Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse

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In the present paper, we describe a novel method which enables the analysis of tissue acylcarnitines and carnitine biosynthesis intermediates in the same sample. This method was used to investigate the carnitine and fatty acid metabolism in wild-type and LCAD^{-/-} (long-chain acyl-CoA dehydrogenase-deficient) mice. In agreement with previous results in plasma and bile, we found accumulation of the characteristic C_{14:1}-acylcarnitine in all investigated tissues from LCAD^{-/-} mice. Surprisingly, quantitatively relevant levels of 3-hydroxyacylcarnitines were found to be present in heart, muscle and brain in wild-type mice, suggesting that, in these tissues, long-chain 3-hydroxyacyl-CoA dehydrogenase is rate-limiting for mitochondrial β -oxidation. The 3-hydroxyacylcarnitines were absent in LCAD^{-/-} tissues, indicating that,

in this situation, the β -oxidation flux is limited by the LCAD deficiency. A profound deficiency of acetylcarnitine was observed in LCAD^{-/-} hearts, which most likely corresponds with low cardiac levels of acetyl-CoA. Since there was no carnitine deficiency and only a marginal elevation of potentially cardiotoxic acylcarnitines, we conclude from these data that the cardiomyopathy in the LCAD^{-/-} mouse is caused primarily by a severe energy deficiency in the heart, stressing the important role of LCAD in cardiac fatty acid metabolism in the mouse.

Key words: acylcarnitine, cardiomyopathy, carnitine biosynthesis intermediate, long-chain acyl-CoA dehydrogenase (LCAD), mitochondrial β -oxidation, tandem mass spectrometry.

INTRODUCTION

Carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) is a vital compound that plays an indispensable role in fatty acid metabolism. Before activated fatty acids, i.e. acyl-CoAs, can be broken down, they must first be transported into the mitochondrial matrix, where β -oxidation takes place. Because cytosolic acyl-CoAs cannot cross the inner mitochondrial membrane, acyl units are transferred from CoASH (reduced CoA) to carnitine and transported as acylcarnitine esters into the matrix by a dedicated carrier protein [1,2]. In the matrix, the acyl groups of the acylcarnitines are transferred back from carnitine to CoASH, and the resulting acyl-CoAs can then undergo β -oxidation. Free carnitine can be transported back to the cytosol for another round of import, but can also be used to transport acyl units out of the mitochondria [1,3,4]. This reversible acylation of carnitine allows replenishment of the free CoA pool when acyl-CoAs accumulate, for example in the case of mitochondrial β -oxidation disorders. Additionally, production of acylcarnitines can be used to export toxic acyl groups as acylcarnitines, first out of mitochondria and cell, followed by excretion from the body via urine or bile [5,6].

Omnivorous humans obtain carnitine both via the diet (meat and dairy products) and through endogenous synthesis. The first step in the carnitine biosynthesis is the hydroxylation of TML (6-*N*-trimethyl-lysine) to HTML (3-hydroxy-6-*N*-trimethyllysine), which is subsequently cleaved to yield TMABA (4-trimethylaminobutyraldehyde). TMABA is converted into γ -butyrobetaine (4-trimethylaminobutyric acid), which then is hydroxylated to carnitine [4]. The role of the carnitine biosynthetic pathway in overall carnitine homeostasis, especially in case of disturbed

fatty acid metabolism, such as in mitochondrial β -oxidation disorders, remains poorly understood.

In recent years, several mouse models for mitochondrial β -oxidation defects have been generated [7] including the LCAD^{-/-} (long-chain acyl-CoA dehydrogenase-deficient) mouse. LCAD is one of five enzymes that catalyse the α,β -dehydrogenation of acyl-CoAs, the initial step of mitochondrial β -oxidation. LCAD has been shown to be involved in the degradation of branched-chain fatty acids originating from peroxisomal catabolism of phytanic acid, but LCAD is also able to handle straight-chain and certain monounsaturated acyl-CoAs [8–10]. The phenotype of the LCAD^{-/-} mouse is most similar to human VLCAD (very-long-chain acyl-CoA dehydrogenase) deficiency, and is characterized by unprovoked sudden death, fasting and cold intolerance, hypoketotic hypoglycaemia, and marked fatty changes in liver and heart [7,11]. No human deficiency of LCAD has been described so far. LCAD^{-/-} mice have been shown to accumulate C₁₂–C₁₄-acylcarnitines in bile and serum [7,12]. Although plasma and bile acylcarnitine levels were determined in LCAD^{-/-} mice, it is not known how LCAD deficiency affects carnitine metabolism in tissues, since no method is available to determine the tissue levels of carnitine metabolites, i.e. (acyl)-carnitines and carnitine biosynthesis intermediates.

