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Mitochondrial 2,4-dienoyl-CoA Reductase Deficiency in Mice Results in Severe Hypoglycemia with Stress Intolerance and Unimpaired Ketogenesis

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Abstract

The mitochondrial β-oxidation system is one of the central metabolic pathways of energy metabolism in mammals. Enzyme defects in this pathway cause fatty acid oxidation disorders. To elucidate the role of 2,4-dienoyl-CoA reductase (DECR) as an auxiliary enzyme in the mitochondrial β-oxidation of unsaturated fatty acids, we created a DECR-deficient mouse line. In Decr−/− mice, the mitochondrial β-oxidation of unsaturated fatty acids with double bonds is expected to halt at the level of trans-2, cis/trans-4-dienoyl-CoA intermediates. In line with this expectation, fasted Decr−/− mice displayed increased serum acylcarnitines, especially decadienoylcarnitine, a product of the incomplete oxidation of linoleic acid (C18:2), urinary excretion of unsaturated dicarboxylic acids, and hepatic steatosis, wherein unsaturated fatty acids accumulate in liver triacylglycerols. Metabolically challenged Decr−/− mice turned on ketogenesis, but unexpectedly developed hypoglycemia. Induced expression of peroxisomal β-oxidation and microsomal ω-oxidation enzymes reflect the increased lipid load, whereas reduced mRNA levels of PGC-1α and CREB, as well as enzymes in the gluconeogenic pathway, can contribute to stress-induced hypoglycemia. Furthermore, the thermogenic response was perturbed, as demonstrated by intolerance to acute cold exposure. This study highlights the necessity of DECR and the breakdown of unsaturated fatty acids in the transition of intermediary metabolism from the fed to the fasted state.


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Introduction

Fatty acids are amphiphatic molecules that have indispensable roles in many cellular functions. In addition to energy storage in the form of triacylglycerols, fatty acids are involved in the synthesis of membrane lipids and in signal transduction and endocrine processes. When carbohydrates are depleted as an energy source during fasting and starvation, triacylglycerol stores are mobilized and acetyl-CoAs produced by hepatic β-oxidation of fatty acids are condensed to ketone bodies to ensure an alternative fuel source for extrahepatic tissues, such as brain, skeletal muscle, and cardiac muscle.

Inherited disorders of mitochondrial β-oxidation are among the most common inborn errors of metabolism affecting infants and children. Although clinical phenotypes vary, the inability to completely utilize fatty acids during periods of increased energy requirement is common to all β-oxidation disorders. Under normal conditions, patients are usually asymptomatic, but when challenged with short-term fasting during infectious illness, severe and even fatal phenotypes arise. Disease states can manifest as one or more of the following characteristics: liver dysfunction, hypoketotic hypoglycemia, organic aciduria, skeletal myopathy, and elevated fatty acid concentrations in the serum and tissues [1].

The presence of cis double bonds in naturally occurring (poly–) unsaturated fatty acids poses problems for β-oxidation, that require a few auxiliary enzymes (for review, see [2]). During degradation, double bonds in odd-numbered positions (e.g., oleic acid) lead to Δ2-enoyl-CoAs, which must be isomerized by an enoyl-CoA isomerase (ECI) (Figure 1, center pathway). Double bonds in even-numbered positions give rise to conjugated Δ1Δ3-enoyl-CoAs, which cannot be hydrated by the enoyl-CoA hydratases for thermodynamic reasons [3]. In eukaryotes, they are reduced by an NADPH-dependent 2,4-dienoyl-CoA reductase (DECR) to 3-enoyl-CoA, which is then isomerized by ECI to trans-2-enoyl-CoA, suitable for further oxidation (Figure 1, left pathway). DECR may also play a role in the degradation of fatty acids containing odd-numbered double bonds because the
**Author Summary**

Fatty acids released from triacylglycerols or obtained from the diet serve as a main energy provider to the heart and skeletal muscle, and when carbohydrates are scarce, fatty acids provide energy for the whole organism. Inherited disorders of mitochondrial β-oxidation are among the most common inborn errors of metabolism affecting infants and children. Under normal conditions, patients are usually asymptomatic; but when challenged with metabolic stress, severe phenotypes arise. Here we describe the generation of a mouse model in which the total degradation of unsaturated fatty acids is prevented by disruption of an auxiliary enzyme of β-oxidation. Although degradation of saturated fatty acids proceeds normally, the phenotype presented here is in many ways similar to mouse models of the disrupted classical β-oxidation pathway, but with additional unique features. The null mutant mice are asymptomatic until exposed to fasting, during which they switch on ketogenesis, but simultaneously develop hypoglycemia. A number of human patients suffer from idiopathic hypoglycemia (hypoglycemia of unknown cause). Our mouse model links this disease state to a specific defect in the breakdown of polyunsaturated fatty acids. Furthermore, it shows that degradation of unsaturated fatty acids is essential for balanced fatty acid and energy metabolism, as well as adaptation to metabolic stress.

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**Results**

**Generation of Decr⁻/⁻ Mice**

A replacement vector was designed as described under Materials and Methods to replace a 0.5-kb region in the Decr locus by homologous recombination. This region containing the first exon of Decr was replaced with a neo selection cassette in targeted RW4 cells (Figure 2A). Correct targeting was verified by Southern blotting. A 1-kb probe hybridizing to the promoter region of the Decr gene, which is not present in the replacement vector, labeled a 5.8-kb fragment in the wild type allele formed after BamHI digestion. Hybridization of the probe to a 4.7-kb fragment of the digested allele from Decr⁻/⁻ mice confirmed the correct insertion of the neo cassette (Figure 2B). Chimeric mice were produced by microinjecting correctly targeted RW4 cells into C57BL/6 blastocysts. Chimeric mice were backcrossed onto C57BL/6 mice to produce Decr⁻/⁻ and finally Decr⁻/⁻ mice. The different mouse genotypes were distinguished by PCR using genomic DNA (Figure 2C).

Immunoblotting of mitochondrial extracts from liver, muscle and heart with an antibody against human DECR revealed a detectable signal from wild type mice, whereas no signal could be detected for homozygous null mutant mice (Figure 2D). The reductase activity (n = 3) measured in liver (muscle) mitochondrial extract was 2.2 ± 0.6 μmol/min per mg of protein (2.6 ± 0.3 μmol/min per mg protein) and 0.5 ± 0.1 μmol/min per mg of protein (trace) for wild-type and Decr⁻/⁻ mice, respectively. It is likely that the observed “residual activity” represents the activity of recently characterized mitochondrial 2-enoyl thioester reductase (EC 1.3.1.38) [17], which functions in mitochondrial fatty acid synthesis and can also reduce 2,4-hexadienoyl-CoA in vitro [18].

