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Brief Report—Endocrine Research

Production and Release of Acylcarnitines by Primary Myotubes Reflect the Differences in Fasting Fat Oxidation of the Donors

Magnus Wolf,* Shili Chen,* Xinjie Zhao, Mika Scheler, Martin Irmler, Harald Staiger, Johannes Beckers, Martin Hrabé de Angelis, Andreas Fritsche, Hans-Ulrich Häring, Erwin D. Schleicher, Guowang Xu, Rainer Lehmann, and Cora Weigert

Division of Endocrinology, Diabetology, Angiology, Nephrology, Pathobiochemistry, and Clinical Chemistry (M.W., H.S., A.F., H.-U.H., E.D.S., R.L., C.W.), Department of Internal Medicine, University Tübingen , 72076 Tübingen , Germany; CAS Key Laboratory of Separation Science for Analytical Chemistry (S.C., X.Z., G.X.), Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 116023 Dalian, China; Institute of Experimental Genetics (M.S., M.I., J.B., M.H.d.A.), Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), 85764 Neuherberg, Germany; Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Zentrum München at the University of Tübingen (H.S., A.F., H.-U.H., E.D.S., R.L., C.W.), 72076 Tübingen , Germany; German Center for Diabetes Research (DZD) (M.S., M.I., H.S., M.H.d.A., A.F., H.-U.H., E.D.S., R.L., C.W.); and Chair of Experimental Genetics (J.B., M.H.d.A.), Center of Life and Food Sciences Weihenstephan, Technische Universität München, 85354 Freising-Weihenstephan, Germany

Context: Acylcarnitines are biomarkers of incomplete β-oxidation and mitochondrial lipid overload but indicate also high rates of mitochondrial fatty acid oxidation. It is unknown whether the production of acylcarnitines in primary human myotubes obtained from lean, metabolically healthy subjects reflects the fat oxidation in vivo.

Objective: Our objective was to quantify the acylcarnitine production in myotubes obtained from subjects with low and high fasting respiratory quotient (RQ).

Methods: Fasting RQ was determined by indirect calorimetry. Muscle biopsies from the vastus lateralis muscle were taken from 6 subjects with low fasting RQ (mean 0.79 ± 0.03) and 6 with high fasting RQ (0.90 ± 0.03), and satellite cells were isolated, cultured, and differentiated to myotubes. Myotubes were cultivated with 125μM ^{13}C-labeled palmitate for 30 minutes and 4 and 24 hours. Quantitative profiling of 42 intracellular and 31 extracellular acylcarnitines was performed by stable isotope dilution-based metabolomics analysis by liquid chromatography coupled to mass spectrometry.

Results: Myotubes from donors with high fasting RQ produced and released significant higher amounts of medium-chain acylcarnitines. High ^{13}C8 and ^{13}C10 acylcarnitine levels in the extracellular compartment correlated with high fasting RQ. The decreased expression of medium-chain acyl-coenzyme A dehydrogenase (*MCAD*) in these myotubes can explain the higher rate of incomplete fatty acid oxidation. A lower intracellular [^{13}C]acetylcarnitine to carnitine and lower intracellular ^{13}C16/^{13}C18 acylcarnitine to carnitine ratio indicate reduced fatty acid oxidation capacity in these myotubes. Mitochondrial DNA content was not different.

Conclusion: Acylcarnitine production and release from primary human myotubes of donors with high fasting RQ indicate a reduced fatty acid oxidation capacity and a higher rate of incomplete fatty acid oxidation. Thus, quantitative profiling of acylcarnitine production in human myotubes can be a suitable tool to identify muscular determinants of fat oxidation in vivo. (***J Clin Endocrinol Metab*** **98: E0000–E0000, 2013)**

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* M.W. and S.C. contributed equally to this work.

Abbreviations: CoA, coenzyme A; FBS, fetal bovine serum; MCAD, medium-chain acyl-CoA dehydrogenase; NEFA, nonesterified fatty acid; RQ, respiratory quotient; UHPLC-ESI-QQQ-MS, ultra high performance liquid chromatography/electrospray ionization triple quadrupole mass spectrometry.

