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Medium-chain fatty acids undergo elongation before β-oxidation in fibroblasts ☆

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Abstract

Although mitochondrial fatty acid β-oxidation (FAO) is considered to be well understood, further elucidation of the pathway continues through evaluation of patients with FAO defects. The FAO pathway can be examined by measuring the 3-hydroxy-fatty acid (3-OHFA) intermediates. We present a unique finding in the study of this pathway: the addition of medium-chain fatty acids to the culture media of fibroblasts results in generation of 3-OHFAs which are two carbons longer than the precursor substrate. Cultured skin fibroblasts from normal and LCHAD-deficient individuals were grown in media supplemented with various chain-length fatty acids. The cell-free medium was analyzed for 3-OHFAs by stable-isotope dilution gas-chromatography/mass-spectrometry. Our finding suggests that a novel carbon chain-length elongation process precedes the oxidation of medium-chain fatty acids. This previously undescribed metabolic step may have important implications for the metabolism of medium-chain triglycerides, components in the dietary treatment of a number of disorders.

Keywords: Fatty acid oxidation; Medium-chain triglycerides; Fatty acid synthesis; Long-chain L-3-hydroxyacyl CoA dehydrogenase

Fatty acid β-oxidation (FAO) is a complex metabolic pathway for which the basic enzymatic steps have been well elucidated [1–5]. The pathway is a cyclical one involving four enzymatic steps that occur inside the mitochondria for fatty acids with carbon chain lengths from 4 to 20 carbons. The process of oxidizing longer-chain length fatty acids takes place in the peroxisomes. Each cycle of FAO removes a two carbon acetyl-CoA unit from the fatty acid chain, resulting in a product that is shorter, and which is directed back through the cycle for removal of additional two carbon units. Intermediates of FAO can be measured in the blood, urine and other body fluids, and in the media of human fibroblasts grown in cell culture. 3-Hydroxy-fatty acids (3-OHFAs) are intermediates of this pathway, and the quantitative measurement of the 3-hydroxy-fatty acids has been used clinically to help diagnose disorders of the pathway involving the 3-hydroxyacyl-CoA dehydrogenase step [6,7], and as a research tool to study treatment options [8,9].

One of the standards of care for individuals who have defects in long-chain FAO enzymes is to supplement their diet with medium-chain fatty acids [10–12]. This therapeutic intervention supplies energy from fats and at the same time bypasses the metabolic block. In the process of studying the effect of adding medium-chain fatty acids to fibroblast cell culture, we made a previously undocumented observation. When medium-chain length fatty acids are

☆ *Abbreviations:* LCHAD, long-chain L-3-hydroxyacyl CoA dehydrogenase; MCTs, medium-chain triglycerides; FAO, fatty acid oxidation; FAS, fatty acid synthesis; 3-OHFAs, 3-hydroxy-fatty acids; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; FA, fatty acid; GC/MS, gas chromatography/mass spectrometry; PBS, phosphate-buffered saline.

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added to culture media of fibroblasts, the resulting 3-hydroxy-fatty acid intermediates are two carbons longer than the precursor substrate. This is true in fibroblasts from individuals with normal FAO, and to a lesser extent in fibroblasts from long-chain L-3-hydroxyacyl CoA dehydrogenase (LCHAD) deficient patients. This study reports on our investigations of this novel observation.

Methods

Samples. Fibroblasts used in this study were from patients who had fibroblasts submitted for FAO studies, but were confirmed as having normal FAO. Eight different normal cell lines were used during the course of the study. Fibroblasts from LCHAD deficient cell lines were as previously described [7]. This study has IRB approval from the University of Texas Southwestern Medical Center of Dallas.

Materials. Low-glucose DMEM and fetal bovine serum were obtained from Life Technologies (Rockville MD, USA). Octanoate, decanoate, laurate, palmitate, fatty acid free bovine serum albumin (BSA), cerulinen, sodium sulfate, sodium acetate, and 2-phenylbutyric acid were obtained from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide, 13-C1-octanoate, 13-C1-decanoate, 13-C1-laurate, 13-C12-laurate, 13-C1-myristate, and 13-C1-palmitate were obtained from Sigma–Aldrich. 3-OHFA stable isotope standards were synthesized and used as previously described [6]. 14-C-acetate was obtained from Amersham (Buckinghamshire, UK). HPLC-grade ethyl acetate and HCl were obtained from Mallinkrodt (St. Louis, MO, USA). Anhydrous ethanol was obtained from Electron Microscopy Services (Ft. Washington, PA, USA). *N*,*O*-bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (99:1) was obtained from Supleco (Bellefonte, PA, USA).