**Clinical Phenotype**

Under standard laboratory conditions, Decr⁻/⁻ mice were indistinguishable from wild type mice. Crossbreeding of Decr⁻/⁻ mice produced progeny in approximately Mendelian ratios, with no gender bias (Table 1). Both male and female mutant mice were viable and fertile. They exhibited weight gain and a life-span similar to that of wild type mice. Analysis of organ weights and histological analysis of major organs, including liver, muscle, heart, kidney, lungs, spleen and intestine, showed no differences between wild type and mutant mice.

**Fasting Intolerance**

A common feature of individuals affected with inborn errors of mitochondrial fatty acid oxidation is that they are asymptomatic under normal conditions. The same phenomenon is observed in several animal models of fatty acid oxidation disorders. Clinical symptoms arise only after metabolic stress, such as prolonged physical exercise or fasting, which is often associated with infectious illness. In order to study the effect of metabolic stress on Decr⁻/⁻ mice, the mice were subjected to fasting for 24 or 48 h.

**Altered Lipid Homeostatic Response**

During and after fasting, the Decr⁻/⁻ mice showed a tendency to be more passive and unresponsive compared with wild type mice. Mice were sacrificed and blood and selected organs were collected for further characterization. No differences were observed in the levels of serum alanine aminotransferase, alkaline phosphatase or glutamyl transferase between wild type and Decr⁻/⁻ mice, indicating intact liver cells. Concentrations of different amino acids in the sera were also comparable (Table S1). The livers of the Decr⁻/⁻ mice were markedly pale, and liver weights, when

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Fatty acids released from triacylglycerols or obtained from the diet serve as a main energy provider to the heart and skeletal muscle, and when carbohydrates are scarce, fatty acids provide energy for the whole organism. Inherited disorders of mitochondrial β-oxidation are among the most common inborn errors of metabolism affecting infants and children. Under normal conditions, patients are usually asymptomatic; but when challenged with metabolic stress, severe phenotypes arise. Here we describe the generation of a mouse model in which the total degradation of unsaturated fatty acids is prevented by disruption of an auxiliary enzyme of β-oxidation. Although degradation of saturated fatty acids proceeds normally, the phenotype presented here is in many ways similar to mouse models of the disrupted classical β-oxidation pathway, but with additional unique features. The null mutant mice are asymptomatic until exposed to fasting, during which they switch on ketogenesis, but simultaneously develop hypoglycemia. A number of human patients suffer from idiopathic hypoglycemia (hypoglycemia of unknown cause). Our mouse model links this disease state to a specific defect in the breakdown of polyunsaturated fatty acids. Furthermore, it shows that degradation of unsaturated fatty acids is essential for balanced fatty acid and energy metabolism, as well as adaptation to metabolic stress.

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determined as a percentage of body weight, were significantly (p < 0.01) greater than that of wild type mice (Figure 3). Hematoxylin and eosin-stained histological liver sections (Figure 4A and 4B) obtained during the fed state showed no differences. Fasted Decr2/2 mice showed normal lobular architecture when compared with wild type mice, except for the presence of hepatocytes with a foamy appearance and centralized nuclei, which are characteristics of microvesicular steatosis (Figure 4C and 4D). Hepatocytes with extensive microvesicular vacuolation were mainly present in periportal and midzonal regions, whereas the majority of hepatocytes in centrilobular regions appeared normal. When Oil red O staining was performed to stain neutral lipids, the sections showed massive and homogeneously distributed micro- and macrovesicular lipid droplet formation, whereas only minor microvesicular lipid droplet accumulation was present in age-matched wild type control sections (Figure 4E and 4F). These data suggested that fasting results in the accumulation of lipids in the livers of Decr2/2 mice.

The amounts of circulating non-esterified fatty acids (NEFA) were analyzed in the sera of Decr−/− and wild type mice. Under normal nutritional conditions, mean serum NEFA levels were comparable between wild type and Decr−/− mice (0.43 ± 0.11 mmol/l, 0.52 ± 0.03 mmol/l, respectively) but after fasting, the Decr−/− mice demonstrated increased serum NEFA levels, reaching 1.28 ± 0.12 mmol/l after 48 h compared with the wild type levels of 0.68 ± 0.16 mmol/l (p < 0.001) (Figure 5A).

Altered Glucose Homeostatic Response

A common symptom associated with inherited defects of mitochondrial fatty acid oxidation is the development of hypoglycemia in response to fasting, a phenomenon also observed in several animal models of disrupted mitochondrial fatty acid oxidation [19–21]. This effect is considered to be caused by glyogen depletion in combination with an impaired gluconeogenic response. In order to analyze whether the defect in mitochondrial oxidation of (poly)unsaturated fatty acids generates a similar hypoglycemic condition, serum glucose levels were determined for wild type and Decr2/2 mice after 24 h and 48 h of fasting (Figure 5B). In the fed state, glucose levels were comparable (11.0 ± 1.4 mmol/l for wild type and 9.5 ± 0.6 mmol/l for Decr−/− mice). Twenty-four-hour fasting had no effect on the serum glucose levels of wild type mice (10.9 ± 0.3 mmol/l), whereas a significant decrease was observed in the levels in Decr−/− mice (6.6 ± 0.2 mmol/l, p < 0.01). After mice were subjected to prolonged fasting (48 h), the glucose levels in Decr−/− mice were further decreased to 2.5 ± 0.3 mmol/l, whereas the decrease in wild type mice resulted in a glucose concentration of 5.9 ± 0.9 mmol/l. These data revealed that Decr−/− mice have an accelerated hypoglycemic response to fasting.

In order to determine whether the hypoglycemic state of the Decr−/− mice after fasting is in part due to more rapid depletion of glycogen stores, the liver and muscle glycogen concentration was measured before and after 6 h, 15 h, and 24 h of fasting (Figure 5C).
In the fed state, liver and muscle glycogen content was similar between wild type and Decr2/2 mice. As expected, fasting resulted in a gradual depletion of glycogen stores, and no significant differences in glycogen content between wild type and Decr2/2 mice were found at any observation time points.