Human skeletal muscle is the predominant tissue for the oxidation of lipids. The capacity of the muscle to oxidize fatty acids is mainly determined by the supply of oxygen to the muscle, by the cellular uptake of fatty acids, and by the mitochondrial oxidative capacity (1). Short-term fasting, such as an overnight fast, already increases lipolysis with concomitant increases in plasma nonesterified fatty acid (NEFA) and fatty acid oxidation. The quotient of CO_2 production and O_2 consumption (respiratory quotient [RQ]) reflects the mix of fat and carbohydrates being oxidized. The high reliance on fat oxidation during fasting results in a low fasting RQ that theoretically should go toward 0.7 (2). High fasting RQ is considered as a risk factor for the metabolic syndrome because it is often found in obese adults (3) and subjects with a family history of type 2 diabetes (4, 5) and is associated with an increased risk to gain weight (6, 7). The improvement of insulin sensitivity in obese, insulin-resistant individuals by weight loss and increased physical activity is associated with a reduction of fasting RQ (8).

An important regulatory step in mitochondrial β-oxidation of long-chain fatty acids is the transport of fatty acids as acylcarnitines into the mitochondrial matrix. The reverse transport of partially oxidized acyl chains leads to elevated plasma levels of medium- and long-chain acylcarnitines. Knowledge about the accumulation of certain acylcarnitine species comes mainly from the diagnosis of genetic defects in lipid oxidation disorders of newborns (9–11). In healthy adults, increased concentrations of medium- and long-chain acylcarnitines are a physiological phenomenon in the fasted state and after endurance exercise (12–15). They are considered to reflect the excess of lipolysis-derived NEFAs in these conditions leading to mitochondrial overload and release of acylcarnitines as intermediates of β-oxidation. Higher levels of acylcarnitines were also associated with obesity and type 2 diabetes, both of which are characterized by increased availability of NEFAs (16).

We hypothesized that genetic and epigenetic determinants of fat oxidation and the fasting RQ also regulate the acylcarnitine production of primary human myotubes and that assessment of de novo synthesis of acylcarnitines can be used as a tool to identify muscular determinants of whole-body fat oxidation. We determined fat oxidation in vivo as RQ after an overnight fast in 12 healthy adults and established primary human myotube cultures from the muscle biopsies of these probands. The myotubes were cultivated with ^{13}C-labeled palmitate, and quantitative profiling of intra- and extracellular acylcarnitines was performed by stable isotope dilution-based metabolomics analysis.

Subjects and Methods

Primary skeletal muscle cells from 12 individuals were studied. These donors (5 females and 7 males, aged 24.1 ± 3.0 years) were lean nondiabetic subjects (body mass index 21.8 ± 1.8 kg/m^2, insulin sensitivity Matsuda index 23.8 ± 8.2) and gave informed written consent to the study. The Ethical Committee of the Tübingen University Medical Department had approved the protocol. Insulin sensitivity was determined by a hyperinsulinemic-euglycemic clamp. Body composition and blood parameters were measured as described (17). Fasting RQ was determined by indirect calorimetry (Supplemental Data, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Primary skeletal muscle cells were grown from satellite cells obtained from percutaneous needle biopsies performed on the lateral portion of quadriceps femoris (vastus lateralis) muscle. Muscle biopsies were performed after an overnight fast between 8:00 and 10:00 AM. First and second passages of subcultured cells were used. Cells were grown in a 1:1 mixture of α-MEM and Ham's F-12 supplemented with 20% fetal bovine serum (FBS), 1% chicken extract, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.5 μg/mL amphotericin B until 70% to 80% confluency. Cells were fused for 6 days in α-MEM containing 5.5mM glucose with 2% FBS, 2mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.5 μg/mL amphotericin B. BSA-bound palmitate was prepared as described recently (18).

Acylcarnitine analyses, quantitative RT-PCR, and duplex PCR

Cells were treated in essential MEM containing 5.5mM glucose, 100μM L-carnitine, 2% FBS, 2mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin with 125μM [^{13}C16]palmitate (99 atom percent ^{13}C) for 30 minutes and 4 and 24 hours. Analyses were performed by stable isotope dilution-based metabolomics analysis by ultra high performance liquid chromatography/electrospray ionization triple quadrupole mass spectrometry (UHPLC-ESI-QQQ-MS). Details are provided in Supplemental Data.