Media preparation. All fatty acids, non-isotopic and isotopic, were dissolved in anhydrous ethanol to a concentration of 100 mmol/L to make each stock fatty acid. The stock fatty acids were then added to 40 g/L bovine serum albumin (BSA) to a final concentration of 1.0, 3.0 or 5.0 mmol/L and incubated at 37 °C for 30 min to allow binding of the fatty acid (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, as previously described [7].

For fatty acid synthesis (FAS) experiments, 14-C-acetate and cold sodium acetate were added to the medium in a final concentration of 1.8 mmol/L sodium acetate with 0.3 μCi/ml 14-C-acetate added. 14-C-Actetate incorporation into lipid material was measured as described below. Cerulinen was diluted in DMSO to a final concentration of 10 mg/ml and added to media at a concentration of 10 μg/ml so that DMSO concentration in the media did not exceed 0.1%. Cells were incubated overnight, or as described for the timed experiments, then the medium was collected and analyzed as previously described for 3-OH-fatty acids [7]. Cells were harvested for measurement of FAS as follows: flasks of cells were washed twice with 2 ml each of PBS and washes were discarded. Two milliliters of 2 M NaOH was added to each flask and cells were allowed to digest for 30 min at room temperature. Flasks were agitated forcefully to remove and break up cells. One hundred microliters of this cell/NaOH mixture was pipetted into labeled microcentrifuge tubes for Lowry protein determinations as previously described [13]. The remainder was transferred to 10 ml glass tubes, capped tightly, and digested at 80 °C for one hour. After digestion, 2.3 ml of 2 M HCl was added to each tube and the tubes were vortexed. Tubes were then extracted twice with 4 ml of ethyl acetate each time, with the top ethyl acetate layer being transferred to 15 ml conical centrifuge tubes. The combined ethyl acetate extractions were then washed with 4 ml of 2 M HCl, followed by 4 ml of de-ionized water. Five milliliters of the washed ethyl acetate extraction was then transferred to scintillation vials and dried down under nitrogen at 37 °C. After drying, 4 ml of scintillation fluid was added and the vials were counted in a liquid scintillation counter.

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 days after subculture. To run an experiment, medium was removed from the cells and 5 ml of the experimental medium was added. Flasks were incubated from 24 to 96 h at 37 °C in a 5% CO_2–95% air incubator. After incubation, media were removed and saved for analysis, and cells were returned to DMEM–10% FBS or used for FAS analysis.

Extraction and analysis. Media sample extraction, derivatization, analysis by GC/MS, and data analysis were performed as previously described [7]. Assay of the 13-C-isotopic medium-chain 3-OHFAs was achieved by analyzing for compounds that were one atomic weight unit higher than their native counterparts. For example, if 13-C1-laurate was used, the sample was analyzed for not only native 3-OH-laurate with an ionic mass/charge (m/z) ratio of 345, but also a 3-OH-laurate with a stable isotope 13-carbon and an m/z ratio of 346.

Results

Previously, we have seen that incubation of skin fibroblasts in culture media that has been supplemented with fatty acids will result in an accumulation of the 3-OH-intermediates of FAO of the same chain length as the added species, as well as shorter 3-OH-species over time. As can be seen in Fig. 1, addition of octanoate to either normal or LCHAD deficient fibroblasts results in increasing accumulation of 3-OH-octanoate over time. Addition of palmitate shows little accumulation of any 3-OH-species over time in normal cells (~200 nM as opposed to >800 nM, Fig. 1) and mainly accumulation of the long-chain forms in cells deficient in the long-chain FAO enzymes.

An intriguing finding that is especially pronounced with normal fibroblasts is that the addition of medium-chain fatty acids to the culture medium results in accumulation of 3-OH-intermediates that are two carbons longer than the added species. Fig. 2 demonstrates that the longer the added species, the more two carbon addition appears to take place, so that addition of laurate results in a greater increase in 3-OH-myristate, than addition of octanoate results in accumulation of 3-OH-decanoate. This effect is more noticeable in normal cells than in LCHAD deficient cells (Fig. 2).

