Fatty acid oxidation in health and disease

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Chapter 2

Functional redundancy of mitochondrial enoyl-CoA isomerases in the oxidation of unsaturated fatty acids

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Abstract

Mitochondrial enoyl-CoA isomerase (ECI1) is an auxiliary enzyme involved in unsaturated fatty acid oxidation. In contrast to most of the other enzymes involved in fatty acid oxidation, a deficiency of ECI1 has yet to be identified in man. We used WT and Eci1-deficient mice to explore a potential presentation of human ECI1 deficiency. Upon fasting, Eci1-deficient mice displayed normal blood β-hydroxybutyrate levels (WT 1.09 mM vs. KO 1.10 mM), a trend to lower blood glucose levels (WT 4.58 mM vs. KO 3.87 mM, P=0.09) and elevated blood levels of unsaturated acylcarnitines, in particular C12:1 acylcarnitine (WT 0.03 μM vs. KO 0.09 μM, P<0.01). Feeding an olive oil rich diet induced an even greater increase in C12:1 acylcarnitine levels (WT 0.01 μM vs. KO 0.04 μM, P<0.01). Overall, the phenotypic presentation of Eci1-deficient mice is mild, possibly caused by the presence of a second enoyl-CoA isomerase (Eci2) in mitochondria. Knockdown of Eci2 in Eci1-deficient fibroblasts caused a more pronounced accumulation of C12:1 acylcarnitine upon incubation with unsaturated fatty acids (12-fold, P<0.05). We conclude that Eci2 compensates for Eci1 deficiency explaining the mild phenotype of Eci1-deficient mice. Hypoglycemia and accumulation of C12:1 acylcarnitine might be diagnostic markers to identify ECI1 deficiency in humans.
Introduction

Mitochondrial fatty acid β-oxidation (FAO) is the most important pathway for the degradation of saturated and unsaturated fatty acids. The β-oxidation cycle of fatty acids consists of four sequential reactions: dehydrogenation, hydration, a second dehydrogenation, and thiol-olytic cleavage. Every cycle shortens the acyl-CoA by two carbon atoms and an acetyl-CoA is generated. In addition to the set of enzymes involved in this β-oxidation cycle, the degradation of unsaturated fatty acids requires the obligatory participation of a set of three auxiliary enzymes: the 2,4-dienoyl-CoA reductase (DECR), the Δ3,5,Δ2,4-dienoyl-CoA isomerase, and the Δ3,Δ2-enoyl-CoA isomerase (ECI) (Hiltunen and Qin, 2000). An important reason for this phenomenon is that the first dehydrogenase in the oxidation cycle has a trans-2-enoyl-CoA intermediate as the product and therefore cannot metabolize directly the cis-3-enoyl-CoA configuration of unsaturated fatty acyl-CoAs. The combined set of three auxiliary enzymes can position different combinations of double bonds in the acyl-CoA to the trans-2 configuration, allowing reentrance into the β-oxidation cycle (Schulz and Kunau, 1987). The action of these three auxiliary enzymes is illustrated by the metabolism of oleic acid (figure 1a) and linoleic acid (figure 1b). Oleoyl-CoA (cis-9-C18:1-CoA) is converted by two cycles of β-oxidation to cis-5-C14:1-CoA, the substrate for long-chain acyl-CoA dehydrogenase (LCAD) that further converts it into trans-2,cis-5-C14:2-CoA. From thereon, the oxidation can occur via an isomerase- or a reductase-dependent pathway, but the major route is via the isomerase-dependent pathway (85%) (Ren and Schulz, 2003). For the reductase-dependent pathway, the auxiliary enzymes Δ3,5,Δ2,4-dienoyl-CoA isomerase and DECR are needed. Both routes require the involvement of ECI (Ren and Schulz, 2003) (figure 1a). ECI catalyzes the conversion of the double bond in cis-3-enoyl-CoA or trans-3-enoyl-CoA to trans-2-enoyl-CoA (figure 1a). In the reductase-dependent pathway, ECI is also responsible for the reverse reaction in which the trans-2,cis-5-C14:2-CoA is converted into trans-3,cis-5-C14:2-CoA (figure 1a). Linoleoyl-CoA (cis-9,12-C18:2-CoA) is converted by three cycles of β-oxidation to cis-3,6-C12:2-CoA. From thereon, the auxiliary enzymes ECI and DECR are needed (figure 1b) (Schulz and Kunau, 1987).

Different ECIs have been purified, cloned, and characterized (Geisbrecht et al., 1999; Müller-Newen and Stoffel, 1991; Palosaari et al., 1990; Stoffel et al., 1964). ECI can be present either as a monofunctional protein or as an integral part of the L-bifunctional protein also known as multifunctional β-oxidation enzyme type 1 (EHHADH) (Palosaari and Hiltunen, 1990), but in all cases, the ECI belongs to the low similarity isomerase/hydratase superfamily of proteins (Müller-Newen et al., 1995; Palosaari et al., 1991). In mammalian cells, three proteins with ECI activity have been characterized, including the mitochondrial ECI localized exclusively in mitochondria (encoded by Ec1, formerly known as Dci (Müller-Newen and Stoffel, 1991; Palosaari et al., 1990; Stoffel et al., 1964)), the peroxosomal ECI localized in mitochondria and peroxisomes (encoded by Ec12 formerly known as Peci (Geisbrecht et al., 1999)), and EHHADH localized in peroxisomes (encoded by Ehhadh (Palosaari and Hiltunen, 1990)). Recently, we discovered a fourth ECI (Eci3), which is a mouse and rat specific protein (Ofman et al., 2006). This ECI is expressed in peroxisomes of mouse kidney and more ubiquitous in peroxisomes of rat tissue.