Statistical analyses

Data are presented as means ± SEM. A 2-sided *t* test was used to compare the 2 groups. $P < .05$ was considered significant. The statistical software package JMP (SAS Institute, Cary, North Carolina) was used.

Results

Lipid oxidation in vivo

The 12 selected probands had a fasting RQ from 0.75 to 0.94 (data not shown). When divided into 2 groups according to their fasting RQ (mean 0.79 ± 0.03 vs mean 0.90 ± 0.03, Table 1), insulin sensitivity, NEFA, percentage of body fat and maximal oxygen consumption of the probands were not different, but lipid and carbohydrate oxidation, which were calculated based on the data of oxygen consumption and carbon dioxide release, were different (Figure 1, A and B).

Acylcarnitine profiling

Primary skeletal muscle satellite cells were obtained from muscle biopsies of the 12 probands. Differentiated myotubes were incubated with 100 μM L-carnitine and

Table 1. Characteristics of the Probands

	Donors With Lower Fasting RQ	Donors With Higher Fasting RQ	*P* Value
Gender (female/male)	1/5	4/2	
Age, y	24.7 ± 1.5	23.5 ± 4.1	.535
Body weight, kg	67.7 ± 6.5	63.7 ± 8.1	.367
Height, cm	175.3 ± 6.7	171.3 ± 8.5	.387
BMI, kg/m^2	22.0 ± 1.3	21.7 ± 2.3	.759
Lean body mass, kg	55.2 ± 5.2	49.3 ± 6.0	.102
Percent body fat	18.3 ± 5.1	22.2 ± 8.8	.380
Fasting glucose, mmol/L	4.7 ± 0.5	4.8 ± 0.4	.760
Fasting insulin, pmol/L	32.5 ± 6.8	37.2 ± 14.8	.499
ISI-Matsuda	23.9 ± 4.7	23.8 ± 11.2	.993
HbA1c, %	5.0 ± 0.3	5.2 ± 0.4	.273
Fasting NEFA, μmol/L	490 ± 185	367 ± 248	.355
Triglycerides, mmol/L	0.76 ± 0.27	0.95 ± 0.29	.244
Cholesterol, mmol/L	4.63 ± 0.75	4.76 ± 1.29	.836
HDL cholesterol, mmol/L	1.50 ± 0.29	1.63 ± 0.44	.561
LDL cholesterol, mmol/L	2.79 ± 0.62	2.91 ± 1.09	.810
VO_2 max, ml/kg/min	46.0 ± 9.7	37.2 ± 10.3	.201
RQ fasting	0.79 ± 0.03	0.90 ± 0.03	<.0001
Lipidox fasting, mg/kg/min	93.5 ± 14.6	37.5 ± 25.7	.0024
CHO fasting, mg/kg/min	78.3 ± 53.1	234.8 ± 64.7	.0036

Abbreviations: CHO, carbohydrate oxidation; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; ISI-Matsuda, insulin sensitivity Matsuda index; LDL, low-density lipoprotein; VO_2max, maximal oxygen consumption.

125μM [^{13}C16]palmitate for 30 minutes and 4 and 24 hours. Stable isotope dilution-based metabolomics analysis by UHPLC-ESI-QQQ-MS was applied to cell lysates and supernatants to study the production and release of acylcarnitines. After 24 hours of incubation, 42 unlabeled and 31 labeled acylcarnitine species were detected in cell lysates, whereas 31 unlabeled and 24 labeled species were detected in the supernatant (Supplemental Table 1). Detected ^{13}C-labeled species include acetylcarnitine, short-chain (C3–C5), medium-chain (C6–C12), and long-chain (>C12) acylcarnitines as well as unsaturated and hydroxylated forms, but not branched-chain acylcarnitines. The abundance of ^{13}C-labeled hydroxylated and monounsaturated acylcarnitines showed that intermediates of β-oxidation derived from the dehydrogenation and hydratation of acyl-coenzyme A (CoA), were also transferred to carnitine and released into the supernatant. Acylcarnitines containing 18 ^{13}C isotopes indicate elongation of palmitate to stearic acid (C18) or eicosanoic acid (C20). C18:1 acylcarnitine can derive as an intermediate from β-oxidation of stearic acid or from oleic acid (C18:1).