To investigate carnitine metabolism and its regulation in normal and pathological conditions, quantification of the metabolites involved in carnitine metabolism in body fluids and tissues is of great importance. Several methods have been described for the measurement of acylcarnitines in plasma, urine, bile and tissues [13–18]. Tissue acylcarnitine analysis, however, has been restricted to the measurement of large subsets, e.g. free carnitine

Abbreviations used: A_{AC}, area under the acylcarnitine peak; A_{IS}, area under the internal standard peak; γ -butyrobetaine, 4-trimethylaminobutyric acid; CAT, carnitine acetyltransferase; CoASH, reduced CoA; CPT2, carnitine palmitoyltransferase 2; HTML, 3-hydroxy-6-*N*-trimethyllysine; LCAD, long-chain acyl-CoA dehydrogenase; TMABA, 4-trimethylaminobutyraldehyde; TML, 6-*N*-trimethyllysine; VLCAD, very-long-chain acyl-CoA dehydrogenase.

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and short-, medium- and long-chain acylcarnitines [13,14]. Until now, no method has been described to determine the whole tissue acylcarnitine spectrum. We devised a novel sample work-up procedure, and slightly modified the established tandem-MS detection method, which now enables the determination of discrete acylcarnitines in tissues by tandem-MS. We also adapted this procedure to measure the levels of the carnitine biosynthesis intermediates in the same tissue sample, using a modification of our recently developed method for the analysis of carnitine biosynthesis intermediates in urine [19].

This new method was used to determine the effects of LCAD-deficiency on carnitine metabolites in tissues and plasma. Our results show considerable differences in tissue carnitine content and acylation between the wild-type and LCAD^{-/-} mice. In addition to the expected C_{14:1}-acylcarnitine accumulation, the acylcarnitine pattern in wild-type and LCAD^{-/-} mice in the different tissues unveils some of the specific metabolic conditions that exist in different tissues.

EXPERIMENTAL

Materials

The [²H₃]carnitine and [²H₃]C₃-, C₈- and C₁₆-acylcarnitine internal standards were obtained from Dr Herman J. ten Brink (VU Medical Hospital, Amsterdam, The Netherlands). The [²H₉]TML internal standard and γ -[²H₃]butyrobetaine were synthesized as described previously [19]. [²H₉]HTML was prepared enzymatically by incubating [²H₉]TML with *Neurospora crassa* TML dioxygenase, which was heterologously expressed in *Saccharomyces cerevisiae* as described by Swiegers et al. [20]. The resulting mixture of [²H₉]HTML and [²H₉]TML was applied to Microcon YM30 filters, and the deproteinized filtrate was used as an internal standard for TML and HTML. All other reagents were of analytical grade.

Mice

B6,129-*Acadl*^{-/-} (LCAD^{-/-}) and B6,129 *Acadl*^{+/+} (wild-type) mice were produced in a breeding colony at the University of Alabama at Birmingham. All experiments were approved by the local ethical committee. They were genotyped as described previously [12]. All experimental mice were male and 7–9 weeks of age at the time of sample collection. All mice were fed with a standard rodent diet (LM-485; Harlan Teklad). Mice were fasted for 7 h during the dark cycle and humanely killed for tissue collection. Tissues were immediately flash-frozen in liquid nitrogen.

Sample preparation for acylcarnitine and carnitine biosynthesis intermediate determination in tissues

Approx. 20 mg of cardiac left ventricle and 60 mg of the other tissues (liver, kidney, brain, muscle and testis) were placed in 1.5 ml Eppendorf vials, and the exact mass of the sample was determined using a microbalance. The internal standards for both the acylcarnitines and the carnitine biosynthesis intermediates were added (see Table 1). [²H₃]Carnitine was used as internal standard for free carnitine, [²H₃]C₃-acylcarnitine for C₂–C₅-acylcarnitines, [²H₃]C₈-acylcarnitine for C₆–C₁₀-acylcarnitines and [²H₃]C₁₆-acylcarnitine for C₁₂–C_{22:OH} acylcarnitine. Because of the lack of a commercially available labelled very-long-chain acylcarnitine internal standard, the [²H₃]C₁₆-acylcarnitine internal standard was used to approximate the levels of the C₂₀–C_{22:OH}-acylcarnitines. [²H₉]TML, [²H₉]HTML and γ -[²H₃]butyrobetaine were used as internal standards for TML, HTML and γ -butyrobetaine re-

Table 1 Internal standards added to the different tissues

Values are in nmol.