To determine whether this accumulation of the two carbon longer form (2 + C) is arising directly from the added substrate, we added stable isotope labeled medium-chain fatty acids containing a 13-C in place of the normal 12-C in position 1 of the fatty acid carbon chain and followed the stable isotope into the 3-OH-intermediate. Table 1 demonstrates the % accumulation of isotope-labeled 3-OH-intermediates from cells that have had medium- and longer-chain fatty acids added to the culture medium. As can be seen, in normal cells the isotopic form is predominately found in the 2 + C intermediate for chain lengths of C10 through C14. In LCHAD deficient cells the 3-OH intermediates are more evenly distributed between the same chain length intermediate and the 2 + C intermediate. This table demonstrates that the increase in the 2 + C intermediates is coming directly from the added substrate, as it shows that when a stable isotope labeled medium-chain fatty acid is added to the fibroblast culture medium the stable-isotope label is detected predominately in the longer metabolite form.

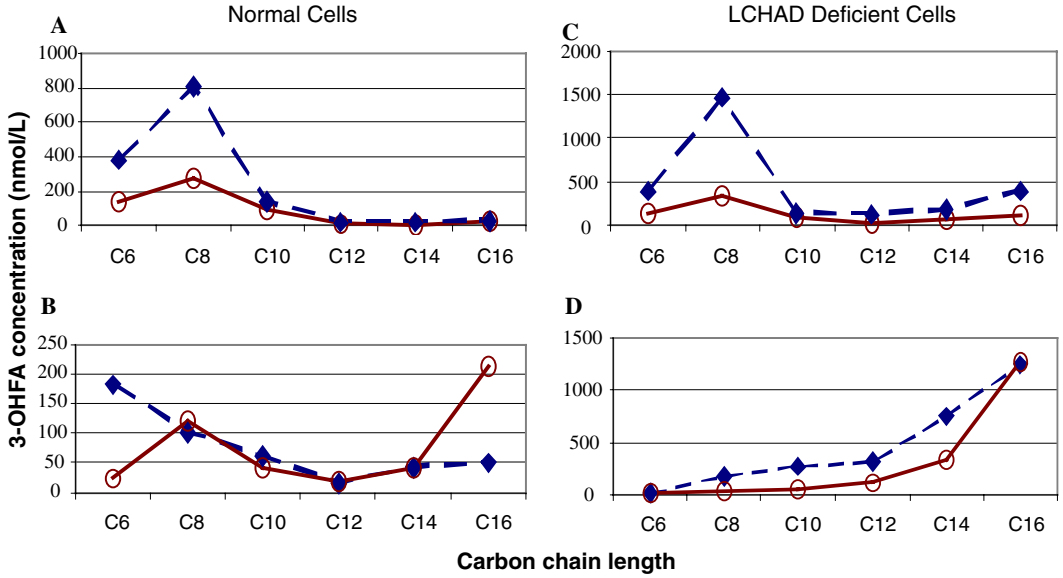

Fig. 1. Accumulation of 3-OH-fatty acid intermediates in the culture medium of normal cells supplemented with octanoate (A) or palmitate (B), and LCHAD deficient cells supplemented with octanoate (C) or palmitate (D). Twenty-four (-○-) and 96 h (- - -♦- - -) time points are depicted in each panel.

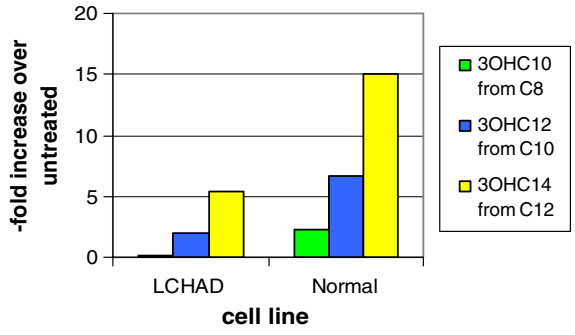

Fig. 2. Increase in accumulation of 3-OH-fatty acids in media from LCHAD deficient and normal fibroblasts incubated in medium containing 500 μmol/L medium-chain fatty acids. Addition of octanoate (C8) causes accumulation of 3-OH-C10. Addition of decanoate (C10) accumulates 3-OH-C12. Addition of laurate (C12) accumulates 3-OH-C14. Results are means of six experiments.

