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Technical Advance **Metabolism**

Animal models suggest that acetylcarnitine production is essential for maintaining metabolic flexibility and insulin sensitivity. Because current methods to detect acetylcarnitine involve biopsy of the tissue of interest, noninvasive alternatives to measure acetylcarnitine concentrations could facilitate our understanding of its physiological relevance in humans. Here, we investigated the use of long–echo time (TE) proton magnetic resonance spectroscopy (^{1}H-MRS) to measure skeletal muscle acetylcarnitine concentrations on a clinical 3T scanner. We applied long-TE ^{1}H-MRS to measure acetylcarnitine in endurance-trained athletes, lean and obese sedentary subjects, and type 2 diabetes mellitus (T2DM) patients to cover a wide spectrum in insulin sensitivity. A long-TE ^{1}H-MRS protocol was implemented for successful detection of skeletal muscle acetylcarnitine in these individuals. There were pronounced differences in insulin sensitivity, as measured by hyperinsulinemic-euglycemic clamp, and skeletal muscle mitochondrial function, as measured by phosphorus-MRS (^{31}P-MRS), across groups. Insulin sensitivity and mitochondrial function were highest in trained athletes and lowest in T2DM patients. Skeletal muscle acetylcarnitine concentration showed a reciprocal distribution, with mean acetylcarnitine concentration correlating with mean insulin sensitivity in each group. These results demonstrate that measuring acetylcarnitine concentrations with ^{1}H-MRS is feasible on clinical MR scanners and support the hypothesis that T2DM patients are characterized by a decreased formation of acetylcarnitine, possibly underlying decreased insulin sensitivity.

Long–echo time MR spectroscopy for skeletal muscle acetylcarnitine detection

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Animal models suggest that acetylcarnitine production is essential for maintaining metabolic flexibility and insulin sensitivity. Because current methods to detect acetylcarnitine involve biopsy of the tissue of interest, noninvasive alternatives to measure acetylcarnitine concentrations could facilitate our understanding of its physiological relevance in humans. Here, we investigated the use of long–echo time (TE) proton magnetic resonance spectroscopy (^{1}H-MRS) to measure skeletal muscle acetylcarnitine concentrations on a clinical 3T scanner. We applied long-TE ^{1}H-MRS to measure acetylcarnitine in endurance-trained athletes, lean and obese sedentary subjects, and type 2 diabetes mellitus (T2DM) patients to cover a wide spectrum in insulin sensitivity. A long-TE ^{1}H-MRS protocol was implemented for successful detection of skeletal muscle acetylcarnitine in these individuals. There were pronounced differences in insulin sensitivity, as measured by hyperinsulinemic-euglycemic clamp, and skeletal muscle mitochondrial function, as measured by phosphorus-MRS (^{31}P-MRS), across groups. Insulin sensitivity and mitochondrial function were highest in trained athletes and lowest in T2DM patients. Skeletal muscle acetylcarnitine concentration showed a reciprocal distribution, with mean acetylcarnitine concentration correlating with mean insulin sensitivity in each group. These results demonstrate that measuring acetylcarnitine concentrations with ^{1}H-MRS is feasible on clinical MR scanners and support the hypothesis that T2DM patients are characterized by a decreased formation of acetylcarnitine, possibly underlying decreased insulin sensitivity.

Introduction

The use of proton magnetic resonance spectroscopy (^{1}H-MRS) in human skeletal muscle has been instrumental in establishing the importance of ectopic fat accumulation in the development of type 2 diabetes mellitus (T2DM). It was found in a large number of studies that intramyocellular lipid (IMCL) levels are inversely related to insulin sensitivity (1, 2). However, the same methodology also resulted in the identification of the so-called athletes' paradox: endurance-trained athletes are, despite being very insulin sensitive, also characterized by high IMCL levels (3). These findings have led to the development of new hypotheses to explain the relation between fat accumulation in muscle and insulin sensitivity.

One of the interesting and novel hypotheses suggests a role for carnitine metabolism (4, 5). It has long been known that carnitine permits mitochondrial import of long-chain fatty acids for subsequent β-oxidation (6). However, it has recently been suggested that carnitine may also play a crucial regulatory role in substrate switching and glucose homeostasis (4, 5, 7). Acetylcarnitine is formed in conditions in which acetyl-CoA formation, either as end product of glycolysis or β-oxidation, exceeds its entry into the tricarboxylic (TCA) cycle. Free carnitine can act as a sink for excess acetyl groups in a reversible reaction catalyzed by the enzyme carnitine acetyltransferase (CRAT) (7). Acetylcarnitine, like other acylcarnitine esters, can readily be exported out of the mitochondria (Figure 1).