**Table 1.** Genotype analysis of progeny resulting from crossbreeding Decr+/− mice.

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Mice with indicated genotype (% of total)</th>
<th>Mice with indicated gender (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
<td>+/−</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>32</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>29 (23)</td>
<td>63 (49)</td>
</tr>
</tbody>
</table>

Figure 2. Targeting of the Decr locus and verification of gene inactivation. (A) Schematic drawing of the targeting strategy showing the wild type allele, targeting vector and targeted allele after homologous recombination. The targeted allele depicts the strategy used to delete exon 1, in which the endogenous sequence is replaced by a neomycin-positive selection cassette (neo). A thymidine kinase cassette (tk) was used for negative selection. Exons are denoted as numbered solid bars (1–9). Recognition sites for restriction endonucleases are marked as B = BamHI, H = HindIII, E = EcoRI, S = SmaI, EV = EcoRV. The genomic fragment used as an external probe for Southern analysis is marked with an “X”, and the expected fragments for the wild type and targeted allele after BamHI digestion are indicated with arrows. (B) Southern blot analysis of mouse liver DNA. Genomic DNA was digested with BamHI and detected using probe X to yield the expected fragments of 5.8 kb for the wild type allele in Decr+/+ mice (+/−), 4.7 kb for the targeted allele in Decr2/2 mice (−/−), and both fragments in heterozygous Decr+/− mice (+/−). (C) Mouse genotypes were determined from tail samples using PCR with primers denoted with arrows a, b, and c in the schematic drawing. The amplified fragment for wild type Decr+/+ mice was 382 bp, 280 bp for homozygous Decr−/− mice, and both fragments were amplified from heterozygous Decr+/− mice. (D) Western blot analysis of mitochondrial homogenates from liver, muscle and heart using an antibody against rat DECR showing the presence or absence of the 33-kDa band corresponding to DECR in Decr+/+ and Decr−/− mice, respectively. Twenty micrograms of protein was loaded in each lane.

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Figure 3. Effects of fasting on organ weights in wild-type and Decr−/− mice. Age-matched male mice were fasted for 24 h, after which they were sacrificed. The wet weight of selected organs (heart, liver, kidney and fat) was determined and compared between wild type and Decr−/− mice. Weights were calculated as a percentage of body weight and are expressed as means ± SE of 4–6 mice per group. Asterisks (*) denote significant differences (p<0.01) between wild type and Decr−/− mice.

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Hypoketonemia is often associated with fasting-induced hypoglycemia and defective mitochondrial fatty acid oxidation. The hypoketotic state is caused by an inability of mitochondria to offer enough acetyl-CoA moieties (products of β-oxidation) for ketone body production during fasting. Hypoketotic hypoglycemia is a condition that is used in the diagnosis of human genetic defects to establish a link between symptoms and a fatty acid oxidation defect.

To study the ketogenic response to fasting, serum β-hydroxybutyrate levels were measured in Decr−/− and wild type mice under the fed state and after 24 h of fasting (Figure 5E). In the fed state, the formation of ketone bodies was very low, as indicated by values of 0.13±0.01 mmol/l and 0.16±0.06 mmol/l for wild type and Decr−/− mice, respectively. Fasting greatly increased serum β-hydroxybutyrate values in the wild type and Decr−/− mice and comparable values of 1.04±0.14 mmol/l for wild type and 1.11±0.10 for Decr−/− mice indicated that the reduced capacity

Figure 4. Histological assessment of liver morphology in fasted and non-fasted wild-type and Decr−/− mice. Light microscopic images of representative paraffin-embedded liver sections from wild type (+/+) and Decr−/− mice (−/−) stained with hematoxylin and eosin (A–D) and cryosections stained with Oil red O (E,F). Liver morphology of non-fasted animals showed no differences between wild type and Decr−/− mice (A,B). Fasting for 24 h revealed no apparent changes in the liver morphology of wild type mice, but induced microvesicular steatosis in Decr−/− mice, as observed by the appearance of foamy hepatocytes with centralized nuclei (C,D). Oil red O staining of neutral fat in representative liver cryosections revealed small, lightly stained vacuoles in the wild type sample (E), whereas a large number of intensely stained vacuoles of varying size can be seen in Decr−/− mice (F), indicating the accumulation of fat. Magnification ×20.

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for the mitochondrial oxidation of (poly)unsaturated fatty acids in Decr<sup>−/−</sup> mice did not prevent a normal ketogenic response.

**Altered Fatty Acid Pattern of Total Lipids**

To further analyze the role of DECR disruption in hepatic lipid accumulation, liver lipids were analyzed using positive ion mass spectrometry, as described under Materials and Methods. Under fed conditions, there were no significant differences in the amount or composition of total liver fatty acids between wild type and null mutant mice, as indicated in Figure 6A. The main fatty acid species were palmitic acid (C<sub>16:0</sub>), stearic acid (C<sub>18:0</sub>), oleic acid (C<sub>18:1</sub>), linoleic acid (C<sub>18:2</sub>), and arachidonic acid (C<sub>20:4</sub>). Figure 6C further shows that under fed conditions, the proportion of saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) in total liver fatty acids was comparable between wild type and Decr<sup>−/−</sup> mice.

Analysis of liver fatty acids after the mice were fasted for 24 h (Figure 6B) indicated that fasting had a minor effect on the lipid content of wild type liver, with an overall increase of 29% in the concentration of fatty acids. The increase in palmitic (C<sub>16:0</sub>) and stearic acid (C<sub>18:0</sub>) concentrations contributed to the increased SAFA concentration, whereas the increased linoleic acid (C<sub>18:2</sub>) concentration contributed to the increased PUFA concentration. There were no significant differences in the amount or composition of total liver fatty acids between wild type and heterozygous mutant mice. In Decr<sup>−/−</sup> mice, however, the overall concentration of fatty acids increased by 108% after fasting. This was in agreement with the lipid accumulation observed in histological sections by Oil red O staining. The most profound changes between fasted wild type and Decr<sup>−/−</sup> mice were observed for the levels of palmitoleic acid (C<sub>16:1</sub>), oleic acid, linolenic acid (C<sub>18:3</sub>) and linoleic acid, which were 2.5- to 3.8-fold higher in Decr<sup>−/−</sup> mice. In comparison to the fed state, the concentrations of MUFA and PUFA increased by 288% and 254%, respectively, in Decr<sup>−/−</sup> mice (Figure 6B). The increased concentrations of MUFA were due to the increase in oleic acid and palmitoleic acid concentrations, whereas the increased PUFA concentrations were due to the increased linoleic acid and linolenic acid concentrations. The effect of fasting was most pronounced for the concentrations of linoleic and linolenic acids, which were increased 5.5-fold and 6.9-fold when compared with the fed state. The concentration of SAFA remained relatively unchanged, although an increase in the concentration of palmitic acid and a decrease in the concentration of stearic acid were observed.

**Acylcarnitine and Dicarboxylic Acid Analysis**

Acylcarnitine profiling is commonly used as a biochemical tool to diagnose various inherited metabolic disorders. For example, the
The initial diagnosis of long-chain fatty acid oxidation disorders is most often performed by analyzing serum or plasma acylcarnitines. Disruption of mitochondrial β-oxidation of long-chain fatty acids leads to intramitochondrial accumulation of acyl-CoA esters, which leak into the bloodstream as acylcarnitines after transesterification with carnitine. This means that the serum acylcarnitine profile reflects acyl-CoA esters that accumulate intramitochondrially and pinpoints the site of metabolic block in the oxidation pathway.