	Liver/heart/testis	Kidney/muscle	Brain	Plasma/urine
[² H ₃]Carnitine	16.25	6.25	6.25	1.175
[² H ₃]C ₃ -acylcarnitine	0.25	0.125	0.125	0.25
[² H ₃]C ₈ -acylcarnitine	0.1	0.05	0.05	0.1
[² H ₃]C ₁₆ -acylcarnitine	0.05	0.25	0.1	0.1
[² H ₉]TML	0.375	0.375	0.375	0.18
[² H ₉]HTML	0.18	0.18	0.18	0.075
γ -[² H ₃]Butyrobetaine	0.075	0.075	0.075	0.11

spectively. The samples were freeze-dried overnight. The freeze-dried tissues were kept on ice and grounded to powder using an Eppendorf micropestle. A 1 ml volume of an 8:2 (v/v) acetonitrile/water solution (80 % acetonitrile) was added to the tissue powder, and this suspension was sonicated twice for 20 s at 2.5 W with a probe-tip vibra-cell sonicator (Sonics & Materials, Danbury, CT, U.S.A.). Subsequently, the samples were centrifuged at 16 000 g for 5 min at 4 °C, and the supernatant was collected. Half of this 80 % acetonitrile supernatant was used for the acylcarnitine analysis, and the other half was used for the analysis of carnitine biosynthesis intermediates (see below).

Acylcarnitine analysis in tissues, plasma and urine

Half of the 80 % acetonitrile supernatant was evaporated under a stream of nitrogen at 40 °C. A 100 μ l volume of propylation reagent, a 4:1 (v/v) mixture of propan-2-ol and acetylchloride, was added to the residue, vortex-mixed and incubated at 65 °C for 15 min. The propylation reagent was evaporated under a stream of nitrogen at 40 °C, and the residue was taken up in 300 μ l of acetonitrile, which was stored at –20 °C until analysis. Plasma and urine samples were prepared as described previously [15], except that propylation reagent was used instead of butylation reagent, and samples were stored in acetonitrile at –20 °C until analysis. On the day of analysis, 70 μ l of the acetonitrile solution was mixed with 30 μ l of distilled water. A Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.), using a Gilson 231XL autosampler and a Hewlett-Packard HP-1100 HPLC pump was used for detection of the acylcarnitines. Collision energies, cone voltages and other mass spectrometric details are described by Rashed et al. [21]. To detect all the propylated acylcarnitines, the scan range for parent ions of *m/z* 85 was enlarged to *m/z* 180–600. For the quantification of the acylcarnitine levels, the area under each acylcarnitine peak (*A*_{AC}) and that under the added internal standard (*A*_{IS}), used for that particular acylcarnitine, was quantified using MassLynx 3.3 (Micromass). If appropriate, the peak areas were corrected for the contribution of naturally occurring isotopes. Assuming identical response to the appropriate internal standard, semi-quantitative analysis of acylcarnitines and hydroxyacylcarnitines was carried out by determining the ratio (*A*_{AC}/*A*_{IS}) and multiplying this by the amount of added internal standard. To compare individual samples, the result was normalized for creatinine concentration in the case of urine, and for wet tissue mass in the case of tissues.

Carnitine biosynthesis intermediate analysis in tissues, plasma and urine

To extract the carnitine biosynthesis intermediates efficiently, the remaining tissue powder pellet (after the 80 % acetonitrile extraction) was extracted for a second time with 500 μ l of an 2:8 (v/v) acetonitrile/water solution (20 % acetonitrile) as described

in the previous section. Half of the 20 % acetonitrile supernatant was pooled with the remaining half of the 80 % acetonitrile supernatant, and this solution was evaporated under a stream of nitrogen at 40 °C. The residue was resuspended in 100 µl of distilled water, and carnitine biosynthesis intermediates were analysed with ion-pair HPLC tandem MS and quantified as described previously [19].

To determine the concentration of the carnitine biosynthesis intermediates in plasma, internal standards (see Table 1) were added to 100 µl of plasma, and this mixture was partly deproteinized by loading it onto a Microcon 30 kDa filter, which was centrifuged at 14000 g for 20 min. An 80 µl volume of filtrate was derivatized and analysed with tandem MS as described previously [19]. This method, without the filtration step, was also used for the analysis of the carnitine biosynthesis intermediates in urine.

Validation

For the determination of the recovery of the internal standards, six pieces of each tissue were prepared, and both acylcarnitine and carnitine biosynthesis intermediate internal standards were added to three of these, after which the extraction procedure was performed as described above for all six samples. Before the first evaporation step, internal standards for acylcarnitines and carnitine biosynthesis intermediates were added to the three samples where addition of internal standard had been omitted. The recovery of the internal standards was used as a measure for the recovery of the endogenous compounds.