The formation of acetylcarnitine helps to keep the mitochondrial acetyl-CoA/free CoA ratio low. A low acetyl-CoA/free CoA ratio is needed to maintain pyruvate dehydrogenation (PDH) activity (8), which is known to control the rate of aerobic carbohydrate oxidation. A compromised capacity to generate acetylcarnitine, either due to reduced CRAT activity or low carnitine concentrations, may reduce PDH activity, hence reducing oxidative degradation of glucose, a major concern in insulin-resistant muscle. Compromised mitochondrial entrance of pyruvate is reflected in decreased metabolic flexibility, an early detectable feature of insulin-resistant muscle. Indeed, *Crat*-knockout mice, which are unable to convert acetyl-CoA to acetylcarnitine, are characterized by decreased glucose tolerance. On the other hand, gain-of-function experiments of CRAT in primary human myotubes showed increased acetylcarnitine efflux and higher PDH activity

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Table 1. Subject characteristics

Group	Age (yr)	BMI (kg/m^2)	VO_2 max (ml/min/kg)	Fat percentage (%)
Endurance-trained athletes	25 ± 4	21.2 ± 1.6	59.6 ± 3.8	13.0 ± 2.1
Lean sedentary subjects	22 ± 4	21.9 ± 2.1	41.0 ± 1.2[A]	17.8 ± 4.7
Obese sedentary subjects	59 ± 7[B]	31.2 ± 1.6[B]	27.7 ± 4.5[B]	34.6 ± 6.8[B]
T2DM patients	64 ± 7[B]	30.5 ± 1.4[B]	24.8 ± 4.7[B]	27.9 ± 6.1[B]

[A]Significant difference between endurance-trained athletes and lean sedentary subjects ($P < 0.05$). [B]Significant difference between endurance-trained athletes/lean sedentary subjects versus obese sedentary subjects/T2DM patients ($P < 0.05$).

(5). Similarly, a reduced availability of free carnitine caused by high-fat overfeeding hampered acetylcarnitine formation in rats, and carnitine supplementation was able to reverse diet-induced mitochondrial dysregulation, including restoration of PDH activity. Furthermore, acetylcarnitine formation was increased with increased levels of free carnitine in skeletal muscle of rats and in primary human skeletal myotubes (4). To test the relevance of acetylcarnitine in insulin resistance and T2DM in humans, it is key to measure acetylcarnitine levels in tissue.

Acetylcarnitine has previously been visualized noninvasively using short–echo time (TE) ^{1}H-MRS by subtracting pre- and postexercise spectra. Because the peak was not visible in the rest spectrum, only an exercise-induced difference signal could be quantified at 2.13 ppm (9). More recently, the observation of an alternative resonance peak of acetylcarnitine at 3.17 ppm has been reported at 7T (10). However, due to the overlap with other trimethyl ammonium (TMA) peaks, this peak also remained only quantifiable after exercise, when it was increased considerably. Furthermore, due to the decreased spectral resolution, this peak was not resolved and therefore remained undetected on clinically available 3T MR systems. We hypothesized though that acquisition strategies using a long TE would yield valuable information about the concentration of acetylcarnitine in vivo. The acetylcarnitine peak at 2.13 ppm is normally covered by broad lipid resonances at short TE. Acquisition strategies using a long TE led to the relative suppression of these broad lipid resonances or short transversal relaxation time (T_2) metabolite signals in general (11, 12). The use of a long TE would enhance the visibility of the acetylcarnitine peak irrespective of the magnetic field strength without being limited to an exercise-induced increase.

We here developed a long-TE acquisition strategy to measure acetylcarnitine levels at rest in a single scan on a clinical 3T MR scanner. Detecting acetylcarnitine levels noninvasively gives the opportunity to study acetylcarnitine levels in relation to pathological conditions such as mitochondrial dysfunction, insulin resistance, and diabetes. To evaluate the physiological relevance of the measurement of skeletal muscle acetylcarnitine levels, we applied the long-TE ^{1}H-MR methodology in skeletal muscle of endurance-trained athletes, lean sedentary subjects, obese sedentary subjects, and T2DM patients. Our results indicate that higher acetylcarnitine concentration is associated with increased insulin sensitivity and better in vivo mitochondrial function, as measured with phosphorus-MRS (^{31}P-MRS), and that no athletes' paradox is present for acetylcarnitine levels.

Results

Subject characteristics. Endurance-trained athletes and lean sedentary subjects as well as obese sedentary subjects and T2DM patients were matched for age and BMI. Age and BMI were different between endurance-trained athletes/lean sedentary subjects and obese sedentary subjects/T2DM patients ($P < 0.01$). Fat percentage also showed a similar distribution, with no significant differences between endurance-trained athletes versus lean sedentary subjects and obese sedentary subjects versus T2DM patients, but with a significantly higher fat percentage in the obese sedentary subject and T2DM patient groups when compared with the lean sedentary subject and endurance-trained athlete groups ($P < 0.01$). Maximal oxygen uptake (VO_2 max) was highest in endurance-trained athletes, and this was significantly different from that of the lean sedentary subject, obese sedentary subject, and T2DM patient groups ($P < 0.01$ for all comparisons). VO_2 max was also significantly higher in the lean sedentary subject group when compared with either the obese sedentary subject or the T2DM patient group ($P < 0.01$), while there was no significant difference between the latter two ($P = 0.87$). All subject characteristics are summarized in Table 1.