To study the effect of Decr gene disruption on the acylcarnitine profile and whether disruption leads to the secretion of specific acylcarnitine species, serum acylcarnitine profiles were determined for non-fasted and fasted wild type and Decr−/− mice by mass spectrometry. Under non-fasted conditions, there were no significant differences in the levels of total serum acylcarnitines between wild type and Decr−/− mice (263±29 nM and 240±16 nM, respectively). In addition, no significant differences were detected in the levels of individual acylcarnitines from C8 to C20 (Figure 7A). Predominant acylcarnitines in the sera were C16 and C18:1 acylcarnitines. Fasting increased the total concentration of acylcarnitines by 2-fold in wild type mice (567±32 nM); however, in Decr−/− mice, a markedly higher 9-fold increase was observed (2150±230 nM). Compared with wild type mice, the levels of all analyzed acylcarnitine species were highly elevated in Decr−/− mice. The increase was most distinct for the level of decadienoylcarnitine (C10:2), the concentration of
which was 44-fold higher in the sera of Decr<sup>−/−</sup> mice compared with wild type controls (Figure 7B).

To analyze the excretion of dicarboxylic acids, another marker for mitochondrial β-oxidation dysfunction, mice were housed in metabolic cages and urine was collected for 24 h (fed state sample). Subsequently, food was removed and collection was continued for another 24 h (fasted sample). Dicarboxylic acid contents were monitored by means of mass spectrometry. A significant excretion of molecules with masses corresponding C7:2, C8:2, C10:2, C10:3, and C14:3 dicarboxylic acids was observed in Decr<sup>−/−</sup> mice after fasting. The excretion of these dicarboxylic acids, especially C10:2 and C14:3, which were not detected in the urine of wild type mice, was also observed in the fed state for Decr<sup>−/−</sup> mice (Table S2 and Figure S1). C8:2 and C10:2, the expected products of linoleic acid degradation, reached concentrations of 154 μM and 72 μM, respectively, while C14:3, which is expected product of α-linolenic acid, was found up to 16 μM. In the urine of wild type mice, their concentrations, with the exception of C8:2, were close to or below detection limit (approx. 5 μM).

Quantitative Real-Time PCR Analysis of Gene Expression

Adaptation to fasting is partially transmitted via altered transcription of genes encoding enzymes that function in multiple pathways. These alterations are directed by transcription factors, coactivators and corepressors that act as sensors of the nutritional status of an organism. To analyze whether disturbances observed in Decr<sup>−/−</sup> mice were accompanied by altered expression of genes encoding proteins involved in mitochondrial and extramitochondrial (peroxisomal and microsomal) fatty acids and carbohydrate metabolism, quantitative real-time PCR method was conducted.

When the expression levels of several mitochondrial β-oxidation enzymes in the liver were compared between wild type and Decr<sup>−/−</sup> mice (Figure 8), a 2-fold increase was observed in the expression level of the rate-limiting enzyme carnitine palmitoyltransferase (CPT-1) in Decr<sup>−/−</sup> mice. Expression levels of other studied mitochondrial β-oxidation enzymes, long chain acyl-CoA dehydrogenase (LCAD) and very long chain acyl-CoA dehydrogenase (VLCAD) were comparable, but were slightly increased in Decr<sup>−/−</sup> mice (Figure 8A). A greater change was observed in the expression levels of genes associated with peroxisomal β-oxidation, because the expression levels of acyl-CoA oxidase (Acox) and peroxisomal multifunctional enzyme 1 (MFE1) were 2.3- and 3.4-fold higher in Decr<sup>−/−</sup> mice, respectively (Figure 8B). In addition, the expression of ECI, which is one of the auxiliary enzymes that functions in the oxidation of polyunsaturated fatty acids with double bonds in odd-numbered positions, was slightly upregulated (1.3-fold). A
significant 2.1-fold increase was also observed in the expression level of cytochrome P450 IVA1 (CYP 4A10), which is a key enzyme in microsomal ω-oxidation (Figure 8C). Normally, enzymes involved in fatty acid synthesis and desaturation are downregulated during fasting. The expression level of acetyl-CoA carboxylase (Acaca), which catalyzes the first step in the fatty acid synthesis pathway, was lower in Decr−/− mice, although it was not significant (Figure 8C).

Interestingly, we observed significantly decreased expression levels of SREBP1 and chREBP in Decr−/− mice (Figure 8C). During fasting, the expression levels of SREBP1 and chREBP in wild type mice after fasting (Figure 8E). Although peroxisome proliferator activated receptor α (PPARα) has a central role in the transcriptional control of genes encoding fatty acid oxidation enzymes, further transcription factors are responsible for the regulation of other metabolic pathways (e.g., sterol regulatory element-binding protein (SREBP), which regulates genes involved in lipogenesis, cholesterogenesis, and glucose metabolism, and carbohydrate responsive element binding protein (chREBP), which mediates the transcriptional effects of glucose on glycolytic and lipogenic genes).

Cold Intolerance and Physical Activity
In order to determine the effects of cold stress, mice were fasted for 20 h and exposed to a cold environment (+4°C) for 4 h. Decr−/− mice exhibited severe cold intolerance during acute cold exposure and the experiment was terminated when body temperature decreased below 25°C. It has been shown that mice with temperatures below 25°C do not recover, and thus this body temperature can be considered terminal without using death as an end-point [24]. After exposure for 2 h, the average body
temperature of \( \text{Decr}^{--/} \) mice (\( n = 5 \)) was 23.4°C compared with 33.0°C for wild type mice, and three of the five \( \text{Decr}^{--/} \) mice demonstrated temperatures that had declined below 25°C (average 21.3°C). The temperatures of the two remaining \( \text{Decr}^{--/} \) mice continued to decline linearly and averaged 21.6°C after 3 h, at which time the experiment was terminated (Figure 9). Shivering was also initially present in \( \text{Decr}^{--/} \) mice but decreased during the experiment and was absent after 2 h, at which time mice became lethargic. In contrast, none of the 5 wild type mice succumbed to cold and, at the end of the 4 h exposure, shivering was clearly present and the average body temperature was 34.5°C. The severely cold intolerant phenotype of \( \text{Decr}^{--/} \) mice was observed only if cold exposure was preceded by fasting (data not shown).