Although a human inherited metabolic disease has been described for most of the mitochondrial FAO enzymes, patients with ECI1 deficiency have yet to be identified. Such patients might have been missed because of unusual or mild phenotypes. To obtain more insight in the role of ECI1 in mitochondrial FAO, we further characterized a previously ge-
generated Eci1-deficient mouse model (Janssen and Stoffel, 2002). This mouse model might be suitable to identify potential presentations of human ECI1 deficiency as well as to define diagnostic markers for ECI1 deficiency. It was reported that Eci1-deficient mice showed hepatic accumulation of lipids with unsaturated fatty acyl groups and developed dicarboxylic aciduria (Janssen and Stoffel, 2002). We compared WT and Eci1-deficient mice on a normal chow diet with overnight fasted mice or mice on a diet containing a high content of unsaturated fatty acids. We found that Eci1-deficient mice have a tendency to develop hypoglycemia and are characterized by a unique and novel aberrant acylcarnitine profile. In addition, we show that the relatively mild phenotypic presentation of Eci1-deficient mice is caused by functional redundancy with Eci2.

**Mitochondrial β-oxidation of oleoyl-CoA (C18:1) and linoleoyl-CoA (C18:2)**

![Diagram of β-oxidation pathways](image)

**FIGURE 1.** Mitochondrial β-oxidation of oleoyl-CoA and linoleoyl-CoA. A. The β-oxidation of oleoyl-CoA (C18:1-CoA) via the isomerase-dependent route and the reductase-dependent route. The major route for oleoyl-CoA oxidation is via the isomerase-dependent route (85%). Figure based on (Ren and Schulz, 2003). B. The β-oxidation of linoleoyl-CoA (C18:2-CoA). Figure based on (Schulz and Kunau, 1987).
Experimental procedures

Materials
[U-13C]-oleic acid (C18:1, cis-9) was purchased from Cambridge Isotope Laboratories, [U-13C]-linoleic acid (C18:2, cis-9,12), myristoleic acid (C14:1, cis-9), dodecenoic acid (C12:1, cis-5), C12:0-CoA, and C14:0-CoA were purchased from Sigma. 3-nonenoi acid was obtained from TCI Europe. The synthesis of 3-nonenoi-CoA was performed as described by Rasmussen et al. (Rasmussen et al., 1990). Purity of the product was determined by high pressure liquid chromatography (HPLC) analysis. [9,10-3H(N)]-palmitic acid and [9,10-3H(N)]-oleic acid were purchased from PerkinElmer and bovine serum albumin (BSA; fatty acid free) from Sigma. The internal standards d3-C0, d3-C3, d3-C6, d3-C8, d3-C10 and d3-C16 carnitine were purchased from Dr. Herman J. ten Brink (VU Medical Hospital, Amsterdam, The Netherlands).

Animal studies
Eci1-deficient mice on a mixed background were obtained from Memorec Biotec GmbH (Köln, Germany) (Janssen and Stoffel, 2002). The colony was maintained by crossing with C57BL/6N (Charles River). After 2 generations of backcrossing with C57BL/6, WT (wild type) and Eci1-deficient mouse were generated via heterozygous breeding pairs. Mice (WT, n=5 and Eci1-deficient mouse, n=7) were housed at 21±1 °C, 40-50% humidity, on a 12h light-dark cycle, with ad libitum access to water and a standard rodent diet. At 6 weeks of age, 50 µl of blood was collected from the vena saphena for the measurements of glucose, acylcarnitines, and ketones. At 7 weeks of age, mice were weighed, placed in a clean cage without food but with access to water, and fasted for 24h. This was followed by blood collection to obtain equivalent measurements in the fasted state. Immediately thereafter, mice were fed an olive oil rich diet (Arie Blok, Woerden, The Netherlands, Diet olive oil, 4021.82, 42 energy % fat; for fatty acid composition see table 1). Regular bleeding via the vena saphena was used for acylcarnitine measurements in blood. At 13 weeks of age, mice were fasted for 24h and anesthetized with an ip injection of 100 mg/kg pentobarbital. Anesthetized mice were euthanized by exsanguination from the vena cava inferior. The heart, liver, and muscles were rapidly excised, weighed, and processed for biochemical and histological analysis. All experiments were approved by the institutional review board for animal experiments at the Academic Medical Center, Amsterdam.

Cell culture
Fibroblasts were obtained from WT and Eci1-deficient mouse (mixed background) ears and cultured in DMEM with glutamine, 10% fetal bovine serum (Gibco), 1% mixture of penicillin, streptomycin, fungizone (Gibco), and incubated in a CO₂ incubator (5% CO₂) at 37°C.  

Table 1: fatty acid content of the olive oil rich diet.