Long-TE spectrum. Enhanced visibility of the acetylcarnitine peak in a long-TE spectrum of the vastus lateralis muscle, as compared with a conventional short-TE spectrum, is apparent from Figure 2. Total scan time per spectrum was 2 minutes.

With long TE, the acetylcarnitine peak at 2.13 ppm could be assigned by simple visual inspection. In contrast, with short TE, the peak was hardly visible due to broad lipid resonances in the region 2.0 to 2.5 ppm obscuring the peak. Also very characteristic for the long-TE spectrum was the sharp, single, and symmetric appearance of the total creatine (t-Cr) peak at 3.03 ppm. This peak was relatively broad and asymmetric in the short-TE spectrum. The residual water signal in the long-TE spectrum was relatively low without the application of sophisticated water suppression techniques.

The T_2 of acetylcarnitine was found to be 265 ± 45 ms (270 ± 29 ms for lean subjects versus 262 ± 37 ms for obese subjects, $P = 0.88$), which is approximately 10 times longer than the T_2 of water at 3T (see also Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI74830DS1). The T_2 of t-Cr was found to be 162 ± 4 ms (157 ± 3 ms for lean subjects versus 166 ± 6 ms for obese subjects, $P = 0.24$), which is in correspondence with the long decay component as reported in a previous study (13).

Results from the phantom experiments are depicted in Figure 3. The MR-based quantification, with the long-TE protocol, correlates perfectly with the known acetylcarnitine concentration in the phantom experiments (Pearson's $r = 0.99$, $R^2 = 0.99$, $P < 0.01$).