As previously mentioned, \( \text{Decr}^{--/} \) mice showed decreased blood glucose and elevated NEFA concentrations after fasting in comparison to wild type mice. This response was more pronounced in both mouse genotypes when the fasting period was followed by acute cold exposure. When cold exposure was terminated, \( \text{Decr}^{--/} \) mice demonstrated glucose and NEFA concentrations of \( 3.0 \pm 0.2 \text{ mmol/l} \) and \( 1.6 \pm 0.02 \text{ mmol/l} \), respectively, whereas the values obtained for wild type mice were \( 6.3 \pm 0.5 \text{ mmol/l} \) and \( 0.77 \pm 0.08 \text{ mmol/l} \).

To analyze the physical activity of mice during fasting and cold exposure, a LabMaster study was conducted. This study showed that during fasting wild type mice maintained a normal activity pattern, in which activity was highly enhanced during the dark exposure, whereas the values obtained for \( \text{Decr}^{--/} \) mice were 6.3 \( \pm 0.2 \text{ mmol/l} \) and 0.77 \( \pm 0.08 \text{ mmol/l} \), respectively, whereas the values obtained for wild type mice were

![Figure 9. Changes in body temperature during cold exposure with prior fasting](https://example.com/figure9)

**Figure 9. Changes in body temperature during cold exposure with prior fasting.** Cold tolerance was tested after 20 h of fasting by exposing individually housed wild type mice (open boxes) and \( \text{Decr}^{--/} \) mice (solid boxes) to a 4°C environment for a maximum of 4 hours or until body temperature dropped below 25°C. Temperature was measured from the shaved mid-dorsal body surface using an infrared thermometer. At each time point, body temperatures are presented as mean values of three measurements from the same individual. doi:10.1371/journal.pgen.1000543.g009

...and 317 \( \pm 65 \text{ counts/15 min} \), respectively (Figure 10D). Average heat production during cold exposure was also measured, and \( \text{Decr}^{--/} \) mice showed a slight but significant decrease in heat production. The average heat production of wild type and \( \text{Decr}^{--/} \) mice was 27.1 \( \pm 1.2 \text{ kcal/h/kg} \) and 23.2 \( \pm 0.8 \text{ kcal/h/kg} \), respectively (Figure 10E).

**Discussion**

The results presented herein indicate that mitochondrial 2,4-dienoyl-CoA reductase activity in mice is indispensable for the complete oxidation of (poly)unsaturated fatty acids and for adaptation to metabolic stress. \( \text{Decr}^{--/} \) mice exhibited hypoglycemia during fasting, with concomitant accumulation of metabolites of unsaturated fatty acids in the liver, sera, and urine. Furthermore, a predisposition to cold intolerance and a reduction in diurnal activity during metabolic stress were observed.

Insufficient adaptation to metabolic stress in \( \text{Decr}^{--/} \) mice is exemplified by the development of microvascular hepatic steatosis after as little as 24 h of fasting. However, levels of serum alanine aminotransferase, alkaline phosphatase, and glutamyl transferase were similar in both \( \text{Decr}^{--/} \) and wild type animals, suggesting that null mutant mice did not develop liver cell membrane injury during the observation period.

In the \( \text{Decr}^{--/} \) mice, analysis of fatty acid composition in total liver lipids revealed a specific accumulation of mono- and polyunsaturated fatty acids, with oleic and linoleic acids being the dominant species. This can be explained in terms of their impaired β-oxidation, resulting in their channeling toward esterification and leading ultimately to hepatic steatosis. The fact that saturated fatty acids, such as palmitic and stearic acids, which, together with mono- and polyunsaturated fatty acids (especially oleic and linoleic acid), are the main components of triacylglycerols in adipose tissue [25], did not accumulate suggest that they were effectively metabolized. The proceeding β-oxidation of saturated fatty acids explains the carbon source for ketogenesis, which was found to be similar in wild type and \( \text{Decr}^{--/} \) mice.

A prominent feature of \( \text{Decr}^{--/} \) mice is the hypoglycemic response to fasting, which seems not to be related to differences in glycogen or amino acid metabolism, but is associated with altered transcriptional control mechanisms in the gluconeogenesis pathway. Indeed, there were no significant differences in liver and muscle glycogen content in wild type and \( \text{Decr}^{--/} \) mice, either in the fed or in the fasted state. This finding suggested that the observed hypoglycemia was not due to a failure in glycogen metabolism. The amino acid profile and levels in the sera of \( \text{Decr}^{--/} \) mice is the hypoglycemic and wild type animals, suggesting that adaptation to fasting was not affected at the hormonal level.

PEPCK and G6Pase are regarded, under *in vivo* conditions, as unidirectional enzymes that, among other factors, control the gluconeogenic flux and show increased activity in the fasted state. The detected low PEPCK and G6Pase mRNA levels can explain, in part, the hypoglycemia observed in \( \text{Decr}^{--/} \) mice via contributing to the decreased flux through the gluconeogenic pathway in the liver. Decreased mRNA levels of coactivator PGC-1α and its inducer CREB, which together drive the expression of PEPCK and G6Pase via HNF4α and FOXO1, suggest that
signaling pathways leading to activation of gluconeogenesis during fasting are compromised in Decr^−/− mice. However, which of the component(s) upstream of PGC-1α or CREB, especially TORC2 (transducer of regulated CREB activity 2), energy sensing kinase AMPK, and salt-inducible kinase SIK, are affected and whether this effect is mediated by certain accumulated PUFA species will be evaluated in further studies (Figure 11). Analysis of the expression levels, activity and phosphorylation status of these factors in Decr^−/− mice could elucidate the regulatory link between gluconeogenesis and the disrupted breakdown of unsaturated fatty acids.

The expression of several genes, including CPT-1, Acox, and CYP4A1, has been shown to be upregulated by polyunsaturated fatty acids [26,27]. In addition, unsaturated fatty acids were previously shown to impact different transcription factors that play a pivotal role in lipid and carbohydrate metabolism. Thus, the
accumulated unsaturated fatty acids and their derivatives in Decr−/− mice can act as regulators of gene expression by functioning as ligands for nuclear receptors, such as PPARγ, which is the major PPAR subtype present in hepatocytes and is involved in regulating genes involved in lipid and carbohydrate metabolism [28, 29]. In accordance, the expression of the PPARγ target genes CPT-1, Acox, and CYP4A1 was markedly higher after fasting in Decr−/− mice compared with wild type mice. Unsaturated fatty acids can also affect gene expression via SREBP1, a factor considered a key regulator of triacylglycerol and fatty acid synthesis [30]. Although it is believed that they function mainly by inhibiting the maturation of lipogenic SREBP1 [31, 32], they have been shown to decrease hepatic SREBP1 mRNA [33]. In line with these observations, the mRNA level of SREBP1 was reduced 3-times in Decr−/− compared with wild type mice after fasting, and the levels of SCD1 and Acaca mRNAs, which are mediated in part by SREBP1, were decreased as well. PUFA has recently been shown to have a suppressive effect on lipogenic and glycolytic gene expression through chREBP [34]. Namely, PUFA ablates chREBP translocation from the cytosol to nucleus and accelerates chREBP mRNA decay [35, 36]. Of note, the Decr−/− mice displayed significantly decreased (4-times lower) levels of chREBP mRNA compared with wild type animals after fasting, likely reflecting the increased chREBP mRNA decay mediated by accumulated PUFA.

