Biochimica et Biophysica Acta 1841 (2014) 987–994

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

# Plasma acylcarnitines inadequately reflect tissue acylcarnitine metabolism☆

Marieke G. Schooneman [a,b], Niki Achterkamp [a], Carmen A. Argmann [d], Maarten R. Soeters [a], Sander M. Houten [b,c,d,*]

[a] Department of Endocrinology and Metabolism, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[b] Department of Clinical Chemistry, Laboratory Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[c] Department of Pediatrics, Emma Children's Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[d] Department of Genetics and Genomic Sciences, Icahn Institute for Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, NY, USA

ARTICLE INFO

*Article history:*
Received 9 January 2014
Received in revised form 12 March 2014
Accepted 9 April 2014
Available online 18 April 2014

*Keywords:*
Acylcarnitines
Insulin resistance
Fatty acid oxidation
Lipotoxicity

ABSTRACT

Acylcarnitines have been linked to obesity-induced insulin resistance. However the majority of these studies have focused on acylcarnitines in plasma. It is currently unclear to what extent plasma levels of acylcarnitines reflect tissue acylcarnitine metabolism. We investigated the correlation of plasma acylcarnitine levels with selected tissue acylcarnitines as measured with tandem mass spectrometry, in both fed and fasted BALB/cJ (BALB) and C57BL/6N (Bl6) mice. Fasting affected acylcarnitine levels in all tissues. These changes varied substantially between the different tissue compartments. No significant correlations were found between plasma acylcarnitine species and their tissue counterparts in both mouse strains, with the exception of plasma C4OH-carnitine in BALB mice. We suggest that this lack of correlation is due to differences in acylcarnitine turnover rates between plasma and tissue compartments and the fact that the plasma acylcarnitine profile is a composition of acylcarnitines derived from different compartments. Therefore, plasma acylcarnitine levels do not reflect tissue levels and should be interpreted with caution. A focus on tissue acylcarnitine levels is warranted in metabolic studies.



## 1. Introduction

The western lifestyle and concomitant obesity epidemic are the main causes of the increasing prevalence of insulin resistance, a condition characterized by decreased insulin sensitivity of tissues such as in liver and muscle. In obesity and overfeeding, high levels of circulating lipids can lead to lipid storage in non-adipose tissue [22]. This ectopic fat deposition can cause disturbances in lipid metabolism which may lead to insulin resistance, a concept referred to as lipotoxicity [10,11,22]. Several lipid intermediates like fatty acyl-CoAs, diacylglycerol (DAG), ceramides, gangliosides and free fatty acids (FFA) have been implicated in the development of insulin resistance [20,28]. Acylcarnitines have been suggested to result from incomplete fatty acid oxidation (FAO) and were also proposed to induce insulin resistance [1,12].

Acylcarnitines comprise an acyl group esterified to L-carnitine, which enables them to cross the mitochondrial membrane. Given the abundance of different acyl groups, the resulting acylcarnitine profile is extensive. Most acyl groups are derived from FAO, but they can also originate from amino acid and glucose metabolism. L-carnitine is mainly absorbed from the diet, but can also be formed through biosynthesis in human liver, kidney and brain. Other tissues must therefore acquire carnitine from the circulation [37]. The plasma acylcarnitine profile is directly influenced by diet and metabolic status such as fasting, which is associated with altered FAO flux [7,15,17,30]. Acylcarnitine profiling has been and remains the golden standard in diagnosing many different inborn errors of metabolism including FAO disorders [5].

Several recent studies show increased levels of multiple acylcarnitines in obese, insulin resistant subjects [11,17,20]. This has led to the hypothesis that accumulating acylcarnitines can interfere with insulin signaling. Long-chain acylcarnitines [20,26,27], ketone body derived C4OH-carnitine [1,20,31] and branched-chain amino acid (BCAA) derived species C3- and C5-carnitine [24,39] have been suggested to induce insulin resistance. In some of these studies, the investigators measured both in plasma and muscle tissue, but the majority focused only on plasma measurements of acylcarnitines and

*Abbreviations:* BALB, Balb/cJ; Bl6, C57BL/6N; DAG, diacylglycerol; FFA, free fatty acids; FAO, fatty acid oxidation; BCAA, branched-chain amino acids; WAT, white adipose tissue; BAT, brown adipose tissue; ACN, acetonitrile; PPAR-α, peroxisome proliferator-activated receptor-α; SCOT, succinyl CoA-oxoacid transferase; PDH, pyruvate dehydrogenase

☆ Funding statement: None.