The variation within one mouse was determined by analysing the acylcarnitine and carnitine biosynthesis intermediate levels in six different pieces of each tissue of a single mouse. The variation between mice was determined by analysing each tissue (in duplicate) in six different mice.

Enzyme assays

CPT2 (carnitine palmitoyltransferase 2) activity was measured as described by Slama et al. [22]. CAT (carnitine acetyltransferase) activity was measured as described by Barth et al. [23]. TML dioxygenase was measured as described by Vaz et al. [24] and the γ -butyrobetaine dioxygenase assay was reported previously by Vaz et al. [25].

Comparison of wild-type and LCAD^{-/-} mice

Carnitine metabolites (acylcarnitines and carnitine biosynthesis intermediates) were analysed in duplicate in tissues of six LCAD^{-/-} mice, and results were compared with the wild-type data obtained in the validation experiments. In a separate experiment, three wild-type and three LCAD^{-/-} mice were fasted as described above, and urine and plasma were collected for carnitine metabolite analysis.

Western blotting

A Multiphor II Nova Blot electrophoretic transfer unit (Amersham Biosciences) was used to transfer proteins on to a nitrocellulose sheet (Schleicher & Shuell, Dassel, Germany) as described by the manufacturer of the transfer unit. After blocking of non-specific binding sites with 50 g/l Protifar and 10 g/l BSA in PBS with Tween 20 (1 g/l) for 1 h, the blot was incubated for 2 h with a 1:5000 dilution of rabbit polyclonal antibody against VLCAD in the same buffer without Protifar. The anti-VLCAD antibody [26] was a gift from Dr T. Hashimoto (Shinshu University, Matsumoto, Japan). Goat anti-rabbit IgG antibodies conjugated

to alkaline phosphatase were used for detection, according to the manufacturer's instructions (Bio-Rad, Hercules, CA, U.S.A.).

Statistical analysis

The Mann–Whitney test was used to evaluate whether significant differences exist between the LCAD^{-/-} and wild-type carnitine metabolite levels.

RESULTS AND DISCUSSION

Method development

To develop a new method for the extraction and efficient measurement of acylcarnitines from tissues, several issues had to be addressed. In initial experiments, aqueous homogenates were prepared followed by simultaneous deproteinization and extraction with acetonitrile. Besides the fact that preparing aqueous homogenates of tissues such as muscle and heart is difficult and not practical for large-scale sample work-up, the extraction efficiency of long-chain acylcarnitines was poor. Freeze-drying of the sample turned out to solve both problems. This procedure results in a brittle sample, which can be ground easily to powder and used for immediate solid–fluid extraction with a high percentage acetonitrile solution. This method resulted in an efficient extraction of all acylcarnitines.

Another problem was the interference of glutamic acid with the quantification of acetylcarnitine, a quantitatively important acylcarnitine. When samples are butylated, as in the commonly used plasma method [15], both acetylcarnitine and glutamic acid produce a parent ion of m/z 260, with a daughter fragment of 85 Da upon collision. Using the parent-scan for m/z 85, the abundance of m/z 260 can result from both compounds. Since tissues (especially brain) contain considerable amounts of glutamic acid, reliable quantitative analysis of acetylcarnitine is impossible using butylation. When samples are propylated instead, acetylcarnitine and glutamic acid produce parent ions which differ by 14 Da in mass: glutamic acid is a dicarboxylic acid and is propylated at two carboxylic groups, which results in a molecule with an $[M + H]^+$ ion of 232 Da. Acetylcarnitine, however, only contains one carboxylic group, and its propylation yields an $[M + H]^+$ ion of 246 Da. Both compounds can now be detected separately in the parent-scan of m/z of 85.

Beside acylcarnitines, we also wanted to analyse the carnitine biosynthesis intermediates in different tissues. Preliminary experiments showed that extraction of carnitine biosynthesis intermediates was most efficient with low concentrations of acetonitrile, whereas extraction with high concentrations of acetonitrile was best for acylcarnitines. Initial extraction of the tissue powder with high concentrations of acetonitrile, however, did not affect the subsequent low-acetonitrile extraction of the carnitine biosynthesis intermediates. This enabled analysis of both acylcarnitines and carnitine biosynthesis intermediates in the same tissue sample.