Table 1
Percent accumulation of 13C-isotopic 3-OH-intermediates

Chain length of added fatty acid	Normal cells 13-C-intermediates (%)[a]		LCHAD deficient cells 13-C-intermediates (%)[a]	
	Same length	+2 carbons	Same length	+2 carbons
C8	69	17	28	13
C10	10	78	59	36
C12	4	74	42	47
C14	5	80	38	33
C16	30	10	57	2
C12 + cerulinen	24	52		

[a] Percentages do not add up to 100% because other chain length forms are present in minor amounts.

In Fig. 3 the top panel demonstrates that this increase in the 2 + C form continues to appear, and that over time the isotope does not appear in the shorter-chain length 3-OHFA, suggesting that the isotope labeled carbon is not being cycled through the FAO pathway. Fig. 3 demonstrates this effect for laurate addition, but we also observed it for other medium-chain fatty acids. We further investigated this phenomenon by adding 13-C12 labeled laurate to the cells. This form of laurate has the stable-isotope on the opposite end, carbon number 12 instead of carbon 1. Fig. 3 bottom panel shows that after 96 h of incubation, isotope from the C12 shows up in the shorter 3-OH intermediates, unlike the isotope from C1 labeled laurate, which stays in the 3-OH-C14 form. Fig. 4 demonstrates the accumulation after 96 h incubation of the isotope labeled intermediates as well as the untagged 3-OH-intermediates, with increasing accumulation of the shorter chain length untagged forms. Since the untagged shorter forms appear from both the carbon 12 labeled and carbon 1 labeled laurates, these results suggest that the laurate is being processed through the FAO pathway, but the C1 carbon is retained in the 3-OH-myristate form during the process, whereas the C12 carbon end of the molecule becomes shorter and shorter.

Since the addition of medium-chain fatty acids has also been reported to generate the synthesis of longer-chain fatty acids [14], we considered the possibility that these two carbons were being added as part of the fatty acid synthetic pathway. We ran a series of experiments to measure fatty acid synthesis (FAS) and then block its action with the FAS inhibitor, cerulinen. Cerulinen has been shown to block FAS by many reports in the literature [15–19]. Table 2 shows the results of the FAS experiments and demonstrates that addition of laurate increases FAS, and addition of cerulinen blocks FAS. The use of cerulinen to block fatty acid synthesis still allowed the accumulation of 2 + C form as shown in the bottom of Table 1, although the accumulation was not as great and more accumulation of the

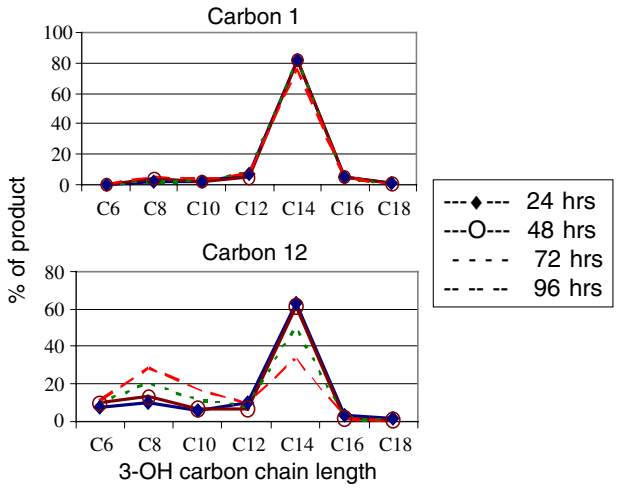

Fig. 3. Percent of 13-C stable isotope-labeled 3-OH-fatty acid intermediates accumulated over time in medium from normal fibroblasts incubated in medium containing 500 μmol/L 13-C1-laurate (top panel) or 13-C12-laurate (bottom panel). Results are means of six experiments.

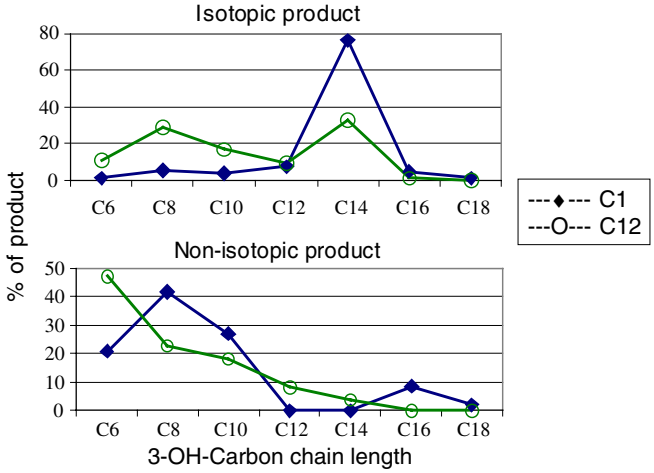

Fig. 4. Percent of both isotope-tagged product (top panel) and non-isotopic product (bottom panel) which accumulated in the medium of normal fibroblasts after 96 h of incubation in medium containing 500 μmol/L 13-C1-laurate or 13-C12-laurate. Results are means of five experiments.

