing from point of ingestion.

We began our experiments with concentrations of medium-chain FA equimolar to the palmitate we had been using for previous work. We increased concentrations as needed for experiments, but did not exceed concentrations that we had measured in patients receiving MCT or what had been previously reported in the literature. Concentrations of 100 and 300 μM were generally used, as these fell into the range found physiologically. Also, because MCT dietary supplements are often used in a 3:1 ratio of octanoate to decanoate, 300 and 100 μM concentrations could easily be used to approximate this ratio.

As can be seen by Figure 1, incubating fibroblasts in a combination of 100 μM palmitate and 300 μM octanoate for 24 h results in accumulation of different chain-length 3-hydroxy-intermediates. LCHAD- and MTP-deficient fibroblasts

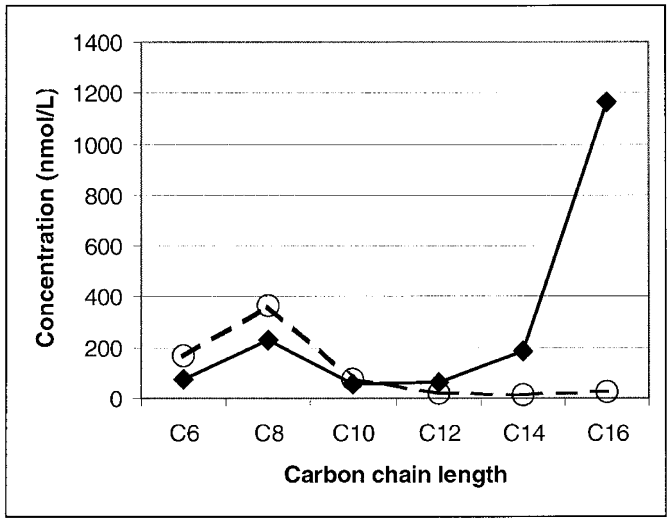

Figure 1. Accumulation of 3-OHFA in medium from LCHAD-deficient (*filled diamond*) *vs* normal (*open circle*) fibroblasts incubated in medium containing 100 μM palmitate and 300 μM octanoate. Results are the mean of eight experiments.

demonstrated a modest accumulation of 3-OH-C8, and a large accumulation of 3-OH-C16, whereas normal cells only accumulate 3-OH-C8 to any appreciable amount. There was no significant difference between metabolite accumulation in LCHAD- and MTP-deficient call lines. The accumulation of the long-chain 3-OH-species by LCHAD-deficient cells was decreased in a dose-dependent manner by increasing the amount of octanoate added, as shown in Figure 2. As can also be seen in Figure 2, 3-OH-C8 concentrations increase as the amount of octanoate increases, a finding that is consistent with the increased 3-OH-C8 concentrations in the serum of patients receiving MCT in their diet. The decrease in long-chain metabolites was also seen when decanoate was added to the medium, but to an even greater degree than with octanoate (Fig. 3). Figure 3 demonstrates that culturing cells in 500 μM octanoate resulted in a 62% decrease in 3-OH-C16 concentration compared with cells receiving no octanoate. Compared with cells receiving no decanoate, 500 μM decanoate resulted in an 84% decrease in 3-OH-C16 concentration.

Because MCT used in dietary treatment are often found in the form of 67% C8 to 23% C10 (~3:1 octanoate:decanoate) with lesser amounts of C6 and C12, we added various combinations of octanoate and decanoate to the medium of the cultured fibroblasts, and incubated them for 24–96 h. Originally, we ran these experiments with only the addition of medium-chain FA to the medium in the absence of additional palmitate. In medium without additional palmitate, any combination of octanoate and/or decanoate, except for octanoate alone, will cause a decrease in the accumulation of 3-OH-C16. We found that over a 96-h time period, a 1:3 ratio of octanoate to decanoate was the best combination for preventing accumulation of 3-OH-C16. At this ratio, there was an approximately 50% decrease in the accumulation of 3-OH-C16 from the accumulation seen in cells cultured in medium with no FA added (Fig. 4). Also shown in Figure 4, if the cells were incubated in medium that was glucose-free or that had palmitate added, only the addition of a 1:1 ratio of octanoate to decanoate with both FA at 300 μM prevented accumulation of 3-OH-C16 at 96 h. This result suggests that when glucose availability is low in LCHAD-deficient cells, or when LCFA are present in elevated amounts, MCT are not as efficient at preventing accumulation of 3-OH intermediates of FAO. This finding has clear implications for the use of MCT with the management of acute metabolic decompensation in LCHAD-deficient patients.