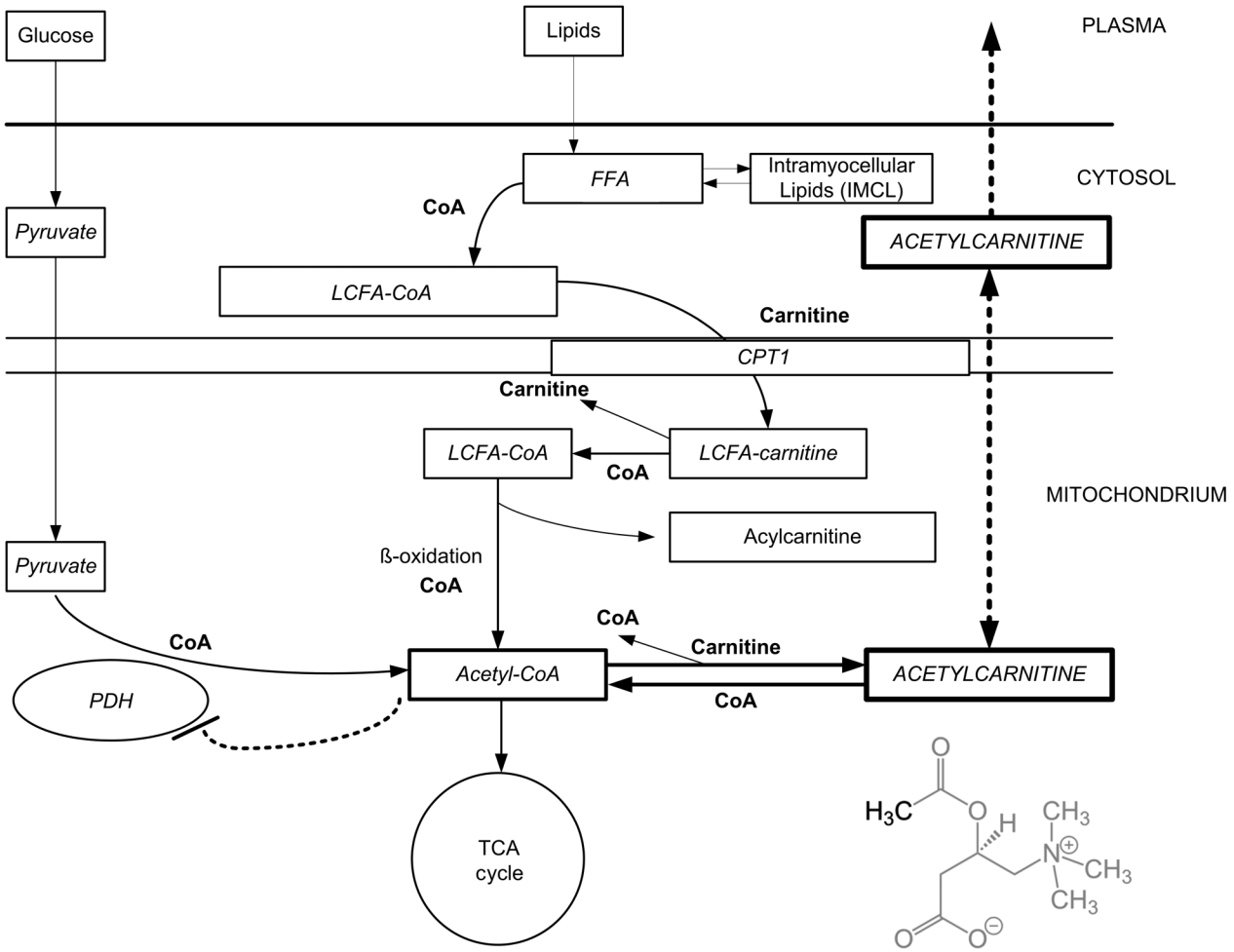

Figure 1. Formation of acetylcarnitine. When acetyl-CoA formation exceeds use by the TCA cycle, carnitine can function as a sink for excess acetyl groups inside the mitochondria, thereby forming acetylcarnitine. This reversible reaction is catalyzed by the enzyme CRAT and leads to the release of free CoA. The formation of acetylcarnitine can help to keep the mitochondrial acetyl-CoA/free CoA ratio low, which is essential to sustaining TCA cycle flux and PDH activity. Acetylcarnitine can be transformed back to acetyl-CoA or can be exported outside the mitochondria. The protons contributing to the resonance of acetylcarnitine at 2.13 ppm in ^{1}H-MRS are highlighted in the molecule structure. CPT1, carnitine palmitoyltransferase 1; FFA, free fatty acids; LCFA, long-chain fatty acids.

Internal reference. As the unsuppressed water signal is used extensively as an internal reference in ^{1}H-MRS, the use of t-Cr as an internal reference was compared with water as an internal reference. The intensity of the water peak was determined from an additionally acquired short-TE spectrum. The ratio of acetylcarnitine over water and the ratio of acetylcarnitine over t-Cr were calculated and plotted in Figure 4, and a very strong correlation was found between the 2 ratios (Pearson's $r = 0.96$, $R^2 = 0.91$, $P < 0.01$, $n = 21$).

Insulin sensitivity. Insulin sensitivity was significantly different among groups (glucose infusion rate [GIR]: 76.8 ± 5.1 μmol/min/kg in endurance-trained athletes, 61.9 ± 5.4 μmol/min/kg in lean sedentary subjects, 30.3 ± 4.6 μmol/min/kg in obese sedentary subjects, and 23.6 ± 2.7 μmol/min/kg in T2DM patients; $P < 0.01$, Figure 5A). Post-hoc analysis revealed that GIRs of endurance-trained and lean sedentary subjects were significantly higher when compared with the values obtained in obese sedentary subjects and T2DM patients ($P < 0.01$).

In vivo mitochondrial function. We determined in vivo mitochondrial function by determining the phosphocreatine (PCr) recovery kinetics after exercise. The PCr recovery rate constant was significantly different among groups, with fastest recovery in endurance-trained athletes and slowest recovery in T2DM patients (endurance-trained athletes: 20.7 ± 3.4 s; lean sedentary subjects: 27.9 ± 0.6 s; obese sedentary subjects: 29.9 ± 1.7 s; T2DM patients: 36.5 ± 2.4 s; $P < 0.01$, Figure 5B). Mitochondrial function was significantly lower in T2DM patients compared with endurance-trained, lean sedentary, and obese sedentary subjects ($P < 0.01$, $P < 0.01$, and $P = 0.04$ respectively), as indicated by the post-hoc analysis.

Acetylcarnitine concentrations (^{1}H-MRS). Two representative long-TE spectra, from an endurance-trained athlete and a T2DM patient, respectively, are depicted in Figure 6. Spectra are scaled to the t-Cr peak to ensure that a difference in the acetylcarnitine peak intensity is apparent by visual inspection.

Overall, acetylcarnitine concentration was significantly different between groups, with highest values in endurance-trained

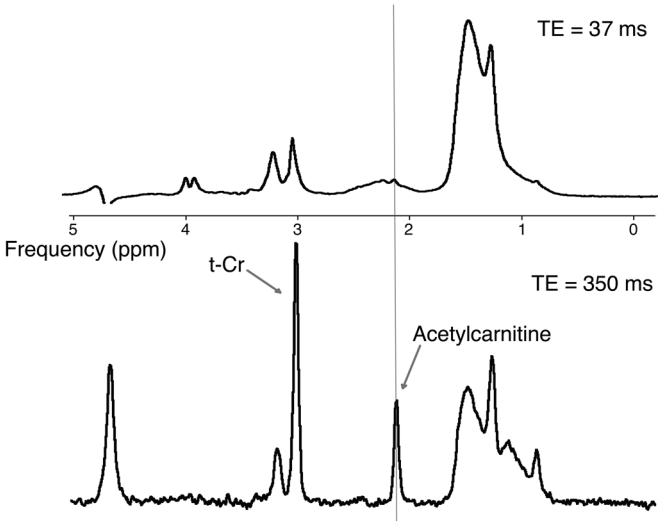

Figure 2. Comparison of short (TE = 37 ms) and long-TE (TE = 350 ms) spectra from the vastus lateralis muscle. Because of the relatively long T_2 of acetylcarnitine (at 2.13 ppm) when compared with overlapping broad lipid resonances, the visibility of the peak is greatly enhanced in the spectrum with the long TE. Because of the relatively short T_2 of water, no water suppression was applied for the long-TE spectrum. Furthermore, because of the long TE, the t-Cr peak (at 3.03 ppm) shows up as a single and sharp peak.