Validation

As an estimation of the recovery of the endogenous acylcarnitines and carnitine biosynthesis intermediates, we investigated the recovery of the internal standards. By adding the internal standards, either at the beginning or at the end of the work-up procedure, and analysing the difference between the internal standard levels in these samples, the recovery of the internal standards was determined for each tissue. The amount of internal standard, added at the end of the procedure, was set at 100 %. For [$^2\text{H}_9$]TML, [$^2\text{H}_9$]HTML and γ - $^2\text{H}_3$]butyrobetaine internal standards, the

Figure 1 Tissue and plasma levels of free carnitine and acetylcarnitine in wild-type and LCAD^{-/-} mice

Grey bars and white bars represent wild-type and LCAD^{-/-} mice respectively. Values are means $\pm$ S.D. for six mice, where each tissue was analysed in duplicate. Only one plasma analysis per mouse was performed due to the small amount of sample available. * $P < 0.05$; # $P < 0.01$.

recovery was more than 75 % in all tissues. The recovery of [²H₃]carnitine and [²H₃]C₃-, [²H₃]C₈- and [²H₃]C₁₆-acylcarnitines was more than 70 % in all tissues except testis, where 40–70 % of all acylcarnitine internal standards were recovered.

The inter-assay variation for the different tissue acylcarnitines and carnitine biosynthesis intermediates was determined to be 30 % or less for most compounds. The intra-assay variation was within acceptable limits (< 30 %). No intra-assay variation could be determined for plasma due to the small quantity of plasma available; however, previous studies have shown that the variation of acylcarnitine analysis in plasma is small [15].

After validation, this new method was used to investigate the differences in carnitine metabolism between wild-type and LCAD^{-/-} mice. First, the results of the acylcarnitine and carnitine biosynthesis intermediates analysis from the wild-type mice will be discussed, after which these will be compared with the results obtained for the LCAD^{-/-} mice.

Tissue distribution of carnitine metabolites in wild-type mice

In the wild-type mouse, considerable differences exist between tissues with respect to the carnitine content and acylation pattern, as can be seen in Figures 1 and 2 (grey bars). Normalized per gram of wet mass, most carnitine (both free and acylated) is found in heart, followed by kidney, muscle, testis, liver and brain. In heart, approx. 25 % of total carnitine is present as acylcarnitines, primarily as acetylcarnitine. In muscle, more than 50 % of total carnitine is present as acylcarnitines, of which half is acetylcarnitine. A striking difference in both the percentage of acylation and the spectrum of long-chain acylcarnitines exists between heart and muscle. The latter tissue contains primarily C₁₆- and C_{18:1}-acylcarnitines, which are present in high amounts (28.7 $\pm$ 7.8 and 19.2 $\pm$ 9.7 nmol/g respectively). Heart, however, contains less acylcarnitine (individual acylcarnitines are in the range

1–3 nmol/g), but these encompass the whole spectrum of medium-/long-chain acylcarnitines, varying from C₁₂- to even C₂₀- and C₂₂-acylcarnitines (Figure 2, grey bars). The accumulation of long-chain acylcarnitines in muscle could perhaps reflect the discontinuous activity of muscle as opposed to the continuous work of the heart and the corresponding energy demand. The muscle acylcarnitines could represent stored energy units which can be readily used upon muscle activity.

An interesting finding was the presence of relatively high amounts of long-chain hydroxyacylcarnitines in heart (Figure 3, grey bars), which, in most cases, were even more abundant than the corresponding acylcarnitines. Hydroxyacylcarnitines were also found in muscle and brain (Figure 3, grey bars). Surprisingly, in brain, C_{12:1-OH}-, C_{14:1-OH}- and C_{18:1-OH}-C_{22:1-OH}-acylcarnitines were present, whereas their corresponding acylcarnitines were not detectable. The observed hydroxyacylcarnitines most likely represent 3-hydroxy β -oxidation intermediates, but mass spectrometric detection does not allow identification of the position of the hydroxy group.

As in previous studies, plasma acylcarnitines represent approx. 40 % of total plasma carnitine and consist mostly of acetylcarnitine and C₁₆-acylcarnitine. Liver has the smallest fraction of acylcarnitines, followed by kidney and brain.

The levels of the carnitine biosynthesis intermediates are shown in Figure 4 (grey bars). The TML content is similar in all tissues except for kidney, where this compound is more abundant. In mice, TML is readily reabsorbed from the urine [27], which could explain the higher levels of this compound in kidney. No HTML was found in any of the tissues studied, which is probably caused by rapid conversion of HTML into TMABA and γ -butyrobetaine. γ -Butyrobetaine levels do not differ much between the studied tissues, but are the lowest in liver. This might be explained by the presence of γ -butyrobetaine dioxygenase in this organ, which rapidly converts γ -butyrobetaine into carnitine.