High fasting RQ is associated with significant accumulation of medium-chain acylcarnitines in the extracellular compartment

^{13}C-labeled acylcarnitines of different chain length and saturation were produced over time and in large part released into the medium leading to their accumulation (Figure 1, C–G). In myotubes obtained from probands with high fasting RQ, medium-chain acylcarnitines showed an increased accumulation in the supernatant (Figure 1D). C8, ^{13}C8, C10, ^{13}C10, and C20 acylcarnitine were significantly increased in the supernatant of these myotube cultures after 24 hours (Figure 1, H and I), other acylcarnitines depicted in Figure 1, H and I, tended toward increased levels ($P < .1$). The correlation of the fasting RQ with ^{13}C8 acylcarnitine ($r^2 = 0.66$; $P = .003$), C8 acylcarnitine ($r^2 = 0.57$; $P = .007$), and ^{13}C10 acylcarnitine ($r^2 = 0.42$; $P = .03$) in the supernatant after 24 hours was significant (data not shown). Whereas the concentration of intracellular acylcarnitines after 30 minutes or 4 or 24 hours was not different between the two RQ groups (Supplemental Figure 1), the sum of ^{13}C-labeled intra- and extracellular medium-chain acylcarnitines was significantly higher in the myotubes of the probands with the high RQ after 24 hours (2395 ± 300 vs 1254 ± 182 pmol/mg protein; $P = .02$; data not shown).

High fasting RQ is associated with low β-oxidation index

Myotubes obtained from probands with a high fasting RQ had a lower intracellular ^{13}C2 (acetylcarnitine) to carnitine ratio after 24 hours (Figure 2A), indicating a lower rate of β-oxidation (10). The ratio of intracellular ^{13}C16 and ^{13}C18 acylcarnitine to free carnitine as an indicator of carnitine palmitoyl transferase 1 activity was also lower in this group (Figure 2B) and correlated with the fasting RQ (Figure 2C).

Low *MCAD* expression in myotubes correlates with high levels of extracellular medium-chain acylcarnitines

Determination of the mitochondrial DNA content in the myotubes as a parameter of mitochondrial density and