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<td>C18:3ω3</td>
<td>0.61</td>
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<tr>
<td>C20:0</td>
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</table>
Antibodies and immunoblotting
The polyclonal antibodies against Eci1 and Eci2 were a generous gift from Dr. H. Schulz. The Eci2 antibody was affinity purified. Secondary antibodies goat anti-rabbit IRD cw800 were from Li-Cor and immunoblot images were obtained using the Odyssey infrared imaging system (Li-Cor biosciences).

Acylcarnitine analysis
Fibroblasts cultured in 12 well plates were incubated for 72 hours at 37°C, 5% CO₂ in MEM medium with 1% mixture of penicillin, streptomycin and fungizone (Gibco) containing 0.4 mM L-carnitine, 0.4% BSA and 100 μM of the indicated fatty acids. After 72 hours, the incubation was stopped by removing the medium from the cells and processed as described (Chegary et al., 2009) using internal standards (50 pmol d3-C3-, 20 pmol d3-C6-, 20 pmol d3-C8-, 20 pmol d3-C10- and 20 pmol d3-C16-acylcarnitine). Semi-quantitative determination of the formed acylcarnitines in the medium was performed using tandem mass spectrometry (Ventura et al., 1999).

Plasma and blood acylcarnitines were measured as described (Vreken et al., 1999) using internal standards (25 μM d3-C0-, 5 μM d3-C3-, 2 μM d3-C6-, 2 μM d3-C8-, 2 μM d3-C10- and 2 μM d3-C16-acylcarnitine).

Fatty acid β-oxidation measurements
Palmitic and oleic acid oxidation were measured by quantifying the production of ³H₂O from either [9, 10-³H(N)]-palmitic acid or [9, 10-³H(N)]-oleic acid as described previously by Manning et al. (Manning et al., 1990). The assay was performed in quadruplicate. The cells were incubated for 2 hours at 37°C in Krebs-Henseleit buffer containing 0.1% (w/v) BSA, 100 μM palmitic or oleic acid and a tracer of tritiated palmitic or oleic acid. Oxidation rate was expressed as nmol of fatty acid oxidized per hour per milligram of cell protein (nmol/h.mg).

Clinical chemistry measurements
Glucose and β-hydroxybutyrate were measured in blood of WT and Eci1-deficient mouse using standard enzymatic methods (Bergmeyer et al., 1986).

Quantitative real-time PCR
RNA was isolated from mouse tissue using Trizol extraction. cDNA was obtained by using the Superscript II Reverse Transcriptase Kit (Invitrogen). Quantitative real-time PCR analysis of Eci1, Eci2, Decr1, Cpt1a, Acadl, Ech1, Ehhadh, Ppargc1a, and Cyp4a10 were performed using the LC480 Sybr Green I Master mix (Roche). All samples were analyzed in duplicate. Data analysis was done using the linear regression as described by Ramakers et al. (Ramakers et al., 2003). For the comparison of the genes, the values were normalized against the housekeeping gene cyclophilin B. Primer sequences are available upon request.

Synthesis of acylcarnitines standards
Commercially available C12:0-CoA and C14:0-CoA were used as substrates. The first step was the enzymatic conversion of C12:0-CoA or C14:0-CoA to trans-2-C12:1-CoA and trans-2-C14:1-CoA via acyl-CoA oxidase (from Arthrobacter sp., Sigma) (reaction A). For the second step, we used purified mitochondrial ECI (a gift of Dr. H. Schulz, Department of Chemistry, City College and Graduate School of the City University of New York, NY), which converted the formed trans-2-C12:1-CoA or trans-2-C14:1-CoA of reaction A to either a cis-3-C12:1-
CoA and cis-3-C14:1-CoA or a trans-3-C12:1-CoA and trans-3-C14:1-CoA (reaction B). The products of these reactions, as well as chemically synthesized cis-5-C12:1- and cis-9-C14:1-CoA, were then converted from a CoA ester to a carnitine ester by incubation with a homogenate of S. cerevisiae overexpressing CPT1a (reaction C) (IJlst et al., 1998; van Vlies et al., 2007).

Acylcarnitine isomer measurements
An ultra-performance liquid chromatography (UPLC) - tandem mass spectrometry (MS/MS) system was used to analyze the acylcarnitine isomers. Samples were applied on a Waters Acquity BEH C18 column (100 mm x 2.1 mm, 1.7 μm). Separation of acylcarnitines was performed at a flow rate of 500 μL/min using gradient elution involving solvent A (0.1% heptfluorobutyric acid in water) and solvent B (100% methanol). Acylcarnitine esters were eluted using solvent B increasing from 50% up to 85% in 7 min followed by a 2 min washing step with 100% solvent B and re-equilibration of the column for 2 min with 100% solvent A in a total run time of 11 min. Acylcarnitine esters were detected using a Waters Quattro Premier XE MS/MS set at the positive electrospray ionization (ESI) mode using nitrogen as nebulizing gas and argon as collision gas at a pressure of 2.5e-3 mbar. Voltages were set at 3.5 kV capillary, 35 V cone and 25 V collision energies. Acylcarnitine esters were measured using the following transitions: m/z 342.3 > 85.0 for C12:1-carnitine and m/z 370.3 >85.0 for C14:1-carnitine. The system was operated by Masslynx software (v4.1).