Comparison of wild-type and LCAD^{-/-} mice

Although free carnitine levels are somewhat decreased in muscle and brain (see Figure 1), the LCAD^{-/-} mice do not display carnitine deficiency, which has been reported in several human β -oxidation disorders, supposedly as a result of the excretion of non-metabolizable acyl units as acylcarnitines [28]. Liver, testis and, especially, kidney even show an increase in free carnitine content. Possibly hepatic carnitine biosynthesis is increased and the renal reabsorption of carnitine is enhanced in LCAD^{-/-} mice, which would counteract the loss of carnitine due to elimination of accumulating acylcarnitines. This is supported by the observation that the kidney has a significantly higher carnitine content, suggesting that carnitine is efficiently reabsorbed. Also, tissue TML levels are generally lower in the LCAD^{-/-} tissues (Figure 4), indicating that this compound is used for carnitine synthesis. The TML levels in kidney are dramatically reduced. This could be a result of increased TML dioxygenase activity, which is present in this tissue. We excluded this possibility by measuring TML dioxygenase activity in wild-type and LCAD^{-/-} kidney, which did not differ between wild-type and LCAD^{-/-} mice [12.0 $\pm$ 2.0 ($n = 6$) and 11.6 $\pm$ 0.7 ($n = 6$) pmol/min $\cdot$ mg respectively]. Alternatively, TML excretion could be enhanced, resulting in lower tissue and kidney TML levels. Unfortunately, urine was not available to measure carnitine and TML excretion. Therefore we repeated the experiment, collected urine and plasma, and analysed carnitine metabolites in these samples. As in the previous experiment, the same metabolic abnormalities were found in the plasma of LCAD^{-/-} mice. Analysis of carnitine biosynthesis intermediates in urine of wild-type and LCAD^{-/-} mice showed

Figure 2 Tissue and plasma acylcarnitine levels in wild-type and LCAD^{-/-} mice

Grey bars and white bars represent wild-type and LCAD^{-/-} mice respectively. Values are means $\pm$ S.D. for six mice, where each tissue was analysed in duplicate. Only one plasma analysis per mouse was performed due to the small amount of sample available. * $P < 0.05$; # $P < 0.01$.

no enhanced excretion of TML in the LCAD^{-/-} group (results not shown), indicating that urinary loss is not the reason for the low tissue and kidney TML levels. Based on this experiment, however, we cannot exclude the possibility that a low, but persistent, urinary loss of TML cumulatively results in TML deficiency.

Except in plasma, no major differences can be seen in tissue γ -butyrobetaine levels between wild-type and LCAD^{-/-} mice (Figure 4). The reduction of plasma γ -butyrobetaine might result

from increased hepatic γ -butyrobetaine uptake and subsequent conversion into carnitine.

Figure 1 shows that there is a profound deficiency of acetylcarnitine in LCAD^{-/-} heart. To a lesser extent, this is also observed in testis, but not in the other investigated tissues, where acetylcarnitine levels are comparable with wild-type values. Normally, the heart's energy need is met primarily by fatty acid β -oxidation. LCAD is highly expressed in heart [29], and the

Figure 3 Hydroxyacylcarnitine levels in heart, muscle and brain in wild-type and LCAD^{-/-} mice

(A) Grey bars and white bars represent wild-type and LCAD^{-/-} mice respectively. Values are means $\pm$ S.D. for six mice, where each tissue was analysed in duplicate. All the differences between wild-type and LCAD^{-/-} mice were determined to be significant ($P < 0.01$). (B) Parent scan of m/z 85, showing the acylcarnitine spectrum of wild-type and LCAD^{-/-} mice in heart. Note the presence of high levels of hydroxyacylcarnitines in the wild-type heart and their dramatic reduction in LCAD^{-/-} mice. The peak at m/z 445 corresponds to the internal standard (I.S.), [2H_3]C₁₆-acylcarnitine.

fact that LCAD^{-/-} mice suffer from cardiomyopathy [11,12] indicates that LCAD plays an important role in cardiac fatty acid metabolism. There are several possible explanations for the shortage of acetylcarnitine in the LCAD^{-/-} heart: (i) carnitine deficiency, (ii) shortage of acetyl-CoA or (iii) decreased activity of CAT, which transfers acetyl groups from CoA to carnitine. Since there is no carnitine deficiency in LCAD^{-/-} hearts (Figure 1), we measured CAT activity in homogenates of three wild-type and three LCAD^{-/-} hearts to discriminate between the two latter possibilities. CAT activity did not differ between wild-type (242 ± 45 nmol/min per mg; $n = 3$) and LCAD^{-/-} heart (259 ± 11 nmol/min per mg; $n = 3$), eliminating the possibility that lower CAT activity caused the observed acetylcarnitine deficiency. This strongly suggests that there is a profound deficiency of acetyl-CoA in LCAD^{-/-} hearts, implying a severe energy shortage which

could result in the observed cardiomyopathy in LCAD^{-/-} mice [12]. Previously, it was speculated that the accumulation of heart acylcarnitines may be an important factor in the development of cardiomyopathy or cardiac arrhythmia in the LCAD^{-/-} mice; however, the total acylcarnitine levels in heart are only marginally elevated. This raises the important point that the deficiency of acetyl-CoA in the LCAD^{-/-} heart is primarily responsible for the occurrence of the cardiomyopathy.