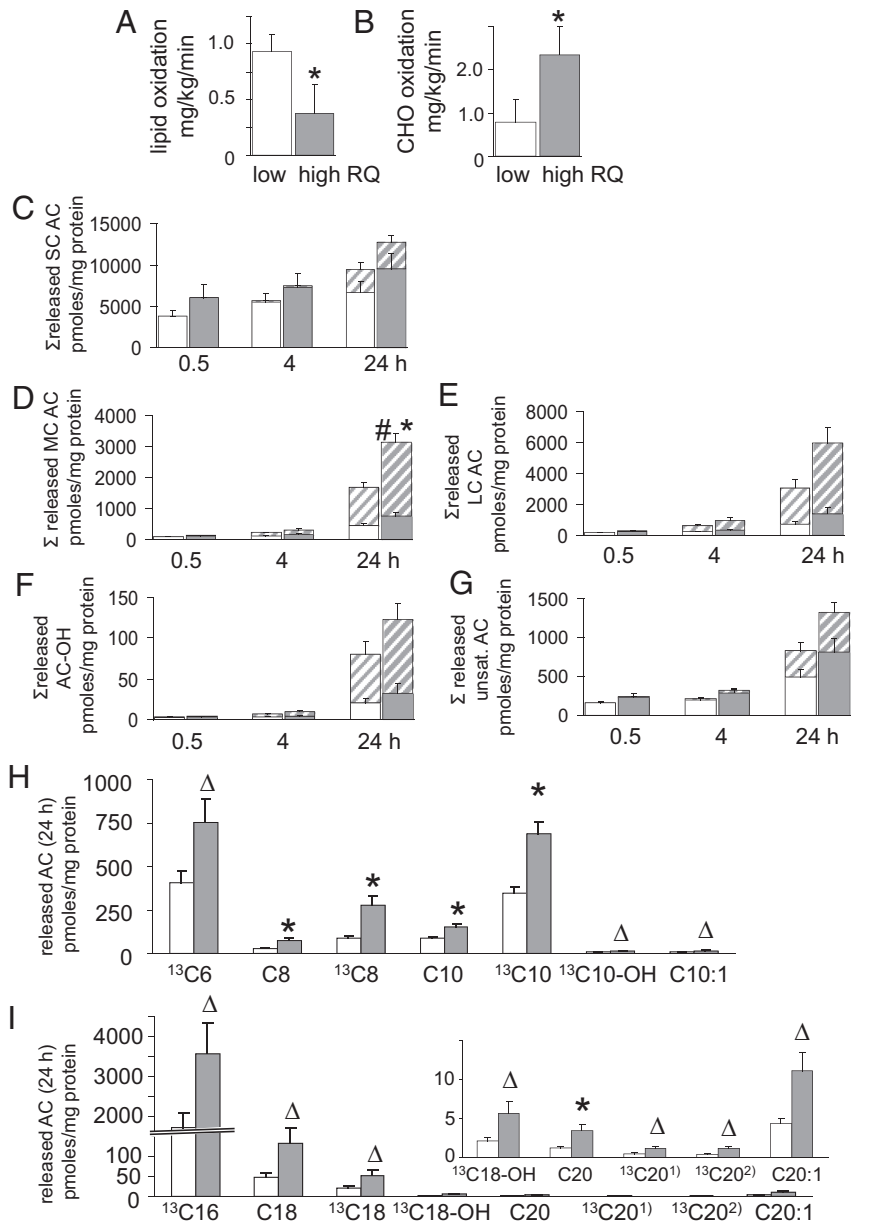

Figure 1. High fasting RQ is associated with significant accumulation of medium-chain acylcarnitines in the extracellular compartment of myotubes. A and B, Oxidation of lipids and carbohydrates (CHO) in overnight fasted donors with low fasting RQ (white columns) and high RQ (gray columns) was calculated based on data of indirect calorimetry and shown as mg/kg/min (mean ± SEM). C–G, Extracellular acylcarnitines were analyzed by UHPLC-ESI-QQQ-MS after 0.5 and 4 and 24 hours incubation with 100 μM L-carnitine and 125 μM [^{13}C16]palmitate. Values are shown as pmol/mg protein (mean ± SEM) of probands with low fasting RQ (white columns) and high RQ (gray columns); ^{13}C-labeled acylcarnitines are shown as a hatched area. Acylcarnitines were categorized according to supplemental Table 2 as short-chain (SC), medium-chain (MC), and long-chain (LC) acylcarnitines (AC). Acylcarnitines that are both hydroxylated and unsaturated (Unsat.) are included in F. * $P < .05$, sum of ^{12}C- and ^{13}C-labeled medium-chain acylcarnitines; # $P < .05$ ^{13}C-labeled medium-chain acylcarnitines of high fasting RQ vs low fasting RQ after 24 hours. H and I, Extracellular medium-chain (H) and long-chain (I) acylcarnitines after 24 hours incubation with a trend or significantly higher levels in myotubes with high fasting RQ (gray columns) vs low fasting RQ (white columns). Values are shown as pmol/mg protein (mean ± SEM). * $P < .05$; Δ $P < .1$, high fasting RQ vs low fasting RQ; 1) C20 AC containing 16 ^{13}C isotopes; 2) C20 AC containing 18 ^{13}C isotopes.

function showed no difference between the two groups (Figure 2D). Genome-wide expression profiling of β-oxidation genes in 6 of the 12 myotube cultures revealed lower expression of medium-chain acyl-CoA dehydrogenase (MCAD), the enzyme responsible for oxidizing C4 to C12 acyl-CoA, in myotubes obtained from subjects with high fasting RQ (0.75-fold down, $P < .05$, data not shown). The difference in *MCAD* mRNA expression was verified by quantitative PCR (Figure 2E). High concentrations of extracellular ^{13}C8 and ^{13}C10 acylcarnitine correlated with low *MCAD* expression ($r^2 = 0.38$; $P = .045$; and $r^2 = 0.47$; $P = .03$, respectively; data not shown), and *MCAD* expression levels showed a clear correlation trend to the fasting RQ (Figure 2F). Moreover, the myotubes obtained from subjects with high fasting RQ had a higher ratio of extracellular ^{13}C8 to ^{13}C10 and to ^{13}C12 acylcarnitine (Figure 2G). These data indicate a lower enzyme activity of MCAD as previously shown in fibroblasts from individuals with genetic MCAD deficiencies (19).