In our experimental setting of acute cold exposure (4 h) without prior acclimatization, it is unlikely that nonshivering thermogenesis plays any major role in maintaining mouse body temperature. The decreased heat production observed in Decr−/− mice in the fasted state can be explained by reduced shivering [37]. Reduced shivering can in turn be explained by the reliance of fast-twitching muscle fibers on glucose, which is depleted in Decr−/− mice under these conditions. Wild type mice display a diurnal activity pattern with higher physical activity during the dark phase, and this pattern is preserved during fasting [38]. This increased dark phase activity, however, was greatly diminished in fasted Decr−/− mice. Of note, no differences in thermoregulation were observed between wild type and Decr−/− mice in the fed state. Because both animal groups generated ketone bodies, which can be utilized as fuel in muscle tissue, the link between simultaneous hypoglycemia and the incapability to maintain body temperature in Decr−/− mice remains an intriguing open question.

By inspecting the acylcarnitine profile of the sera of Decr−/− mice, one can deduce which paths are used by unsaturated fatty acids during their degradation. The mitochondrial β-oxidation of unsaturated fatty acids with preexisting double bonds at even-numbered positions, such as petroselinic acid (C_{18:1}, Δ^9), is predicted to halt in Decr−/− mice during the fourth turn of the β-oxidation cycle after cis-4-decenoyl-CoA is dehydrogenized to trans-2-cis-4-decadienoyl-CoA (Figure 1, left section). If the double bond is in an odd-numbered position, such as in oleic acid (C_{18:1}, Δ^11), mitochondrial oxidation can proceed to completion via the ECI-dependent route (Figure 1, middle section) or can be halted during the third β-oxidation cycle at the level of trans-2-cis-4-tetradecenoyl-CoA, which can be generated from the 2,5-tetradecenoyl-CoA intermediate (Figure 1, right section) by the combined activity of ECI and DECI [39]. For polysaturated fatty acids having double bonds at odd- and even-numbered positions, such as in linoleic acid (C_{18:2}, Δ^9,Δ^12), the lack of DECR activity results in blocking of the β-oxidation of these fatty acids either when intermediates of odd-numbered bonds are routed to the reductase-dependent pathway or

Figure 11. PUFA and activation of gluconeogenesis. Simplified schematic presentation of the identified kinase cascades regulating gluconeogenesis. The activation cascade leads to phosphorylation of CREB (cAMP-responsive element binding protein), which, together with TORC2 (transducer of regulated CREB activity 2), drives the expression of coactivator PGC-1α. Transcription of the key gluconeogenic enzymes PEPCK (phosphoenolpyruvate carboxykinase) and G6Pase (glucose-6-phosphatase) is induced when PGC-1α associates with HNF4α (hepatic nuclear factor 4α) and FOXO1 (forkhead box transcription factor). Nuclear translocation of TORC2, which is needed for activation of the gluconeogenic program, is controlled by phosphorylation by activated AMPK (AMP-activated protein kinase) and SIK (salt-inducible kinase). Observed changes in Decr−/− mice are indicated with red arrows. Possible targets of accumulated PUFA or their derivatives are also indicated.

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when the even-numbered double bond reaches the Δ⁴ position during acyl chain shortening via β-oxidation.

The distinctive accumulation of trans-2,cis-4-decadienoylcarnitine (C₁₀:2) in the sera of Decr−/− mice can be derived from the incomplete oxidation of unsaturated fatty acids of the ω-6 series. Among them, linoleic acid is the most abundant species in the animal body, whereas others, such as petroselinic acid, are substantially less frequent. Because no intermediates of the reductase-dependent pathway for unsaturated fatty acids with double bonds at odd-numbered positions were observed, the data suggest that the major route for β-oxidation of these fatty acids proceeds via the isomerase-dependent pathway in vivo. If the reductase-dependent pathway were preferred, it would lead to accumulation of tetradecatrienoylcarnitine (C₁₄:3, Δ⁴,6,9) and tetradecadienoylcarnitine (C₁₄:2, Δ⁴,6,9) from the incomplete oxidation of linoleic and oleic acids, respectively. In line with this notion, the accumulation of C₁₄:2 metabolites (e.g., intermediates of linoleic and oleic acids, respectively) in the sera of Decr−/− mice was identified by a 5' probe upstream of the targeted locus [5]. Consistent with the characteristics of the clinical case, the same dominant carnitine species was observed in Decr−/− mice, although hyperlysinemia was not observed. An open question, therefore, remains as to whether the primary cause of patient death was due to DECR deficiency or whether the patient suffered another disease that remained undiagnosed.

One mouse model is known in which mitochondrial β-oxidation of unsaturated fatty acids is halted at the level of their ω-6 or trans-5-enoyl-CoA intermediates due to disruption of ECI [16]. Similar to Decr−/− mice, Eci−/− mice are asymptomatic under fed conditions but upon fasting, accumulate unsaturated acyl groups in ester lipids and develop hepatic steatosis. ECI deficiency also led to dicarboxylic aciduria, with an accumulation of medium chain unsaturated dicarboxylic acids. However, whether Eci−/− mice have a hypoglycemic response to fasting or show cold intolerance, as observed for Decr−/− mice, was not reported. In addition, production of ketone bodies was not reported in Eci−/− mice. Thus, a lack of information prevents a thorough comparison of Eci−/− and Decr−/− mice.

In the present study, we examined the physiological consequences of disruption of the mitochondrial β-oxidation of unsaturated fatty acids at the level of 2,4-dienoyl-CoA reductase. This mouse model provides for the first time a hint that the breakdown of PUFA is essential for switching on gluconeogenesis during fasting. Decr−/− mice may serve as a model for studying the mechanism responsible for idiopathic hypoglycemia with unpaired ketogenesis in humans. Analysis of the expression profile of selected transcription factors and cofactors revealed the involvement of CREB and PGC1α upstream of the reduced expression of PEPCK and G6Pase in Decr−/− mice. Among accumulated acylcarnitines in the sera, trans-2,cis-4-decadienoylcarnitine (C₁₀:2), which is a potential novel metabolic marker for screening patients with inborn errors in polyunsaturated fatty acids breakdown, was found.