The deficiency of acetyl-CoA could be caused by low levels of mitochondrial free CoA, which in turn could result from the accumulation of non-metabolizable acyl-CoAs. A lack of free CoA would inhibit pyruvate dehydrogenase and thereby the formation of acetyl-CoA. Alternatively, if the mitochondrial free CoA levels are sufficient to sustain oxidation of pyruvate, the acetyl-CoA formed by pyruvate dehydrogenase would immediately enter the

Figure 4 Carnitine biosynthesis intermediates in plasma and tissues of wild-type and LCAD^{-/-} mice

Grey bars and white bars represent wild-type and LCAD^{-/-} mice respectively. Values are means $\pm$ S.D. for six mice, where each tissue was analysed in duplicate. Only one plasma analysis per mouse was performed due to the small amount of sample available. * $P < 0.05$; # $P < 0.01$.

citric acid cycle (because of the energy shortage in the LCAD^{-/-} heart caused by impaired β -oxidation), resulting in low levels of acetyl-CoA.

The most prominent difference between the wild-type and the LCAD^{-/-} tissue and plasma acylcarnitines (Figure 2) is the increase of the characteristic C_{14:1}-acylcarnitine in the LCAD^{-/-} samples. In plasma, C₁₂- and C₁₄-C_{18:1}-acylcarnitines are also elevated, but to a lesser extent than C_{14:1}-acylcarnitine, which is in agreement with previous observations [11,12]. Although the levels of C_{14:1}-acylcarnitine in tissues are considerably higher than in wild-type mice, this accumulation is not as dramatic as observed in plasma, suggesting that excess C_{14:1}-acylcarnitines are transported out of the tissues into the circulation.

Figure 5 Western blot analysis of VLCAD in heart, muscle, kidney and liver of three wild-type and three LCAD^{-/-} mice

Each lane represents 100 µg of tissue homogenate.

In LCAD^{-/-} muscle, a highly significant accumulation (approx. 5-fold higher than wild-type levels; $P < 0.01$) of C₉-, C_{9:1}- and C₁₁-acylcarnitines was observed (results not shown). Although we cannot distinguish between straight-chain and branched-chain acylcarnitines using our tandem-MS method, it is likely that these acylcarnitines represent 2,6-dimethylheptanoyl-carnitine, 2,6-dimethylheptenoyl-carnitine and 4,8-dimethylnonanoyl-carnitine respectively, which could originate from peroxisomal catabolism of phytanic acid, since LCAD has been shown previously to be involved in the complete degradation of these compounds [8,11]. Further studies with a diet enriched in branched-chain fatty acids could confirm that these acylcarnitines indeed accumulate due to LCAD deficiency. These studies are currently under way.

In addition to the elevated tissue levels of C_{14:1}-acylcarnitine, C₁₄-C₁₈-acylcarnitines were also elevated to variable extents in all LCAD^{-/-} tissues, except for heart and muscle, which show similar or even decreased levels respectively (Figure 2). Heart does show a decrease of the very-long-chain acylcarnitine levels, e.g. C₂₀-C₂₂, in the LCAD^{-/-} mouse. There are several mechanisms which could account for the decreased levels of (very-) long-chain acylcarnitines in muscle and heart, including: (i) carnitine deficiency, (ii) reduced activity of CPT2, which transfers acyl groups from CoA to carnitine, (iii) increased VLCAD activity, (iv) increased triacylglycerol synthesis or (v) excretion of acylcarnitines from tissues into the circulation.

Since both muscle and heart are not carnitine deficient (Figure 1), CPT2 activity was measured in muscle and heart homogenates of three wild-type and three LCAD^{-/-} mice. CPT2 activity in wild-type muscle (18.0 ± 3.3 nmol/min per mg; $n = 3$) was similar to that in LCAD^{-/-} muscle (18.5 ± 6.5 nmol/min per mg; $n = 3$). CPT2 activity even was slightly elevated ($P < 0.05$) in LCAD^{-/-} heart (218 ± 20 nmol/min per mg; $n = 3$) when compared with wild-type heart (157 ± 8 nmol/min per mg; $n = 3$), excluding this as a cause of the decreased levels of (very-) long-chain acylcarnitines in LCAD^{-/-} heart and muscle.