* Corresponding author at: Department of Genetics and Genomic Sciences, Icahn Institute for Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai, 1425 Madison Avenue, Box 1498, New York, NY 10029, USA. Tel.: +1 212 659 9222x89222.
*E-mail address:* sander.houten@mssm.edu (S.M. Houten).

http://dx.doi.org/10.1016/j.bbalip.2014.04.001
1388-1981/

correlations between plasma and tissue were not investigated. Insulin resistance, however, occurs on a cellular level of the different organ and tissue compartments, and these compartments may play distinct roles in the development of whole body insulin resistance. Therefore it is important to understand if and how the carnitine and acylcarnitine pool in tissues is represented in the plasma compartment, and whether plasma acylcarnitine profiles accurately reflect acylcarnitine profiles in any of the tissues implicated in insulin resistance.

In a previous study, we showed that changes in plasma acylcarnitine levels upon fasting in lean healthy men did not reflect changes in acylcarnitine levels in muscle tissue [30]. The aim of this study was to investigate if and in what way acylcarnitines in plasma reflect acylcarnitine profiles in mouse liver, muscle, heart, white adipose tissue (WAT) and brown adipose tissue (BAT). Therefore we studied the correlation of plasma acylcarnitines with the acylcarnitines in these tissues, in both fed and fasted Balb/cJ (BALB) mice and a more insulin resistant model, C57BL/6N (Bl6) mice. We show that plasma acylcarnitine levels poorly reflect tissue acylcarnitine levels.

## 2. Methods

### 2.1. Animal studies

Mice were approximately 10 weeks of age at the time of the experiment. BALB mice were acquired from Harlan Laboratories and Bl6 mice were acquired from Charles River Laboratories. Mice were housed under standard conditions and fed a chow diet. After one week of acclimatization both the BALB and Bl6 groups (22 mice per strain) were split into two, of which one group was fed ad libitum whereas the other half was fasted overnight for approximately 17 to 19 h to increase FAO and induce insulin resistance. Between 9 AM and 11 AM on the study day, all animals were anesthetized with pentobarbital (100 mg/kg i.p.). In the subsequent dissection, venous blood sampling was performed by cannulation of the caval vein. Liver, soleus muscle, gastrocnemius muscle, quadriceps femoris muscle, heart, gonadal white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) were dissected and frozen in liquid nitrogen, later to be stored at −80 °C.

All experiments were approved by the institutional review board for animal experiments at the Academic Medical Center (Amsterdam, The Netherlands).

### 2.2. Laboratory analyses

For plasma acylcarnitine analysis, 25 μl of plasma was mixed with 50 μl of internal standard mixture (25 μl of 5 μM [3,3,3-$^2H_3$]C3-carnitine and 2 μM [6,6,6-$^2H_3$]C6-, [8,8,8-$^2H_3$]C8-, [10,10,10-$^2H_3$]C10- and [16,16,16-$^2H_3$]C16-carnitine in acetonitrile (ACN), and 25 μl of 26 μM [methyl-$^2H_3$]-L-carnitine in 10% ACN) [38]. The plasma samples were deproteinized by addition of 250 μl ACN and subsequent vortex mixing. Next, samples were centrifuged for 10 min at 4 °C at a speed of 20,000 *g*. The supernatant was transferred into 4 ml glass vials and evaporated under a stream of nitrogen at 40 °C. After evaporation, 100 μl butylation reagent (4:1 mixture of 1-butanol and acetylchloride) was added and incubated for 15 min at 60 °C. Again evaporation was performed at 40 °C. The residue was dissolved in 100 μl ACN, vortex mixed and transferred to Gilson vials for tandem mass spectrometric analysis (Waters/Micromass Quattro Premier XE).