Figure 5 demonstrates that when using a preparation containing a greater concentration of decanoate than octanoate, there is less accumulation of the medium-chain 3-OH-intermediates, 3-OH-C8 and -C10. Octanoate by itself or in combination with decanoate caused an increase in accumula-

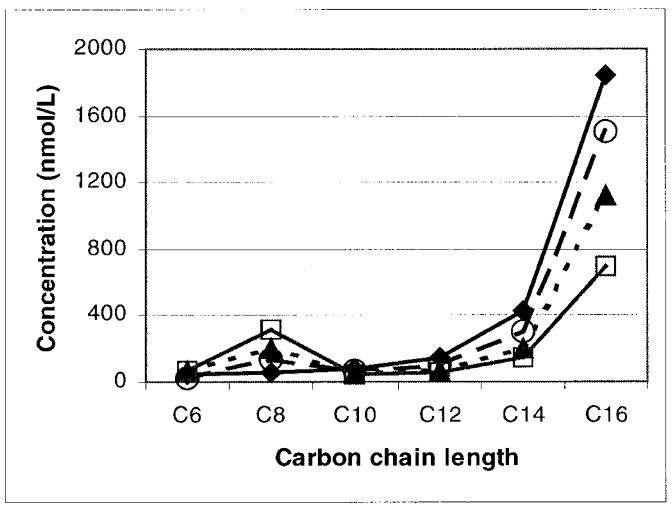

Figure 2. Change in accumulation of 3-OHFA in medium from LCHAD-deficient cells incubated in 100 μM palmitate and increasing concentrations of octanoate. Octanoate concentrations are 0 μM (*filled diamond*), 100 μM (*open circle*), 300 μM (*filled triangle*), and 500 μM (*open square*). Results are the mean of 12 experiments.

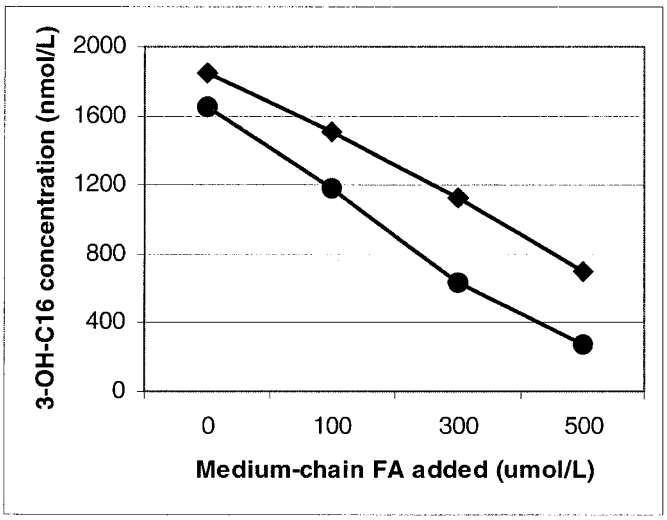

Figure 3. Dose-dependent decrease in accumulation of 3-OH-C16 in medium from LCHAD-deficient cells incubated in 100 μM palmitate and increasing concentrations of either octanoate (*filled diamond*) or decanoate (*filled circle*). Results are the mean of 12 experiments.

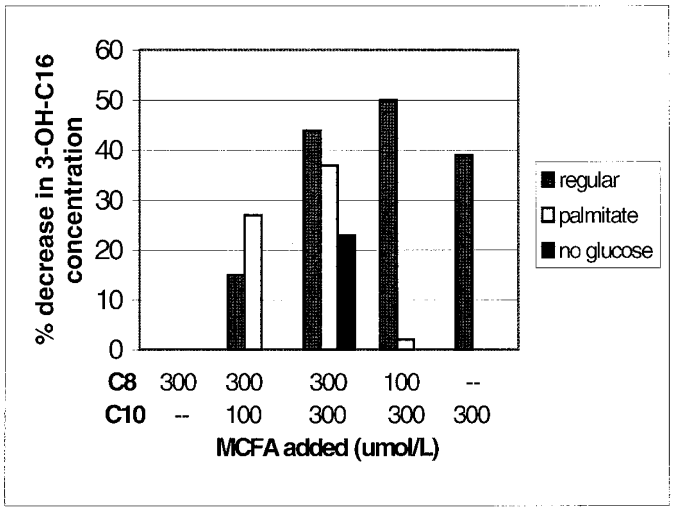

Figure 4. Percentage decrease in accumulation of 3-OH-C16 in medium of LCHAD-deficient cells. Cells are incubated 96 h with combinations of octanoate and decanoate in regular DMEM + 10% FBS, in DMEM + 10% FBS with 100 μM palmitate added, or in no glucose DMEM + 10% FBS. Changes are relative to cells in medium containing no medium-chain FA. Absence of bars on the graph indicates no change in concentration.