Quantification of cis-3-C12:1 and cis-5-C14:1 acylcarnitine by HPLC-MS/MS
Quantification of cis-5-C14:1 acylcarnitine was performed using HPLC-MS/MS as described by Minkler et al. (Minkler et al., 2011; Minkler et al., 2008). cis-5-C14:1 acylcarnitine, d3-cis-9-C14:1 acylcarnitine, and trans-2-C12:1 acylcarnitine were synthesized by small modifications to a standard method (Brendel and Bressler, 1967), purified by cation exchange SPE and preparative HPLC, and standardized by precisely determining their total carnitine content (Minkler et al., 2008). Multiple-point calibration curves were constructed using d3-cis-9-C14:1 acylcarnitine as the internal standard for cis-5-C14:1 and trans-2-C12:1 over 200-fold concentration ranges, ensuring accurate and precise absolute quantification of cis-5-C14:1 acylcarnitine. Quantification of cis-3-C12:1 acylcarnitine was performed using the calibration curve generated from trans-2-C12:1 and assuming that the responses of the two C12:1 acylcarnitines are identical.

Subcellular fractionation of mouse kidney
Subcellular fractionation of mouse kidney was performed as described previously (Ofman et al., 2006). Kidney was obtained from WT and Ec1-deficient mice on a mixed background. Fractions of 1 mL were taken from the bottom of the gradient and were assayed for the marker enzymes glutamate dehydrogenase (GDH) (mitochondria), catalase (peroxisomes),
phosphoglucoisomerase (cytoplasm), β-hexosaminidase (lysosomes) and esterase (micro-somes) as described previously (Wanders et al., 1984; Wanders et al., 1986). Protein concentration was measured according to the method of Bradford (Bradford, 1976), using BSA as standard.

**ECI activity measurements**
The ECI enzyme activity assay was performed by incubating protein samples for 5 minutes at 37°C in a medium containing: 100 mM TRIS buffer (pH 7.4) and 100 μM trans-3-nonenoyl-CoA in a final volume of 100 μl. The reaction was stopped by the addition of 10 μl 2M HCl and neutralized by adding 10 μl 2 M KOH/0.4 M MES. Acetonitrile (ACN) was added to the mixture to a final concentration of 15% (v/v), followed by centrifugation for 5 minutes at 10,000g. The reaction products were analyzed by reverse phase HPLC using a Supelcosil™ LC-18-DB semi-prep column (Supelco analytical). The column was developed isocratically using eluents containing 82% buffer A (10% ACN and 90% 16.9 mM NaPhosphate pH 6.9) and 18% buffer B (70% ACN and 30% 16.9 mM NaPhosphate pH 6.9) for 35 minutes at a flow rate of 3 mL/min followed by a wash step of 100% buffer B for 7 minutes at a flow rate of 3mL/min. Absorbance of the eluate was continuously recorded using a spectrophotometer set at 260 nm. The substrate, trans-3-C9:1-CoA, yields one peak in the chromatogram. Incubation with purified isomerase yields two additional peaks in the chromatogram, the major product trans-2-C9:1-CoA, and a small peak, which we believe is cis-3-C9:1-CoA that can be formed by the reverse reaction. In cell or tissue homogenates, the product trans-2-C9:1-CoA is further converted into L-3-hydroxy-C9:1-CoA as a result of the 2-enoyl-CoA hydratase (crotonase) activity. Therefore, we expressed ECI activity as the sum of the products formed by ECI and 2-enoyl-CoA hydratase, i.e. trans-2-C9:1-CoA and L-3-hydroxy-C9:1-CoA.

**Knockdown of Eci2 in mouse fibroblasts**
For the production of virus containing shRNA against Eci2, HEK293 cells were transfected with pLKO.1-TRC cloning vector, which contains a puromycin resistance cassette and a forward and a reverse oligo annealed (oligonucleotides used are mentioned below), psPAX2 (packaging plasmid for producing virus particles), and pMD2.G (envelope plasmid for producing virus particles) using the lipofectamine 2000 according to the protocol of the manufacturer (Invitrogen). Cells were grown overnight and medium containing the virus with shRNA1, shRNA2, or shRNA3 was collected. WT and Eci1-deficient fibroblasts were exposed to the medium containing the shRNA and were selected by adding 5 μg/mL puromycin. Oligonucleotides used for the knockdown of Eci2, fw shRNA1 primer Mus musculus (Mm) pECI 5’ CCGG AAGCTAAGACTCTATGCACTG CTCGAG CAGTGCATAGTCTTAGCTT TTTTGT 3’, rev shRNA1 primer Mm pECI 5’ AATTCAAAAA AAGCTAAGACTCTATGCACTG CTCGAG CAGTGCATAGTCTTAGCTT TTTTGT 3’, fw shRNA2 primer Mm pECI 5’ CCGG AAGACATCCTGGTGAACCTCTCG CTCGAG CAGAAGTTACCAGGATGTCTT TTTTGT 3’, rev shRNA2 primer Mm pECI 5’ AATTCAAAAA AAGACATCCTGGTGAACCTCTCG CTCGAG CAGAAGTTACCAGGATGTCTT TTTTGT 3’, fw shRNA3 primer Mm pECI 5’ CCGG AAGCCTCTGGTGGGTAGTA CTCGAG TACTACGCAACCAGGCTT TTTTG 3’, rev shRNA3 primer Mm pECI 5’ AATTCAAAAA AAGCCTCTGGTGGGTAGTA CTCGAG TACTACGCAACCAGGCTT TTTTG 3’. All three sets led to knockdown of Eci2 and identical biochemical changes. The results for oligo set number 3 are shown as the knockdown was slightly more efficient for these oligonucleotides.
Statistics
Statistical analysis was performed using Graphpad Prism 5. Data are displayed as the mean ± SD. Differences were evaluated using a two sided t-test or a one-way analysis of variance (ANOVA) with Bonferroni’s Multiple Comparison Test. Statistical significance is indicated as follows: *P < 0.05, **P < 0.01 and ***P < 0.001.

