Peroxisomes contribute to the acylcarnitine production when the carnitine shuttle is deficient

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ABSTRACT

Fatty acid β-oxidation may occur in both mitochondria and peroxisomes. While peroxisomes oxidize specific carboxylic acids such as very long-chain fatty acids, branched-chain fatty acids, bile acids, and fatty dicarboxylic acids, mitochondria oxidize long-, medium-, and short-chain fatty acids. Oxidation of long-chain substrates requires the carnitine shuttle for mitochondrial access but medium-chain fatty acid oxidation is generally considered carnitine-independent. Using control and carnitine palmitoyltransferase 2 (CPT2)- and carnitine/acylcarnitine translocase (CACT)-deficient human fibroblasts, we investigated the oxidation of lauric acid (C12:0). Measurement of the acylcarnitine profile in the extracellular medium revealed significantly elevated levels of extracellular C10- and C12-carnitine in CPT2- and CACT-deficient fibroblasts. The accumulation of C12-carnitine indicates that lauric acid also uses the carnitine shuttle to access mitochondria. Moreover, the accumulation of extracellular C10-carnitine in CPT2- and CACT-deficient cells suggests an extramitochondrial pathway for the oxidation of lauric acid. Indeed, in the absence of peroxisomes C10-carnitine is not produced, proving that this intermediate is a product of peroxisomal β-oxidation. In conclusion, when the carnitine shuttle is impaired lauric acid is partly oxidized in peroxisomes. This peroxisomal oxidation could be a compensatory mechanism to metabolize straight medium- and long-chain fatty acids, especially in cases of mitochondrial fatty acid β-oxidation deficiency or overload.

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1. Introduction

Fatty acid β-oxidation is an important source of energy production in mammals and may occur in both mitochondria and peroxisomes. Although the mechanisms of mitochondrial and peroxisomal β-oxidation are similar, they serve different functions in the cell, evidenced by the different clinical manifestations associated with inherited disorders in mitochondrial [1] or peroxisomal β-oxidation [2]. After transport to the cell by proteins such as the plasma membrane fatty acid binding protein (FABPpm), the fatty acid translocase (FAT/CD36) and members of the fatty acid transport protein family (FATP), fatty acids may be activated to the respective acyl-CoA esters by one of the acyl-CoA synthetases existing in the cell [3]. These enzymes have different substrate specificities and tissue expression and are present at the cytoplasmic side of the plasma and mitochondrial membranes, within the mitochondrial matrix, in peroxisomes and in the endoplasmic reticulum [4]. Peroxisomal and mitochondrial β-oxidation essentially differs in substrate specificities and transport of substrates and products of β-oxidation across the membrane. Short- and medium-chain fatty acids allegedly undergo oxidation only in mitochondria and the β-oxidation of long-chain fatty acids is also believed to occur predominantly in this compartment. Peroxisomes oxidize specific carboxylic acids such as very long-chain fatty acids, branched-chain fatty acids, bile acids, and fatty dicarboxylic acids (DCAs). The fatty acids designated to undergo peroxisomal β-oxidation likely enter peroxisomes as acyl-CoA esters and the candidates to perform this transport are the ATP-binding cassette (ABC) transporters, proteins that couple ATP hydrolysis to substrate transport [5]. Mammalian peroxisomes contain three different half ABC transporters, including the adrenoleukodystrophy protein (ALDP or ABCD1), the ALD-related protein (ALDRP or ABCD2) and the 70-kDa peroxisomal membrane protein (PMP70 or ABCD3) [5,6]. Although the individual functions of the peroxisomal ABC transporters remain to be established, it has been suggested that

PMP70 is involved in the transport of long-chain fatty acids (LCFAs) across the peroxisomal membrane due to an increase in the rate of palmitic acid β-oxidation in cells overexpressing this protein [7]. Once in the peroxisome, acyl-CoAs are initially oxidized until the carbon chain is shortened to C8- or C6-CoA. These medium-chain substrates are the end products of peroxisomal β-oxidation. After conversion to acylcarnitines, by carnitine octanoyltransferase (CrOT) or peroxisomal carnitine acetyltransferase (CrAT), these intermediates are exported from the peroxisome by a still unknown mechanism for further oxidation to acetyl-CoA in mitochondria. It has been shown that peroxisomes can handle long-chain acyl-CoAs with decreasing affinity to medium-chain substrates [8], but the contribution of these organelles to the oxidation of straight medium- and long-chain fatty acids is not clear.

Mitochondrial oxidation of LCFAs (generally considered from C14 to C20) requires conjugation with carnitine. After cytosolic activation of the LCFAs to the respective acyl-CoA esters, they are able to cross the mitochondrial outer and inner membranes by the action of the carnitine shuttle. This system is composed by carnitine palmitoyltransferase 1 (CPT1), carnitine/acylcarnitine translocase (CACT) and carnitine palmitoyltransferase 2 (CPT2). CPT1 converts the long-chain acyl-CoAs into the respective acylcarnitines which are further translocated through the inner mitochondrial membrane via CACT. In the mitochondrial matrix CPT2 reconverts them to the respective acyl-CoA which can enter the β-oxidation pathway for degradation and energy production [1]. Contrary to LCFAs, the oxidation of medium-chain fatty acids (MCFAs; usually considered from C6 to C12) is believed to be largely independent of the carnitine shuttle [9–13]. Indeed MCFAs are able to cross the inner and outer mitochondrial membranes by diffusion and are activated to CoA esters by medium-chain acyl-CoA synthetases localized in the matrix [14]. Nevertheless, due to the broad specificity of the cytosolic long-chain acyl-CoA synthetases, MCFA esterification may also occur in the cytosol, as suggested in previous studies showing MCFA elongation [15,16]. Esterification of MCFAs in the cytosol would involve the carnitine shuttle in the transport of these intermediates into mitochondria for further oxidation. Some studies point to stimulation of MCFA oxidation by carnitine [17–19], as well as altered plasma and urinary concentrations of carnitine esters following medium-chain triglyceride ingestion [20–22]. This suggests that carnitine may influence MCFA metabolism.