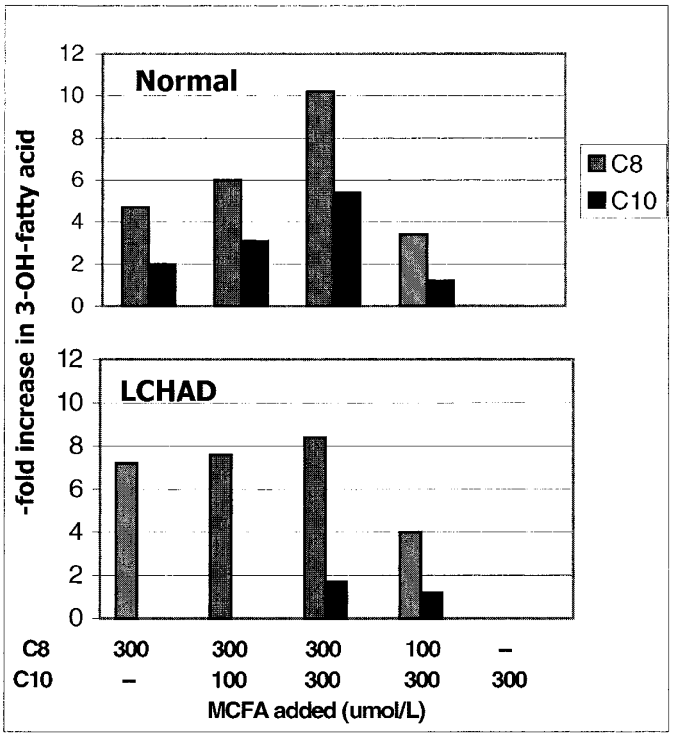

Figure 5. Amount of increase in accumulation of medium-chain 3OHFA in medium of normal cells (*top*) and LCHAD-deficient cells (*bottom*) incubated in DMEM + 10% FBS medium supplemented with medium-chain FA. Changes are relative to cells in medium containing no medium-chain FA. Absence of bars indicates no change in concentration.

tion of 3-OH-C8. Decanoate by itself shows no increase in accumulation of 3-OH-C10 or -C8, although it seems to add to the 3-OH-C8 and -C10 accumulation seen when the two are used in combination. Again, these results are consistent with those seen with patients receiving MCT in their diet. These patients show a significant, though transient, accumulation of 3-OH-C8, and very little to no change in 3-OH-C10.

DISCUSSION

Treatment of LCHAD- and MTP-deficient individuals typically involves two components. One component is prevention of the onset of fasting, or any other metabolic decompensated state that may result in the attempted utilization of FA as fuel. The second component is dietary management by providing appropriate protein, carbohydrate, and an efficacious mixture of essential LCFA and MCT to bypass the metabolic block. LCFA, especially α-linolenic and linoleic, are essential for the proper growth and development of an individual. These LCFA are precursors for the synthesis of DHA (docosahexanoic acid) and arachidonic acid, respectively, which must be present for normal neurologic and retinal development. Individuals who have LCHAD deficiency require these essential LCFA. Although it may be necessary to restrict the amount of total LCFA, it may also be necessary to supply additional essential FA, or their synthetic products such as DHA. The incomplete catabolism of the LCFA results in toxic intermediates. LCHAD deficiency has been proposed to interfere with the synthesis of DHA (8), and the toxic intermediates may contribute to this interference.

Previous studies have suggested that an overabundance of MCT in the diet may be used to synthesize LCFA in premature infants and in rats (16, 17). Our study does not support this premise in human fibroblasts. Supplementation with medium-chain FA results in a decrease in the accumulation of the intermediates of long-chain FAO in fibroblast culture medium, and we have previously reported that increased concentrations of LCFA increases the accumulation of long-chain 3-OH-metabolites (11). Thus, the results of the current study suggest that LCFA are not being synthesized from the medium-chain FA supplied to these cells.

MCT dietary supplementation is generally considered the standard of care for LCHAD-deficient individuals. The results of this study suggest that MCT may be beneficial not only for bypassing the metabolic block, but also for actually inhibiting accumulation of toxic long-chain intermediates, by an as yet undetermined mechanism. We believe this observation may be related to interactions between the mitochondrial matrix enzymes responsible for metabolism of medium-chain FA species and the membrane-associated long-chain metabolizing enzymes. This is a focus of our ongoing studies. The present study also suggests that a mixture of medium-chain fats that has a higher ratio of decanoate to octanoate may be more efficacious than the mixture currently supplied in MCT formulations.

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