Results

Mild phenotypic presentation of Eci1-deficient mice
Janssen and Stoffel established that upon fasting, the Eci1-deficient mouse accumulates lipids with unsaturated fatty acyl groups in the liver and develops dicarboxylic aciduria (Janssen and Stoffel, 2002). Other characteristic derangements observed in inherited FAO defects such as an aberrant acylcarnitine profile or hypoketotic hypoglycemia were not reported. Initially, we studied WT and Eci1-deficient mouse fibroblasts and determined the impact of Eci1 deficiency on FAO in these fibroblasts. We compared the oxidation rate of oleic acid to palmitic acid and surprisingly found no difference (Table 2). This indicates that in vitro in fibroblasts, oxidation of unsaturated fatty acids is only minimally affected by Eci1 deficiency. To characterize the impact of Eci1 deficiency on the metabolism of unsaturated fatty acids in vivo, we compared WT and Eci1-deficient mice on a normal chow diet in the fed state and after an overnight fast. Upon fasting, Eci1-deficient mice showed a trend to lower blood glucose levels when compared to WT mice (P = 0.09) (figure 2b). Ketone body (i.e. β-hydroxybutyrate) levels in the fed and fasted state were not different between Eci1-deficient and WT mice (figure 2b). Next, we determined the acylcarnitine profile in blood, plasma, liver, and heart of WT and Eci1-deficient mice. Tandem mass spectrometric analysis of the acylcarnitines in body fluids and tissue is the gold standard for the clinical diagnostics of FAO disorders. In the fed condition, Eci1-deficient mice showed a slight accumulation of C10:1, C12:1, and C14:1 acylcarnitines in blood (figure 2c) and plasma (data not shown). After the overnight fast, Eci1-deficient mice displayed an increase in the accumulation of unsaturated acylcarnitines. In blood, we detected the accumulation of C10:1, C12:2, C12:1, C14:2, C14:1 and C16:2 acylcarnitines (figure 2c and 2d). In liver and heart, a slight increase in C12:1 and C14:1 acylcarnitines was observed (figure 2d). Thus Eci1-deficient mice are characterized by a tendency to develop ketotic hypoglycemia and a unique and novel aberrant acylcarnitine profile.

An olive oil rich diet increases unsaturated acylcarnitine accumulation in Eci1-deficient mice
To further define the impact of Eci1 deficiency on the oxidation of unsaturated fatty acids, WT and Eci1-deficient mice were fed an olive oil rich diet containing 42 % of the energy derived from fat (for fatty acid composition see Table 1). Biochemical parameters in blood were measured at several consecutive time points during this feeding regimen. Eci1-defi-

<table>
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<tr>
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<td>nmol/mg.h</td>
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</tr>
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<tr>
<td>WT</td>
<td>10.6</td>
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<tr>
<td>Eci1 KO</td>
<td>12.3</td>
<td>12.4</td>
<td>0.99</td>
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FIGURE 2. Metabolite levels in blood, plasma, liver and heart of WT and Eci1-deficient mice. A. Immunoblot analysis of Eci1 in WT and Eci1-deficient mouse hearts showing the absence of Eci1 in the Eci1-deficient mice. B. Glucose levels (in mmol/L) in blood of overnight fasted WT and Eci1-deficient mouse on a chow diet. Blood β-hydroxybutyrate levels in WT and Eci1-deficient mice on a chow diet in the fed and fasted state (in mmol/L). C. Acylcarnitine profiles in blood of WT and Eci1-deficient mice in the fed and overnight fasted state (in µM). D. Acylcarnitine profiles in liver and heart of WT and Eci1-deficient mice on a chow diet in fasted state. The ratio of the acylcarnitines with acetylcarnitine is displayed. E. Levels of C12:1 and C14:1 acylcarnitine measured in blood of WT and Eci1-deficient mice in the fed and fasted state on a chow diet and in the fed state when fed an olive oil rich diet (ORD) for different duration as indicated. Numbers indicate fold increase. Error bars indicate SD *P<0.05, **P<0.01.
FIGURE 3. UPLC-MS/MS identifies isomers of C12:1 and C14:1 acylcarnitine in blood of Eci1-deficient mice. A. UPLC-MS/MS revealed the accumulation of cis-3-C12:1 acylcarnitine and cis-5-C14:1 acylcarnitine in Eci1-deficient mouse blood. Blood of Eci1-deficient mice is compared with blood from WT and LCAD KO mice. The identity of the peaks is confirmed by comparison of the elution profile with chemically and enzymatically prepared acylcarnitine standards. B. Absolute quantification of cis-3-C12:1 and cis-5-C14:1 acylcarnitine in blood of WT and Eci1-deficient mice using the method of Minkler (Minkler et al., 2008; Minkler et al., 2011).
C12:1 and C14:1 acylcarnitines accumulate in the Eci1-deficient mice