athletes and lowest values in T2DM patients (endurance-trained athletes: 1.58 ± 0.30 mmol/kg wet weight (kgww); lean sedentary subjects: 1.28 ± 0.22 mmol/kgww; obese sedentary subjects: 0.70 ± 0.22 mmol/kgww; T2DM patients: 0.42 ± 0.19 mmol/kgww; $P < 0.01$, Figure 7A). Post-hoc analysis revealed that these differences in acetylcarnitine concentrations reached statistical significance between T2DM patients and endurance-trained athletes ($P = 0.01$).

Significant correlations of the individual acetylcarnitine concentrations with BMI (Pearson's $r = -0.56$, $R^2 = 0.31$, $P < 0.01$), age (Pearson's $r = -0.55$, $R^2 = 0.30$, $P < 0.01$), VO_2 max (Pearson's $r = 0.57$, $R^2 = 0.32$, $P < 0.01$), and fat percentage (Pearson's $r = -0.45$, $R^2 = 0.20$, $P < 0.01$) were found. Acetylcarnitine and PCr recovery rate correlated near significance (Pearson's $r = -0.30$, $R^2 = 0.09$, $P = 0.08$).

Carnitine metabolism in human muscle material. To further examine underlying molecular mechanisms for the group differences in acetylcarnitine levels, we took muscle biopsies after an overnight fast from 11 endurance-trained athletes, 6 lean subjects, 5 obese subjects, and 9 T2DM patients. We first determined liquid chromatography–mass spectrometry–based (LC-MS–based) levels of acetylcarnitine (endurance-trained athletes: 593.4 ± 106.3 pmol/kg tissue; lean sedentary subjects: 585.1 ± 111.4 3 pmol/kg tissue; obese sedentary subjects: 336.0 ± 76.7 pmol/ kg tissue; T2DM patients: 341.0 ± 73.2 pmol/kg tissue; $P = 0.129$) and total carnitine (endurance-trained athletes: 3758.2 ± 487.8 pmol/kg tissue; lean sedentary subjects: 3311.0 ± 727.8 pmol/kg tissue; obese sedentary subjects: 2584.0 ± 496.0 pmol/kg tissue; T2DM patients: 2587.0 ± 555.3 pmol/kg tissue; $P = 0.355$), which were not significantly different among groups, although in both cases, a pattern of increased concentrations in endurance-trained athletes was found. Acetylcarnitine as measured with ^{1}H-MRS did not significantly correlate with LC-MS–based acetylcarnitine concentrations (Pearson's $r = 0.28$, $R^2 = 0.08$, and $P = 0.131$) nor with the total carnitine concentrations as measured in the biopsies (Pearson's $r = 0.101$, $R^2 = 0.01$, and $P = 0.587$).

However, since it is known that acetylcarnitine levels can fluctuate over the day, we also determined the activity of CRAT, which is the most important enzyme responsible for acetylcarnitine formation. Interestingly, CRAT activity was significantly higher in the endurance-trained athletes when compared with the other groups (endurance-trained athletes: 91.9 ± 7.5 nmol/mg protein/min; lean sedentary subjects: 65.4 ± 3.8 nmol/mg protein/min; obese sedentary subjects: 53.4 ± 3.5 nmol/mg protein/min; T2DM patients: 52.6 ± 4.7 nmol/mg protein/min; $P < 0.01$). Moreover, MR-derived acetylcarnitine concentration correlated with CRAT activity (Pearson's $r = 0.37$, $R^2 = 0.134$, and $P = 0.04$).

Association between acetylcarnitine and insulin sensitivity. A significant correlation between MR-based acetylcarnitine concentration and insulin sensitivity was found (Pearson's $r = 0.59$, $R^2 = 0.35$, $P < 0.01$) based on the individual data. When we performed a per-group correlation analysis (Figure 7B), the correlation between the acetylcarnitine concentration group means and the insulin sensitivity group means was very strong and highly significant (Pearson's $r = 0.99$, $R^2 = 0.99$, $P < 0.01$). Furthermore, CRAT activity was positively correlated with insulin sensitivity (Pearson's $r = 0.74$, $R^2 = 0.55$, $P < 0.01$ for CRAT).