Discussion

Quantitative acylcarnitine profiling by stable isotope dilution-based metabolomics analysis demonstrate that myotubes obtained from subjects with a high fasting RQ accumulated higher levels of certain medium- and long-chain acylcarnitine species in the extracellular compartment, particularly C8 and C10 acylcarnitines. Because the intracellular acylcarnitine levels were similar between the 2 RQ groups, the data indicate that the produced acylcarnitines were released from the myotubes and prevented their intracellular accumulation. This is also evident from a 100-fold higher amount of medium-chain acylcarnitines in the extracellular compartment compared with intracellular levels.

Theoretically, high levels of acylcarnitines can indicate high fatty acid load. This was shown as increased im acylcarnitine levels and elevated plasma levels in mice fed a

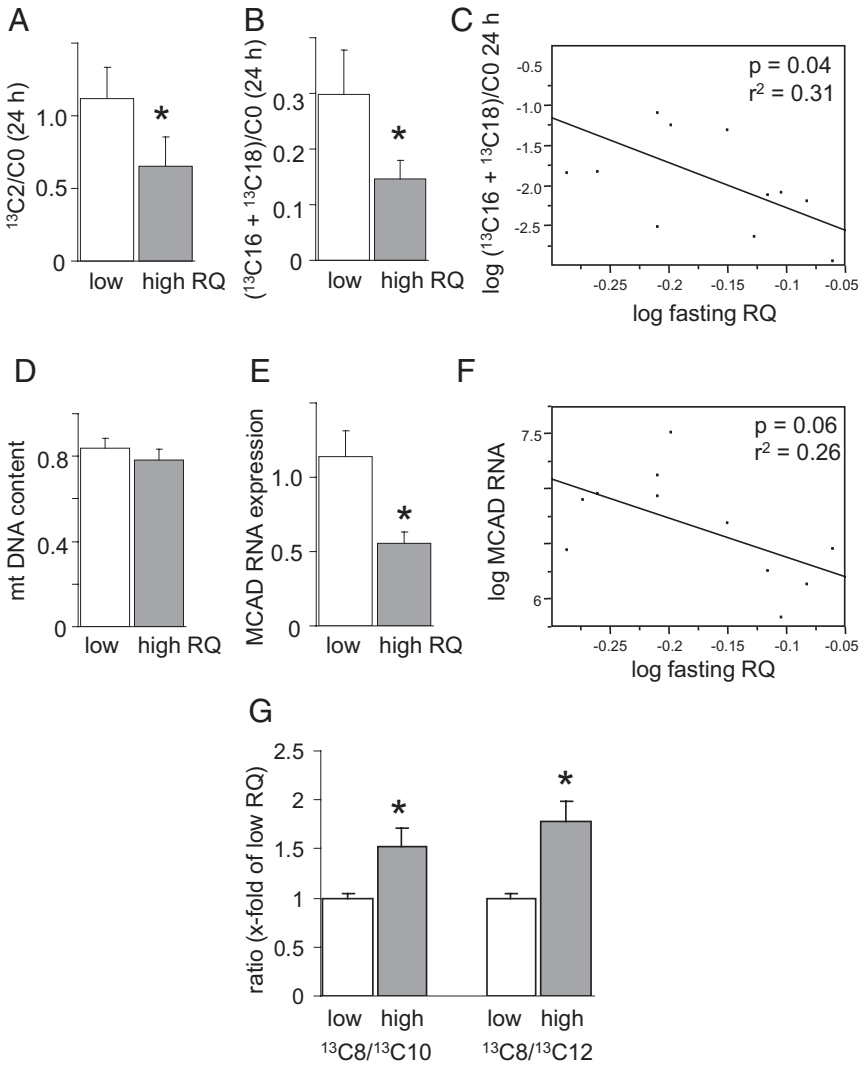

Figure 2. Low MCAD expression in myotubes correlates with high levels of extracellular medium-chain acylcarnitines. A, Ratio of intracellular ^{13}C-labeled acetylcarnitine vs carnitine measured after 24 hours of incubation with 125 μM [^{13}C16]palmitate. Values are shown as arbitrary units (mean ± SEM) of probands with low fasting RQ (white columns), or high RQ (gray columns). * $P < .05$, high fasting RQ vs low fasting RQ. B, Ratio of intracellular ^{13}C-labeled C16 (16 ^{13}C) and C18 (18 ^{13}C) acylcarnitine vs carnitine measured after 24 hours incubation with 125 μM [^{13}C16] palmitate. C, Relationship between fasting RQ and the ratio of intracellular ^{13}C-labeled C16 (16 ^{13}C) and C18 (18 ^{13}C) acylcarnitine vs carnitine after 24 hours. D, Mitochondrial (mt) DNA content in myotubes measured by duplex PCR. E, *MCAD* mRNA levels were determined in myotubes after incubation with 125 μM palmitate for 24 hours. F, Correlation of fasting RQ with *MCAD* mRNA levels after palmitate treatment for 24 hours. G, Ratio of extracellular ^{13}C-labeled C8 vs extracellular ^{13}C-labeled C10 or ^{13}C-labeled C12 acylcarnitine measured after 24 hours incubation with 125 μM [^{13}C16]palmitate. Values are shown as arbitrary units (mean ± SEM). The ratio of probands with low fasting RQ was set as 1 (white columns) and the ratio of probands with high RQ as fold change (gray columns). * $P < .05$, high fasting RQ vs low fasting RQ.