The acylcarnitine profile of overnight fasted mice and mice fed an olive oil rich diet showed an increase in the accumulation of C12:1 and C14:1 acylcarnitine in blood and liver (figure 2c, 2d and 2e). Taking the isomerase- and the reductase-dependent route into account (Ren and Schulz, 2003), we hypothesized that the isomers accumulating were the cis-3-C12:1 and the trans-3-C14:1 acylcarnitines (figure 1). To identify the different isomers of C12:1 and C14:1 acylcarnitine, we used chemically and enzymatically prepared isomers of C12:1 and C14:1 acylcarnitine and analyzed them by UPLC-tandem mass spectrometry. Using this approach, we were able to discriminate between the cis-5, cis-3, trans-3, and trans-2 isomers of C12:1 acylcarnitine, and the cis-9, cis-5, cis-3, trans-3 and trans-2 isomers of C14:1 acylcarnitine (figure 3a). In the blood of Eci1-deficient mice, we detected 4 peaks for C12:1 acylcarnitine identified as cis-5-C12:1 acylcarnitine (peak #2), cis-3-C12:1 acylcarnitine (peak #3), and trans-3-C12:1 acylcarnitine (peak #4, figure 3a, top panel). The fourth peak marked by a question mark may be cis-9 or cis-7-C12:1 acylcarnitine. As expected and consistent with the isomerase-dependent pathway, cis-3 C12:1 acylcarnitine was the most abundant isomer.

For C14:1 acylcarnitine, 2 peaks were identified: cis-9-C14:1 acylcarnitine (peak #1) and cis-5-C14:1 acylcarnitine (peak #2). This accumulation of cis-5-C14:1 acylcarnitine is unexpected and not consistent with the reductase-dependent pathway. We have previously identified cis-5-C14:1 acylcarnitine as the primary accumulating acylcarnitine in the LCAD KO mice (Chegary et al., 2009). The accumulation of cis-5-C14:1 acylcarnitine in Eci1-deficient mice was confirmed by a direct comparison with blood from LCAD KO mice (figure 3a). Thus, accumulation of cis-5-C14:1 acylcarnitine is consistent with inhibition at the level of LCAD. In addition, we accurately quantified cis-3-C12:1 (assumed to have the same response as the standard trans-2-C12:1) and cis-5-C14:1 acylcarnitine (for which we have standardized calibrants). Both acylcarnitines were significantly increased in the blood of Eci1-deficient mice (figure 3b, supplemental figure 1). These data suggest that the breakdown of cis-9-C18:1 occurs primarily via the isomerase-dependent pathway. The accumulation of cis-5-C14:1 indicates that in Eci1-deficient mice, FAO is inhibited also at the level of LCAD. We found no metabolites indicative of the reductase-dependent pathway.

Absence of compensatory upregulation of other ECIs in Eci1-deficient mice

Given the mild phenotypic presentation of Eci1 deficiency in mice, we performed expression analysis to identify potential compensatory mechanisms, with a special focus on the other ECIs and the peroxisome proliferator-activated receptor α (PPARα) signaling pathway. Expression levels of PGC1α (Ppargc1a), Eci2, Ech1, Cpt1a, Cyp4a10, and Decr1 were similar in livers of fasted WT and Eci1-deficient mice (figure 4). There was a small increase in the expression of Acadl, Pdk4 and Ehhadh in the Eci1-deficient mouse liver, suggesting that the PPARα pathway was more active (figure 4). Janssen and Stoffel also observed an increase of selected PPARα target genes after 24h and 48h of fasting, which supports our finding (Janssen and Stoffel, 2002). Despite this finding, these data show that the mild phenotypic
presentation of Eci1-deficient mice is not due to activation of compensatory pathways, as the expression of none of the other ECIs was upregulated at the transcriptional level. This suggests that similar expression levels of other ECIs are sufficient to compensate for Eci1 deficiency.

**Residual ECI activity in mitochondria of Eci1-deficient mice**

It is known that Eci2, formerly known as the peroxisomal ECI, is also localized to the mitochondria (Zhang et al., 2002). To prove that mitochondria harbor more than one ECI, we performed subcellular fractionation of WT and Eci1-deficient mouse kidney and measured the ECI activity in the different fractions. In WT kidney, we detected ECI activity in the mitochondrial fraction. In the mitochondrial fraction of Eci1-deficient kidney, however, there was still substantial residual ECI activity (figure 5). Therefore, we conclude that there is ad-
ditional ECI activity in the mitochondria of the Eci1-deficient mouse.