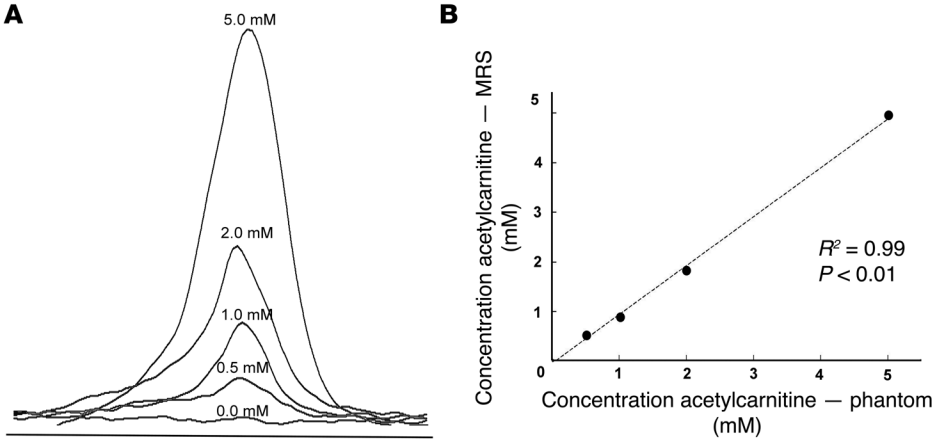

Figure 3. Phantom experiments. Five phantoms were prepared with 0, 0.5, 1.0, 2.0, and 5.0 mM acetylcarnitine. The acetylcarnitine peak at 2.13 ppm for the different concentrations is shown in **A**. The MR-based concentrations of acetylcarnitine are plotted versus the known acetylcarnitine concentrations in **B**. The measured concentrations are in concordance with the known phantom concentrations, as indicated by the very strong correlation (Pearson's $r = 0.99$, $R^2 = 0.99$, $P < 0.01$).

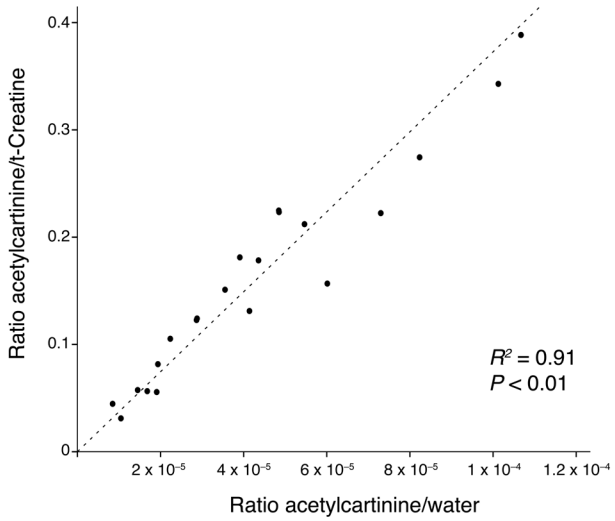

Figure 4. Comparison between the use of t-Cr and water as internal reference. Data are expressed as the T_2 uncorrected ratios of acetylcarnitine over the reference metabolite. The fitted line is forced to go through the origin (dotted line). The correlation between both ratios is strong and highly significant.

A stepwise linear regression analysis with BMI, age, fat percentage, and VO_2 max and insulin sensitivity as possible determinants of acetylcarnitine was performed on the individual data and revealed that all variables, other than insulin sensitivity, were excluded from the model, further illustrating the association between acetylcarnitine levels and insulin sensitivity (included variable: insulin sensitivity, $P < 0.01$; excluded variables: BMI, $P = 0.416$; age, $P = 0.157$; fat percentage, $P = 0.606$; PCr recovery rate, $P = 0.803$; VO_2 max, $P = 0.756$).

Discussion

The potential of in vivo ^{1}H-MRS in skeletal muscle has not been fully exploited. Due to the small chemical shift range in which the metabolites resonate, many of the metabolite resonances are overlapping, thereby obscuring their presence. Here, we show that the use of a long TE can help to enhance the visibility of metabolites with a relative long T_2, such as acetylcarnitine. In conventional short-TE spectroscopy, the acetylcarnitine peak at 2.13 ppm is covered by broad lipid resonances, but it is apparent from our spectra that acetylcarnitine appears as a single, sharp, and symmetrical peak when a long TE is used at 3T. The long-TE approach that we propose here creates the opportunity to noninvasively measure muscle acetylcarnitine levels on clinically available MR systems, which can boost the research into our understanding of the (patho)physiological relevance of this metabolite. To investigate a potential role in insulin sensitivity, we determined acetylcarnitine concentrations in 4 groups, spanning a wide range of values of insulin sensitivity. We carefully phenotyped these groups in terms of insulin sensitivity and in vivo mitochondrial function and indeed found an increase in insulin sensitivity and in vivo mitochondrial function across the groups from T2DM patients to trained subjects. With our long-TE approach, we were indeed able to robustly measure differences in skeletal muscle acetylcarnitine concentrations among the 4 groups. Insulin sensitivity was strongly associated with the measured skeletal muscle acetylcarnitine concentration, with higher acetylcarnitine concentrations in subjects with higher insulin sensitivity.