Table 2
Results of fatty acid synthesis experiments

Condition	Mean activity (nmol 14-C acetate/24 h/mg protein)	% change in activity from baseline
Baseline FAS	18.44	
+Cerulinen	4.25	77% decrease
+Laurate	35.52	193% increase
+Laurate and cerulinen	2.87	84% decrease 92% decrease from laurate alone

same chain length form was seen. These results suggest that this 2 + C form may occur as a branch point between FAO and FAS, but the addition is not occurring as part of the FAS pathway alone. Cerulinen has also been reported to inhibit FAO to some degree by the build up of malonyl-CoA [18], and we did see some apparent inhibition of FAO in these cells with a minor accumulation of all chain length 3-OH-intermediates (results not shown). The decreased accumulation of the 2 + C form may be due to this slight FAO inhibition.

Discussion

Normal oxidative metabolism of fatty acids by mitochondria utilizes predominantly long-chain C16, C18 forms. Medium-chain triglycerides (MCT) have been in use as an artificial dietary supplement since they were first introduced in the 1950s for the treatment of lipid absorption disorders. Since that time they have been used for treatment of a variety of medical conditions, including such diverse conditions as epilepsy and obesity [20,21]. Medium-chain length fatty acids in the form of MCT are commonly used in intravenous nutrition [22,23] and have been especially useful as therapeutic intervention dietary supplements in preterm and low birth weight infants [24,25] and in individuals with defects of long-chain fatty acid oxidation [10]. Because they are utilized in so many therapeutic regimens without fully understanding the metabolic processes, understanding and defining the pathways involved in metabolizing these compounds represent important fields of study.

A sophisticated diagram of fatty acid β-oxidation can be found elsewhere [3]. Basically, fatty acids with carbon chain lengths of C12 and below are oxidized by mitochondrial matrix enzymes, while longer chain lengths are oxidized by enzymes bound to the inner mitochondrial membrane. The process that transfers substrate from membrane to matrix is completely unknown. The observation that medium chain-length fatty acids accumulate a 2 + C intermediate of FAO and this particular accumulation is not seen in longer than C14 or shorter than C8 chain lengths was a surprising observation to us which is always reproducible. The observation suggests that a previously undocumented process involving the matrix enzymes or the transfer of substrate between membrane-bound and matrix enzymes must be involved. The transfer point is the chain length at which fatty acids stop being oxidized by membrane-bound enzymes and begin to be oxidized by matrix enzymes and is probably at C14–C12. The results of our study indicate that in this process lies an additional step, which adds two carbons to the fatty acid prior to continuing the fatty acid oxidative process.

The fact that LCHAD deficient fibroblasts consistently show this effect to a much lesser extent than normal fibroblasts also suggests that a step of the FAO pathway rather than the FAS pathway is involved and that the membrane-bound long-chain FAO enzymes may be responsible. Alternately, it is known that shorter-chain fatty acids do not require carnitine and the carnitine cycle to enter mitochondria, but the exact mechanism of this carnitine-independent

transport is unknown. Activation by chain elongation may be an essential component of the pathway for medium-chain fatty acid utilization. Because FAO takes place inside the mitochondria, related pathways for transport of fatty acids into and out of the mitochondria may also be involved in handling of the fatty acids and the intermediates of this pathway. It is possible that a 2 + C form is being generated in the process of release from the cell. However, it seems unlikely that this would occur for C10–C14 chain lengths and not for shorter or longer-chain length intermediates.

In conclusion, we have shown that in cell culture conditions a process that is independent of FAS is required to chain-elongate medium-chain fatty acids prior to oxidation. Any genetic or acquired abnormalities in this process will have a significant impact on the success of energy generation from MCT feeding regimens. Future studies will be designed to identify the components of this novel metabolic pathway of energy metabolism.

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