**Eci2 is responsible for the residual ECI activity in Eci1-deficient fibroblasts**

To prove that Eci2 is responsible for the residual ECI activity in the mitochondria of the Eci1-deficient mouse, we knocked down Eci2 expression in Eci1-deficient fibroblasts. Eci2 protein levels were decreased by 90% in WT and Eci1-deficient fibroblasts as shown by western blot analysis (figure 6a). Furthermore, we measured ECI activity in these fibroblasts (figure 6b). Considerable residual ECI activity was detected in the Eci1-deficient fibroblasts and the WT fibroblasts with a knockdown for Eci2. The most deficient ECI activity was observed in the Eci1-deficient fibroblast, in which Eci2 was knocked down. This small residual ECI activity in the Eci1-deficient fibroblasts with a knockdown for Eci2 may be due to EHHADH, which is localized in the peroxisomes of these fibroblasts, or incomplete Eci2 knockdown. In order to determine the impact of combined Eci1 and Eci2 deficiency on FAO in these fibroblasts, we incubated the fibroblasts with [U-13C]-oleic or [U-13C]-linoleic acid (figure 6c). The formation of acylcarnitines was measured in the culture medium. Upon incubation with oleic acid, the Eci1-deficient fibroblasts have slightly elevated levels of C12:1 and C14:1 acylcarnitines, which became more pronounced after the knockdown of Eci2 (figure 6c). Furthermore, in these fibroblasts, the cis-3-C12:1 and cis-5-C14:1 isomers were responsible for the accumulation of C12:1 and C14:1 acylcarnitines, consistent with the results obtained in the blood of the Eci1-deficient mouse (supplemental figure 1). Knockdown of Eci2 in WT fibroblasts does not lead to a significant increase in acylcarnitines upon oleic acid incubation. Upon incubation with linoleic acid, Eci1-deficient fibroblasts have slightly elevated levels of C10:1, C12:2, C14:2 and C18:2 acylcarnitines. Combined Eci1 and Eci2 deficiency prominently increases C12:2 accumulation and also leads to a further increase of C10:1, C14:2 and C18:2 acylcarnitines (Fig. 6c). Knockdown of Eci2 in WT fibroblasts does not lead to a significant

![FIGURE 6. Eci2 is responsible for the residual ECI activity in the mitochondria of the Eci1-deficient mouse. A. Western blot analysis using antibodies against Eci1 and Eci2 revealed a knockdown for Eci2 of 90% in the WT and Eci1-deficient mouse fibroblasts. B. ECI activity measurements in WT and Eci1-deficients fibroblast with or without Eci2 knockdown. C. Acylcarnitine profile in medium of WT and Eci1-deficient fibroblasts with or without Eci2 knockdown incubated with [U-13C]-oleic or [U-13C]-linoleic acid. Error bars indicate SD *P<0.05, **P<0.01, ***P<0.001.](image-url)
increase in acylcarnitines upon linoleic acid incubation. From these data we conclude that Eci2 is responsible for the residual ECI activity in mitochondria of the Eci1-deficient mouse.

**Discussion**

Several mouse models for FAO disorders have been generated and characterized. Mice deficient in one of the enzymes of FAO may present with a similar phenotype as human patients. For example, the LCAD KO mouse serves as a model for human very long-chain acyl-CoA dehydrogenase deficiency because these mice display hypoketotic hypoglycemia, accumulation of specific acylcarnitines, as well as cardiac hypertrophy (Bakermans et al., 2011; Chegary et al., 2009; Cox et al., 2001; Kurtz et al., 1998). Although a human inherited metabolic disease has been described for most of the mitochondrial FAO enzymes, patients with a deficiency in one of the ECIs have yet to be identified. These patients might have been missed because of unusual or mild phenotypes. To obtain more insight in the role of EC1 in mitochondrial FAO, we further characterized a previously generated Eci1-deficient mouse model (Janssen and Stoffel, 2002). This mouse model might be suitable to identify potential presentations of human EC1 deficiency as well as to define diagnostic markers for EC1 deficiency. We focused on the acylcarnitine accumulation, as this currently constitutes the main tool in the diagnostics of FAO disorders. Initially, we used Eci1-deficient mouse fibroblasts. In these fibroblasts, the rate of oleic acid and palmitic acid oxidation was not different when compared to WT fibroblasts, indicating a mild metabolic defect. Next, we studied Eci1-deficient mice. Overnight fasting or feeding an olive oil rich diet caused accumulation of C12:1 and C14:1 acylcarnitines in blood and tissues of Eci1-deficient mice. Moreover, Eci1-deficient mice display mild fasting-induced ketotic hypoglycemia. Interestingly, Decr1-deficient mice, in which another auxiliary FAO enzyme is defective, also have ketotic hypoglycemia, albeit more severe (Miinalainen et al., 2009). This suggests that defects in different auxiliary enzymes are characterized by ketotic hypoglycemia. Similar to humans, mouse models for FAO defects such as Decr1- and LCAD-deficient mice have a prominent fatty liver (Chegary et al., 2009; Miinalainen et al., 2009). In contrast to the initial observation by Janssen and Stoffel (Janssen and Stoffel, 2002), Eci1-deficient mice do not have hepatic steatosis. This might be caused by a difference in the duration of the fasting period; Janssen and Stoffel analyzed after 48h of fasting, while we performed an overnight fast. Cardiac hypertrophy, which is present in LCAD-deficient mice (Kurtz et al., 1998), was absent in both Eci1- and Decr1-deficient mice ((Miinalainen et al., 2009) and data not shown). Thus overall, the phenotypic presentation of Eci1 deficiency in mice is relatively mild.