high-fat diet, suggesting the secretion of acylcarnitines from the skeletal muscle in vivo (16). In our myotube cultures, the applied fatty acid load was identical and calculation of [^{13}C]acetylcarnitine to carnitine ratio revealed a lower β-oxidation rate in the myotubes with high accumulation of medium- and long-chain acylcarnitines. Therefore, we conclude that the higher acylcarnitine levels found in the extracellular compartment indicate a lower capacity of complete β-oxidation in the myotubes due to genetic/epigenetic determinants. This may be one factor leading to a higher prevalence on carbohydrate oxidation during fasting in the respective donors with a high fasting RQ. Notably, the difference in acylcarnitine production is evident only after prolonged fatty acid oxidation (24 hours), which is comparable to the physiological situation of an overnight fast. It was not detected after 30 minutes or 4 hours, thus excluding a primary defect in the β-oxidation in these myotubes.

The detected differences in the mRNA expression levels of *MCAD* can partially account for the extracellular accumulation of medium- and long-chain acylcarnitines. The reason for the different *MCAD* expression in the myotube cultures is not yet clear. Some rare mutations in the gene encoding *MCAD* may lead to reduced expression and abundance of MCAD, which is the major determinant of its enzymatic activity. More than 80 mutations have been identified to cause reduced enzymatic activity by altering the structure of the protein or its half-life (11). In general, defective MCAD activity leads to accumulation of plasma C8 acylcarnitine. Our data suggest that low expression levels of MCAD in the muscle of apparently healthy subjects can cause reduced reliance on fat oxidation in the fasted state due to a limited capacity for the oxidation of medium-chain acyl-CoA. As consequence, a low MCAD expression would increase the risk of reduced metabolic flexibility to lipids, weight gain, and development of the metabolic syndrome.

Our data support the concept that the metabolic phenotype of the biopsy donor can be conserved in the respective primary myotube cultures as it was demonstrated for fatty acid oxidation defects in type 2 diabetic patients (20) and metabolic flexibility in healthy donors (21). Furthermore, our data show that assessment of extracellular acylcarnitine levels of primary hu-

man myotubes can be used to identify muscular determinants of whole-body fat oxidation.

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Address all correspondence and requests for reprints to: Cora Weigert, Department of Internal Medicine, Division of Pathobiochemistry and Clinical Chemistry, University of Tübingen , Otfried-Mueller-Strasse 10, 72076 Tübingen , Germany. E-mail: Cora.Weigert@med.uni-tuebingen.de; or Guowang Xu, CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China. E-mail: xugw@dicp.ac.cn.

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