The observed changes in the acylcarnitine pattern in muscle and heart could also result from increased VLCAD activity, which has a broad substrate specificity ranging from C₁₄- to C₂₄-CoA [26]. Therefore the amount of VLCAD protein in heart, muscle, kidney and liver was analysed by Western blot using an anti-VLCAD antibody (Figure 5). The last two tissues were included to compare the heart and muscle VLCAD levels with tissues where long-chain acylcarnitines did accumulate. As expected, there was no difference in the VLCAD protein levels in kidney and liver between the two genotypes. In LCAD^{-/-} heart, however, there is a small

increase in VLCAD protein levels, which could contribute to the decreased levels of very-long-chain acylcarnitines. The expression of VLCAD in muscle is very low, and, in contrast with heart, does not differ between wild-type and LCAD^{-/-} mice, suggesting that other mechanisms are responsible for the decreased long-chain acylcarnitine levels in muscle.

Another possible mechanism to dispose of accumulating acyl-CoA esters is the production of triacylglycerols. Indeed, this is supported by the observations that triacylglycerol droplets accumulate in heart and liver of fasted LCAD^{-/-} mice [12]. Although it is likely that, in the fasted LCAD^{-/-} heart, triacylglycerols are synthesized to reduce the build-up of non-metabolizable acyl units, no accumulation of triacylglycerols is seen in muscle of fasted LCAD^{-/-} mice (P. A. Wood, unpublished work), which is in agreement with the fact that muscle has a very low capacity to synthesize triacylglycerols [30]. Since we have excluded the other mechanisms that could account for the decrease in LCAD^{-/-} muscle long-chain acylcarnitine levels, the only remaining possibility is that these acylcarnitines are secreted into the circulation. Although there is no experimental evidence to support this, the fact that the acylcarnitines which are lacking in LCAD^{-/-} muscle are found in plasma (Figure 2) agrees with this hypothesis.

Closer examination of the acylcarnitine profiles revealed that the hydroxyacylcarnitines observed in wild-type heart, muscle and brain were nearly absent in LCAD^{-/-} mice (Figure 3). The absence of these hydroxyacylcarnitines in the LCAD^{-/-} mice indicates that they most likely represent 3-hydroxyacylcarnitines and are indeed derived from 3-hydroxy-fatty acyl-CoAs. CPT2 is very likely to be responsible for the formation of these 3-hydroxyacylcarnitines, since it has been shown previously that this enzyme is capable of handling 3-hydroxy-fatty acyl-CoAs as substrates [31]. The production of hydroxyacylcarnitines from exogenous hexadecanoate or its corresponding carnitine/CoA-ester was shown previously in rat muscle [32] and heart [33]. In the present study, we show the presence of endogenous hydroxyacylcarnitines in these two mouse tissues and brain. The fact that these hydroxyacylcarnitines are present in the wild-type situation suggests that, in these three tissues, the dehydrogenation of 3-hydroxyacyl-CoA, catalysed by long-chain hydroxyacyl-CoA dehydrogenase, is rate-limiting. In an LCAD^{-/-} situation, however, where only VLCAD is able to catalyse the first step of the β -oxidation, the formation of the enoyl-CoA determines the flux through the β -oxidation pathway, which results in reduction of the hydroxyacylcarnitine levels.

In conclusion, we developed and validated a novel method which enables the analysis of tissue acylcarnitines and carnitine biosynthesis intermediates in the same sample. This method was used to investigate the carnitine and fatty acid metabolism in wild-type and LCAD^{-/-} mice. As was reported previously for plasma and bile [11,12], we found accumulation of the characteristic C_{14:1}-acylcarnitine in all investigated tissues. The most striking finding of the present study was the severe deficiency of acetyl-carnitine in heart, which most likely results from an acetyl-CoA deficiency. Since there was no carnitine deficiency and only a marginal elevation of potentially cardiotoxic acylcarnitines, we conclude that cardiomyopathy in the LCAD^{-/-} mouse is caused primarily by a severe energy deficiency in the heart, stressing the important role of LCAD in cardiac fatty acid metabolism in the mouse. LCAD^{-/-} muscle showed accumulation of odd-number acylcarnitines (C₉ and C₁₁). These acylcarnitines could represent peroxisomal oxidation products of phytanic acid, which are confirmed LCAD substrates. This is in agreement with previous data, which show that LCAD is involved in branched-chain fatty acid metabolism [8,11].

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