Besides the enhanced visibility of the acetylcarnitine peak, the long-TE approach offers additional advantages compared with the conventional short-TE methodology. The methyl t-Cr peak is visible as a single sharp peak in long-TE spectra, making it an excellent internal reference compound. In short-TE spectroscopy, this t-Cr peak at 3.03 ppm is contaminated by extramyocellular lipid (EMCL) signals around 2.95 ppm and is influenced by residual dipolar coupling (14). A longer TE results in a destructive interference of phase differences introduced by residual dipolar coupling in the t-Cr peak (13) and the suppression of the EMCL signal. The use of a long TE also diminishes the need for water suppression. In short-TE measurements, the water signal is approximately 10^3 to 10^5 times as abundant as other metabolite signals and can obscure much smaller resonances, which are present in the millimolar range. Normally, sophisticated water suppression methods are used that unavoidably distort signals near the water resonance. Water intrinsically has a short T_2, leading to a very low signal with longer TE. It should also be mentioned that the use of a long TE leads to spectral simplification and a flat baseline, which make fitting procedures straightforward.

Short TE is normally preferred in ^{1}H-MRS, as it helps minimize the effect of T_2 relaxation, thereby yielding a higher signal-to-noise ratio (SNR). In our protocol, we used a relatively large voxel size to compensate for the loss in SNR with long TE. Using a larger voxel did not lead to increased overlap of the acetylcarnitine peak

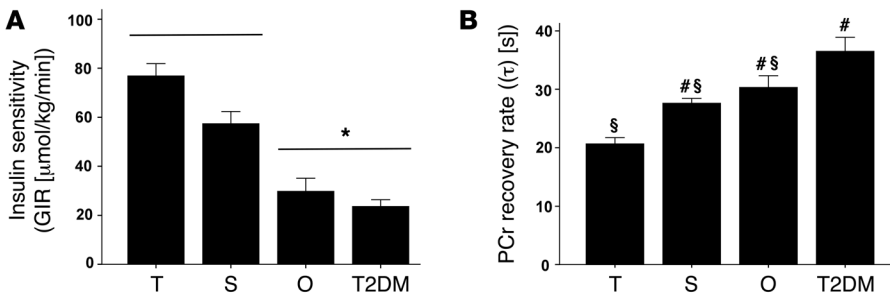

Figure 5. Peripheral insulin sensitivity and PCr recovery rates per group. (**A**) Insulin sensitivity is given as GIR (μmol/kg/min). There are significant differences between endurance-trained athletes/lean sedentary subjects and the obese sedentary subjects/T2DM patients. (**B**) Mitochondrial function is depicted as PCr recovery rate τ (s). The rate was significantly higher in T2DM patients when compared with endurance-trained athletes, lean sedentary subjects, and obese sedentary subjects. T, endurance-trained athletes; S, lean sedentary subjects; O, obese sedentary subjects; T2DM, T2DM patients. *$P < 0.05$, endurance-trained athletes/lean sedentary subjects versus obese sedentary subjects/T2DM patients; #$P < 0.05$, compared with endurance-trained athletes; §$P < 0.05$, compared with T2DM patients. Data are expressed as mean ± SEM.

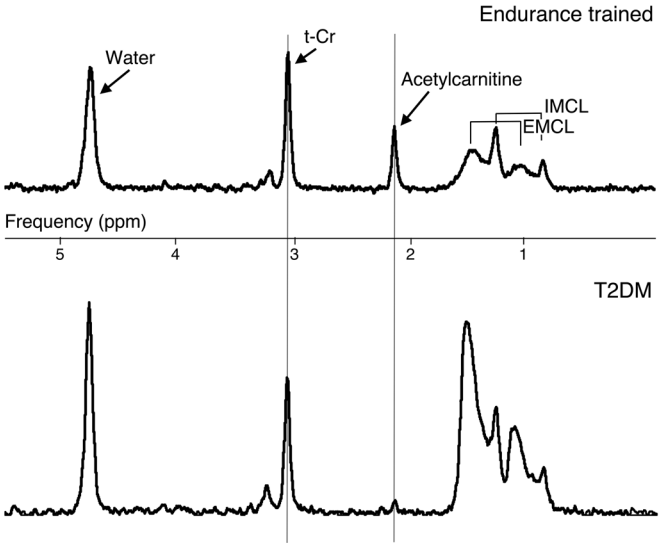

Figure 6. Typical long-TE spectra collected from an endurance-trained athlete and a T2DM patient. Spectra are scaled to the t-Cr peak. Most apparent is the difference between the acetylcarnitine peak intensities in both spectra. EMCL signals are higher in the T2DM patients, as was expected.

with other resonances and inherently led to better tissue averaging. In our experience, this is a robust protocol to obtain spectra with reasonable SNR within a relatively short time.