By using UPLC-MS/MS, we found that cis-3-C12:1 and cis-5-C14:1 acylcarnitine accumulate and not trans-3-C14:1 acylcarnitine. Accumulation of trans-3-C14:1 acylcarnitine was expected if the oleic acid would be oxidized via the reductase-dependent pathway (figure 1a). Therefore, we conclude that the breakdown of oleic acid is primarily proceeding via the isomerase-dependent pathway as was originally proposed by Ren and Schulz (Ren and Schulz, 2003), which is in line with the accumulation of cis-3-C12:1 acylcarnitine. In the murine FAO pathway, the cis-5-C14:1-CoA is known to be the substrate for LCAD, concluded from the fact that its acylcarnitine ester, cis-5-C14:1 acylcarnitine, is the primary metabolite accumulating in the LCAD KO mouse (Chegary et al., 2009). We speculate that the accumulation of the primary substrate for Eci1 (cis-3-C12:1-CoA) inhibits the enzyme activity of LCAD, resul-
ting in elevated levels of cis-5-C14:1-CoA and as a consequence cis-5-C14:1 acylcarnitine. In humans, expression of LCAD is very low and VLCAD is the major enzyme involved in metabolism of cis-5-C14:1-CoA, therefore a potential human ECI1 deficiency may not present with C14:1 acylcarnitine accumulation.

In peroxisomes, Eci2 is the major monofunctional isomerase; however, Eci2 also is known to be localized in the mitochondria (Zhang et al., 2002). Eci1 deficiency in mice leads to a mild phenotype, suggesting that Eci2 can compensate for Eci1 deficiency in mitochondria. It is important to realize that this compensation can occur without an increase in the expression levels of Eci2 (figure 4). Indeed, when we knocked down Eci2 in the Eci1-deficient mouse fibroblasts, more pronounced accumulation of the unsaturated acylcarnitines was observed when incubated with either oleic acid or linoleic acid. When Eci2 was knocked down in WT mouse fibroblasts, no accumulation of unsaturated fatty acids was observed. This further substantiates that both ECIs can compensate for each other’s deficiency and thus are at least in part functionally redundant in unsaturated FAO.

Despite this functional redundancy, our data prove that both Eci1 and Eci2 play a role in the oxidation of oleic acid, with Eci1 being slightly more important. In addition, we also prove that Eci1 and Eci2 are involved in the oxidation of linoleic acid, again with a high degree of functional redundancy. Based on the pathway proposed by Schulz and Kunau (Schulz and Kunau, 1987), ECI activity is crucial for the conversion of two metabolites in linoleic acid degradation (figure 1b; cis-3,cis-6-C12:2-CoA and trans-3-C10:1-CoA). Indeed, upon incubation with [U-13C]-linoleic acid, we observed accumulation of labeled C10:1 and C12:2 acylcarnitine. Therefore, we speculate that these acylcarnitines are the cis-3,cis-6-C12:2 acylcarnitine, and the trans-3-C10:1 acylcarnitine. Since standards for these specific isomers are not yet available, we could not confirm the identity of these acylcarnitines.

The questions that remain are: why are there two ECIs present in the mitochondria, and why there are no unsaturated acylcarnitines accumulating in WT fibroblasts in which Eci2 has been knocked down? A previous study, in which the substrate specificity of the mitochondrial ECIs was characterized, provided evidence that Eci1 is the dominant enzyme in catalyzing the conversions of cis-3-enoyl-CoA to trans-2-enoyl-CoA and trans-2,cis-5-enoyl-CoA to trans-3,cis-5-enoyl-CoA, whereas Eci2 contributes significantly to the conversion of trans-3-enoyl-CoA to trans-2-enoyl-CoA (Zhang et al., 2002). This is in line with our data and suggests that the oxidation of oleic and linoleic acid is more dependent on the isomerase-dependent pathway, with Eci1 as the most important isomerase. Eci2 could be more important for the oxidation of trans fatty acids such as elaidic acid (trans-9-C18:1). As trans fatty acids are only present in small amounts in rodent chow and our diet, they do not contribute to the observed acylcarnitine profiles. Furthermore, we cannot exclude that EHHADH (located in peroxisomes) could partially compensate for Eci1 and Eci2 deficiency as well. This, however, seems unlikely, as EHHADH is predicted to be involved in catalyzing the reaction of trans-2,cis-5-enoyl-CoA to trans-3,cis-5-enoyl-CoA (Zhang et al., 2002).

In conclusion: in mice a deficiency of Eci1 leads to a mild metabolic phenotypic presentation due to a high degree of functional redundancy of Eci1 and Eci2. Therefore, human EC11 deficiency might have been missed because of a very mild presentation or no disease presentation at all. Based on our studies, we suggest screening patients with a mild ketotic hypoglycemia for an accumulation of C10:1, C12:2 and C12:1 acylcarnitines, which are specific for Eci1-deficiency in mice. This might lead to future identification of ECI1-deficient patients.
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References


Chapter 2


SUPPLEMENTAL FIGURE 1. C12:1 and C14:1 acylcarnitine isomers identified in blood and fibroblast medium of WT and Eci1-deficient mice using HPLC-MS/MS as described by Minkler et al. (Minkler et al., 2008; Minkler et al., 2011). Acylcarnitine standard compounds (calibrators) used for the quantification of C12:1 and C14:1 acylcarnitine isomers. Using this HPLC-MS/MS method, we were not able to separate the trans-2 and trans-3 isomers of C12:1 and C14:1 acylcarnitines. Separation of these compounds was accomplished using the UPLC-MS/MS method specifically optimized for separation of these compounds (figure 5).