Materials and Methods

Generation of Decr Knockout Mice

The genomic clone BACM:109-18E (from 129/SvJ strain) corresponding to the mouse Decr locus was obtained from Genome Systems (St Louis, MO, USA). A 3.3 kb EcoRI-HindIII fragment upstream of the first exon was cloned into a Bluescript vector modified with SalI and ClaI sites flanking the polylinker. A 4.4 kb Smal-EcoRV fragment from the first intron was cloned into a Bluescript vector modified with AciI and PstI sites flanking the polylinker. For the replacement vector, SalI-ClaI and AciI-PstI cleaved fragments were ligated to the corresponding sites of the pPGKneo/TK-2 vector (Figure 2A), where they flanked the PGKneo cassette. The neomycin resistance (neo) and thymidine kinase (TK) genes were used for positive and negative selection, respectively.

Linearized replacement vector was electroporated into RW4 embryonic stem (ES) cells (129/SvJ, Tyr⁺/Tyr⁺) that were subsequently grown under G418 and ganciclovir selection. Correctly targeted ES cell clones were identified by Southern analysis of genomic DNA, for which the BamHI restriction fragment length polymorphism created by homologous integration was identified by a 5'-probe upstream of the targeted locus (Figure 2B). Germline chimeric mice were produced by microinjecting ES cells from positive clones into C57BL/6 blastocysts at the Biocenter Oulu Transgenic core facility. Genotyping was performed by PCR analysis of tail DNA samples using forward primers for the wild type allele (5'-TGC GTT CITT TGC TGG...
given, the values represent means and their use in the present study was approved by the relevant ethics committee. Temperature (average temperature of 9.6°C (20 hours) prior to the experiments. Plexiglas cages were placed in a controlled environment to maintain constant conditions.

Mice were individually housed in Plexiglas home cages, fresh air was supplied at a constant flow of 0.33 l/min, and O2 consumption and CO2 production were measured continuously by indirect open circuit calorimetry system with a dual array of infrared photo beams (LabMaster, TSE Systems, Bad Homburg, Germany). Before the experiments were carried out, mice were acclimated to our new environment to ensure their adaptation to the experimental conditions.

During the experiment, mice were individually housed in Plexiglas cages and food was withdrawn for 6 to 48 hours; water was provided $ad$ libitum. Cold tolerance was tested by exposing individually housed fasted (20 h) or non-fasted mice to +4°C for a maximum of 4 hours or until their body temperature dropped below 25°C. Temperature was measured from the shaved mid-dorsal body surface using a ThermoScan thermometer (PRO 4000, Braun, Kruenberg, Germany), as described earlier [46].

Oxygen consumption, CO2 production, energy expenditure, determination of food and water intake, and activity (total activity, ambulatory and fine movement and rearing) were measured simultaneously and continuously in housing cages utilizing an indirect open circuit calorimetry system with a dual array of infrared photo beams (LabMaster, TSE Systems, Bad Homburg, Germany). Before the experiments were carried out, mice were acclimated to their new environment in training cages similar to the actual experimental cages for seven days. During the experiment, mice were individually housed in Plexiglas home cages, fresh air was supplied at a constant flow of 0.33 l/min, and O2 consumption and CO2 production were measured and compared with room air values. Data were collected every 30 min for 72 h. The respiratory exchange ratio was calculated by dividing the volume of CO2 production (VCO2) by the volume of oxygen consumption (VO2), and energy expenditure (heat production) was calculated using the software provided with the instrument. For fasting studies, mice were similarly acclimated to the experimental conditions and their baseline (mice fed $ad$ libitum) was analyzed over 24 hours. Food was then removed at 8 am and fasting was continued for 48 hours; the mice were analyzed continuously, as described above. For the cold exposure study in the LabMaster system, mice were fasted overnight (20 hours) prior to the experiments. Plexiglas cages were placed in a refrigerated cold cabinet (Helkama, Finland) with a controlled temperature (average temperature of 9.6°C) for 2 hours. Data were collected every 15 minutes and analyzed using Microsoft Excel and GraphPad Prism version 4.03.

All animals were handled in strict accordance with good animal practice and their use in the present study was approved by the University of Oulu committee of animal experimentation. When provided, the values represent means ± S.E.

Immunoblotting and Activity Measurements

To isolate mitochondria from heart and skeletal muscle tissues, 200–500 mg of tissue was cut into small pieces in 10 volumes (v/v) of isolation buffer (100 mM KCl, 50 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM EDTA). The solution was replaced with 10 volumes of isolation buffer containing 2 mg/ml of the bacterial protease Nagarse (Sigma, St. Louis, MO, USA) and the tissue samples were incubated on ice for 5 min. Samples were washed with 10 volumes of isolation buffer and subsequently homogenized in 10 volumes of isolation buffer containing 2 mM ATP with a motorized glass-tallon homogenizer. The suspension was centrifuged at 3000 x g for 4 min and the resulting supernatant was further centrifuged at 17000 x g for 10 min to pellet the mitochondria. The resulting pellet was suspended in 1.4 volumes of suspension buffer (10 mM Tris-Cl, pH 7.8, 250 mM sucrose, 0.2 mM EDTA). To produce the mitochondrial homogenate from liver tissue, 500 mg of tissue was homogenized in 10 volumes of isolation buffer, followed by centrifugation as described above and resuspension in suspension buffer. To minimize peroxisomal contamination, mitochondrial purification was completed by isopycnic density gradient ultracentrifugation on a self-generating Percoll (Sigma) gradient, as previously described [17]. For immunoblotting, samples containing 20 μg of protein were transferred to a nitrocellulose membrane after SDS-PAGE and detected using polyclonal antibody against rat 2,4-dienoyl-CoA reductase [47] as the primary antibody and goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, CA, USA) as the secondary antibody, followed by ECL Western Blotting Detection Reagents (Amer sham Biosciences, Piscataway, NJ, USA).

The 2,4-Dienoyl-CoA reductase activity was assayed in mitochondrial extracts by spectrophotometric measurement of NADPH consumption at 22°C using 60 μM 2,4-hexadienoyl-CoA as substrate, as previously described [10].

Blood Chemistry

Blood samples were collected from anesthetized mice by orbital bleeding in Multivette collection tubes (Sarsted, Numbrecht, Germany) and serum was separated by centrifugation after 15 min. After being bled, mice were sacrificed by cervical dislocation and tissues were weighed and collected for further analysis. Serum cholesterol, triacylglycerols, albumin, alkaline phosphatase, alanine aminotransferase, glutamyl transferase, β-hydroxybutyrate and amino acids were analyzed by the clinical laboratory of the University Hospital of Oulu, Finland. Serum glucose, insulin, and serum cytokines were determined by enzymatic colorimetric methods. Insulin levels were measured with the insulin ELISA kit (Chryslt Chem Inc., IL, USA) using mouse insulin as a standard. Glucagon was determined using the glucagon RIA kit (Linco Research Inc, MO, USA).