Here we show that the carnitine shuttle contributes significantly to the oxidation of the MCFA lauric acid (C12:0), as evidenced by its impairment in CPT2- or CACT-deficient cells. In this situation, lauric acid is directed instead to the peroxisome where it undergoes additional cycles of β-oxidation. This suggests that peroxisomes may have a role in the oxidation of straight medium- and long-chain substrates, particularly when mitochondrial fatty acid β-oxidation is defective or overloaded.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), minimal essential medium (MEM), penicillin, streptomycin and Hepes were obtained from Gibco Invitrogen (Carlsbad, California, USA). Fetal bovine serum and L-glutamine were from BioWhittaker Lonza (Verviers, Belgium). Lipofectamine 2000, Trizol and the Superscript II Reverse Transcriptase kit were purchased from Invitrogen (Carlsbad, CA, USA). The LC480 Sybr Green I Master mix was from Roche (Mannheim, Germany). The [U-^{13}C]-palmitic acid (C16:0) was obtained from Advance Research Chemicals (Catoosa, OK, USA) and D3-lauric acid (C12:0) was from Cambridge Isotope Lab (Andover, MA, USA). The internal standards D3-propionyl- (D3-C3-), D3-octanoyl- (D3-C8-) and D3-palmitoyl-carnitine (D3-C16-carnitine) were synthesized by Herman ten Brink (VU Medical Center, Amsterdam, The Netherlands). For immunofluorescence analysis the biotinylated donkey anti-rabbit Ig was purchased from Amersham and the streptavidin-labeled fluorescein

isothiocyanate (streptavidin-FITC) was obtained from Dako (Denmark). Puromycin, human serum albumin (HSA), bovine serum albumin (BSA), ferrocenium hexafluorophosphate, L-cysteine, L-carnitine and acetylchloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Cell culture conditions

Human skin fibroblasts were obtained from anonymized controls ($n = 4$) and patients with established deficiencies at the level of CPT2 ($n = 1$; p.[R124X];[R124X]), CACT ($n = 2$; c.241G > A apparent homozygous on cDNA and c.[3G > A];[417 + 1G > T]), MCAD ($n = 2$; p.[K329E]; [K329E] and p.[S245L]; [S245L]), PEX5 ($n = 1$; p.[N526K]; [N526K]) and PEX19 ($n = 1$; p.[M255NfsX24]; [M255NfsX24]).

HEK293 cells and primary skin fibroblasts were cultured in DMEM with 4.5 g/L glucose, 584 mg/L L-glutamine and 25 mM Hepes, supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 100 mg/mL fungizone, in a humidified atmosphere of 5% CO_2, at 37 °C.

2.3. Knockdown of peroxisomal biogenesis factor 13

Peroxisomal biogenesis factor 13 (PEX13) shRNA containing viruses were produced in HEK293 cells using MISSION shRNA targeting the gene coding for PEX13 (Sigma-Aldrich TRCN0000083622). HEK293 cells were co-transfected with pMD2G, pMDL/RRE, pRSV/REV and the shRNA TRC vector targeting PEX13 using Lipofectamine 2000. After 24 h the medium was refreshed. In the next day the virus-containing medium was collected, centrifuged and stored at −80 °C prior to transduction in order to prevent contamination with HEK293 cells. Primary skin fibroblasts from controls and CPT2- and CACT-deficient cell lines were incubated with the virus-containing medium. After 24 h, the medium was refreshed and puromycin was added to a concentration of 5 μg/mL to select for transduced cells.

2.4. Immunofluorescence analysis of peroxisomes

Immunofluorescence analysis was performed essentially as described before [23]. Briefly, cultured fibroblasts were washed twice with 10 mg/mL BSA in phosphate buffered saline (PBS) and fixed for 20 min with 2% paraformaldehyde in PBS/0.1% Triton X-100. Cells were washed twice with PBS/0.1% Triton X-100, and free aldehyde groups were blocked by incubation for 10 min in 0.1 M NH_4Cl in PBS. Cells were washed three times with 10 mg/mL BSA in PBS and incubated for 45 min with anti-catalase [24]. Cells were washed three times with 10 mg/mL BSA in PBS, incubated for 30 min with biotinylated donkey anti–rabbit Ig and stained with streptavidin-FITC.

2.5. Incubation of human fibroblasts with fatty acids

The acylcarnitine profiling in human fibroblasts was performed essentially as described before by Ventura et al. [25] with minor modifications. Fibroblasts were seeded in 48 well plates (approximately 12.5 μg protein per well) and incubated overnight at 37 °C. The following day, after washing the cells with PBS, the incubation mixture was added to each well and incubated in a humidified CO_2 incubator (5% CO_2, 95% air) at 37 °C. Incubation mixtures contained MEM supplemented with BSA (essentially fatty acid free), 0.4 mM L-carnitine and 120 μM D3-lauric acid (C12:0) or [U-^{13}C]-palmitic acid (C16:0) to a final volume of 250 μL. Fatty acids were previously solubilized in ethanol. For the inhibition studies, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA) and L-aminocarnitine (L-AC) were added to the incubation mixture to a final concentration between 0–10 μM and 0–2000 μM, respectively. After 72 h, the medium was collected and the cells were washed and resuspended in 100 μL of PBS with 1 g/L Triton X-100 to measure