Tissues were freeze-dried overnight and weighed afterwards. ACN (800 μl) and 50 μl of internal standard mixture (25 μl of 5 μM [3,3,3-$^2H_3$]C3-carnitine and 2 μM [6,6,6-$^2H_3$]C6-, [8,8,8-$^2H_3$]C8-, [10,10,10-$^2H_3$]C10- and [16,16,16-$^2H_3$]C16-carnitine in ACN, and 25 μl of 325 μM [methyl-$^2H_3$]-L-carnitine in 20% ACN) were added to the tissue [35]. The sample was homogenized by shaking twice with a 4 mm metal ball using a TissueLyser II (Qiagen) for 30 s at frequency of 30/s. Samples were centrifuged for 10 min at 4 °C at a speed of 20,000 *g*. The supernatant was transferred into 4 ml glass vials and evaporated under a stream of nitrogen at 40 °C. After evaporation, 100 μl propylation reagent (4:1 mixture of 1-propanol and acetylchloride) was added to the residue, vortex mixed and incubated for 15 min at 60 °C. The derivatization reagent was evaporated under nitrogen and the residue was dissolved in 100 μl ACN, vortex mixed and transferred to Gilson vials for tandem mass spectrometric analysis.

To detect the acylcarnitines the scan range for butylated samples was 215–515 (m/z), and for propylated samples 200–750 (m/z). The common daughter ion of 85 was detected, which results in a spectrum of parent ions corresponding to $(M + H)^+$. The area under each acylcarnitine peak (AAC) and that under the IS (AIS) was quantified using MassLynx 4.1. The ratio AAC/AIS was determined and multiplied by the amount of added internal standard to carry out a semi-quantitative analysis of acylcarnitines and hydroxyacylcarnitines. The results of tissue were normalized for dry tissue mass to compare individual samples.

### 2.3. Statistical analysis

We specifically analyzed acylcarnitine species which we considered quantitatively, dietary or metabolically relevant. We therefore chose to analyze several short-chain species such as C0, C2 and C4 which reflect the free carnitine pool and breakdown products from FAO, glucose and amino acid metabolism. We also analyzed C4OH-carnitine which in human muscle is mainly a ketone body derived acylcarnitine (D-stereo isomer of hydroxybutyrylcarnitine) [31] and several long-chain species of which C16 and C18:1-carnitine are derivatives of palmitate and oleate. Also, we analyzed C3- and C5-carnitine. C3-carnitine, or propionylcarnitine, can be derived from the BCAAs valine or isoleucine. C5-carnitine (isovalerylcarnitine or 2-methylbutyrylcarnitine) can be derived from the BCAAs leucine or isoleucine [39]. The data for each individual mouse are available as supplemental table 1 (Table S1).

Acylcarnitine measurements were processed with Masslynx software version 4.1, and further analyzed using Microsoft Office Excel 2003. MetaboAnalyst 2.0 was used for hierarchical clustering and heatmap generation [40,41]. We used the complete clustering algorithm with the Spearman correlation coefficient as the distance measure. Data were processed with range scaling methods (mean-centered and divided by the range of each variable). Final statistical analysis was performed using SPSS statistical software program version 20.0. Data were evaluated by statistical correlations using bivariate Spearman's correlation coefficients (two-tailed) and with independent T-tests. Data were considered significant with a P value $<0.05$ (marked as *) or $<0.01$ (marked as **).

For each of the four datasets (Bl6 or BALB, fasted or fed), we constructed an unweighted signed metabolite coexpression network using the approximately 15 unique metabolites measured across 8 different tissues. We followed the R tutorial as outlined previously [13,43]. In brief, in an unweighted signed coexpression network, nodes represent metabolites and the nodes are connected if the corresponding metabolites are positively co-expressed across the tissue samples according to a hard cutoff (positive Spearman's rank correlation coefficient $>0.75$). This conservative threshold was determined based on the fact that the majority of the metabolite pair-wise correlations were highly significant at this cutoff. While coexpression networks can often use the absolute value of the correlation coefficient as an unsigned co-expression similarity measure, we employed a signed co-expression measure ($s_{ij}$) between metabolite expression profiles $x_i$ and $x_j$, for which we used a simple transformation of the correlation: $s_{ij} = (1 + \mathrm{cor}(x_i x_j) / 2)$.

For the unweighted networks, we then generated an adjacency matrix which contains binary information with entries of 1 or 0 depending on whether or not 2 nodes are adjacent (connected) to one another. To group metabolites with coherent expression profiles into modules, we used average linkage hierarchical clustering, which uses the topological overlap measure (TOM) as dissimilarity. The topological overlap of two nodes reflects their similarity in terms of the commonality of the