Glycogen Analysis

Glycogen content was determined by the phenol–sulfuric acid method modified from Lo et al. [48]. Portions of frozen liver and muscle (50–90 mg) were weighed and placed in test tubes containing 1.0 ml of 5 M KOH solution saturated with sodium sulfate. The tubes were placed in a boiling water bath for 30 min to obtain a homogenous solution. Tubes were cooled on ice for 5 min, and glycogen was precipitated by the addition of 1 ml of 95% ethanol and incubation on ice for 30 min. Samples were centrifuged at 840 x g for 30 min, after which the supernatants were removed and the precipitates were dissolved in 3 ml of distilled water. Aliquots of the glycogen solutions, including standards, were made up to 1 ml in water. One milliliter of 5% phenol solution and 5 ml of a concentrated sulfuric acid solution were added in rapid succession to each tube. The tubes were allowed to stand for 10 min at room temperature, their contents thoroughly mixed, and the tubes were further incubated in the water bath (25°C) for 10 min, followed by measurement of their absorbances at 490 nm.

Mass Spectrometric Analysis of Total Liver Fatty Acids and Serum Acylcarnitines

Mass spectral analyses were performed using an APEX II FTICR-MS equipped with an Apollo ESI source (Bruker...
Determination of the Dicarboxylic Acid Content of Urine

Creatinine content was determined in the urine samples using the method of Popper et al. [49]. The samples were diluted with phosphate buffered saline to a creatinine concentration of 1 mM. Methanol solutions (1 mM) of various dicarboxylic acids were used as standards. Each sample was measured twice: once with standards containing odd-numbered carbon atoms (C5-, C7-, C9-, and C11-dicarboxylic acid) and tetradecanedioic acid as a reference and once with the standard mixture containing even-numbered carbon atoms (C6-, C8-, C10-, and C12-dicarboxylic acid) and tetradecanedioic acid as a reference. When tested separately the concentration of tetradecanedioic acid in the urine samples of mice was below the detection limit of the assay used, allowing the use of this acid as a reference (see below).

For quantitative determination of the dicarboxylic acids, 10 µl of standard solution was evaporated under a stream of nitrogen in eppendorf tubes, followed by the addition of 200 µl of the normalized urine sample. Non-carboxylic acid lipids were removed by extraction with three times 0.5 ml diethylether after increasing the pH with 350 µl 0.3 M NaOH. Carboxylic acids were extracted three times with 0.5 ml diethylether after the pH was decreased with 50 µl 6 M HCl. The acid extract was evaporated in a stream of nitrogen at room temperature. The residue was dissolved in 100 µl of 20 mM N,N'-carbonyldimiazole in acetonitrile and incubated at room temperature for 1 h. Thirty microliters of a solution containing 21 µl of acetonitrile, 3 µl of acetic acid, and 6 µl of phenylethylamime was added, and the samples were incubated for 1 h at room temperature. Finally, the entire sample was evaporated under a stream of nitrogen at 60°C. To desalt the sample, 25 µl of RP-18 (bed-vol.) (ICN, Eschwege, Germany) was prewashed with 0.5 ml methanol, resuspended in 0.5 ml of 0.1% TFA in acetonitrile (9/1, v/v), and added to the evaporated assay-mixture, followed by mixing and centrifugation. The supernatant was then discarded and the residue was again washed with 0.5 ml of 0.01% TFA in acetonitrile (9/1, v/v), and extracted with 200 µl methanol. For the MS measurements, 50 µl of the extract was diluted in 50 µl of MeOH/H2O/ACOH (49.5/49.5/1, v/v/v). Mass spectral data were recorded by accumulation of 256 scans at 256K resolution in positive mode. The intensities of the signals in the MS spectras were determined using the Xmass software (Bruker, Bremen) in order to compare the concentrations of dicarboxylic acid amides in the mouse urines. The intensity of the peak corresponding to the mass of tetradecanedioic acid amide was used as reference for the calculation of the concentrations of other metabolites. The intensity of the signals corresponding to the masses of the other standard substances was used as control for the similar behavior of dicarboxylic acid compounds with different chain length.

Real-Time Quantitative PCR

For real-time quantitative PCR analysis of Decr and several other genes involved in fatty acid metabolism, cDNA was produced using a First Strand cDNA Synthesis Kit (MBI Fermentas, Heidelberg, Germany) from total RNA isolated from mouse liver samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR was performed with a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using fluorogenic probe-based Taqman chemistry with Taqman Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Primers and FAM-labeled probes were designed using Primer Express software (Applied Biosystems) and the sequences available in Genbank and were purchased from Sigma-Genosys (Sigma-Genosys, Haverhill, UK). For relative quantification of gene expression, the results were normalized with GAPDH as an endogenous control for each sample and analyzed using 7500 System Software (Applied Biosystems).

Histological Analysis

For light microscopy analysis, samples from various tissues were fixed in 4% paraformaldehyde in potassium phosphate buffer (100 mM, pH 7.4), embedded in paraffin, sectioned and stained with hematoxylin and eosin. To stain the lipids, tissue samples were embedded in O.C.T. compound (Tissue-Tek, Zoeterwoude, Netherlands) and frozen in liquid nitrogen. Ten micrometer cryosections were cut from frozen samples with a Reichert-Jung 2800 Frigocut cryomicrotome and stained with Oil red O and hematoxylin using standard methods.
Supporting Information

Figure S1 Urine dicarboxylic acid profiles. Urine of the wild type (dotted line) and Dec−/− / mice (solid line) was collected for 24 h (fed sample) and collection was continued for 24 h after food removal (fasted sample). Pooled samples (5 mice/group) were normalized to urine creatinine and analyzed with mass spectrometry using tetradecanedioic acid (C14:0) as a reference. (A) Urine dicarboxylic acid profile without fasting. (B) Urine dicarboxylic acid profile after fasting.

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Table S1 Amino acid analysis from sera of fasted mice. Sera of mice (5 mice/group) was collected for 24 h (fed sample) and collection was continued for 24 h after food removal (fasted sample). Pooled samples (5 mice/group) were normalized to urine creatinine and analyzed with mass spectrometry using tetradecanedioic acid (C14:0) as a reference. (A) Urine dicarboxylic acid profile without fasting. (B) Urine dicarboxylic acid profile after fasting.

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Table S2 Urine dicarboxylic acid analysis. Urine of the wild type and Dec−/− / mice (5 mice/group) was collected for 24 h (fed sample) and collection was continued for 24 h after food removal (fasted sample). Pooled samples were normalized to urine creatinine and analyzed with mass spectrometry using tetradecanedioic acid (C14:0) as a reference.

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