The choice of the methyl t-Cr peak as the internal reference metabolite in this study, instead of the unsuppressed water signal, was based on 2 considerations. In short-TE spectra, analysis and fitting of the dipolar coupled t-Cr resonance is difficult due to the appearance of satellite peaks due to the residual dipolar coupling effect (13). The phase modulations due to the residual dipolar coupling effect are averaged out when using a long TE, resulting in disappearance of the satellite peaks of the t-Cr triplet. The sharp and symmetric appearance of the t-Cr peak at long TE increases reliable fitting of this metabolite. This enables measurement of both the t-Cr and the acetylcarnitine peaks in a single acquisition. Furthermore, it has been reported that the concentration of the t-Cr pool is rather stable, approximately 30 mmol/kgww in muscular tissue, and shows little interindividual and regional variations, which makes it an excellent reference metabolite (15).

Water has extensively been used as an internal reference in MR spectroscopy of skeletal muscle. In this study, we therefore compared the use of the t-Cr peak to the use of water as internal reference. The water signal was measured in a separately recorded short-TE spectrum. We found a very strong correlation between the ratio of acetylcarnitine/t-Cr and acetylcarnitine/water. This indicates that the t-Cr peak is a reliable internal reference and that differences found between groups cannot be explained by the use of a different internal reference. This makes it possible to measure the acetylcarnitine concentration in a single scan. To calculate the absolute concentrations in skeletal muscle, we used fixed T_2 of both acetylcarnitine and t-Cr in all subjects, as T_2 relaxation measurements were not different between lean and obese subjects. Furthermore, it is suggested that a 2-component modulation pattern exists for the t-Cr peak due to the dipolar coupling, giving rise to short and long decay components (short decay in the range of 0 to 35 ms) (13). When correcting the t-Cr peak intensity based on a single exponential T_2 decay function, this could lead to a systematic overestimation of the acetylcarnitine concentration measured. To avoid this, we used a fixed correction factor for the short decay component, and we report acetylcarnitine concentrations of 1.3 to 1.6 mmol/kgww in lean (sedentary and trained) subjects, which is in accordance with present and earlier results of biopsy studies, reporting comparable concentrations of acetylcarnitine (16–18) when converted to mmol/kgww (ranging from ~0.5 to 1.6 mmol/kgww).

The phantom measurements that we performed in this study confirmed that the long-TE MR measurement can accurately determine acetylcarnitine concentrations, with the use of t-Cr as a reference metabolite. However, when comparing the acetylcarnitine concentrations as derived from MRS and LC-MS, we did not find a significant correlation, although a similar pattern across the groups was visible. However, it is important to note that the muscle biopsies were taken early in the morning, while our MR experiments were performed in the late afternoon to be more closely synchronized with the hyperinsulinemic-euglycemic clamp. This time difference may explain the lack of correlation, since acetylcarnitine concentrations are known to vary significantly during the day (19).

As mentioned above, using our long-TE spectroscopy approach, we found that acetylcarnitine levels are highest in endurance-trained athletes and lowest in insulin-resistant T2DM patients.

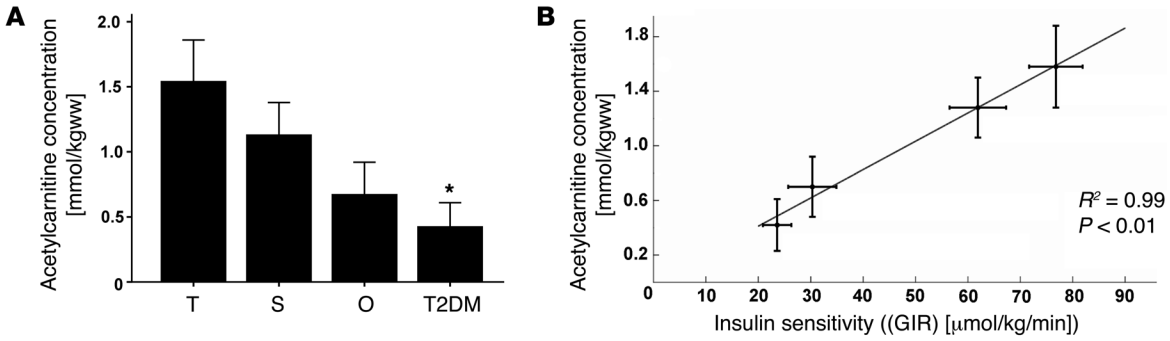

Figure 7. Skeletal muscle acetylcarnitine concentrations per group and the association between mean acetylcarnitine levels and mean insulin sensitivity. (**A**) The acetylcarnitine concentration is given in mmol/kgww. Post-hoc analysis showed a significant lower acetylcarnitine concentration in T2DM patients when compared with endurance-trained athletes. (**B**) Correlation between group means of acetylcarnitine concentration (given in mmol/kgww) and GIR (μmol/kg/min). *$P < 0.05$, compared with endurance-trained athletes. All results are expressed as mean ± SEM.

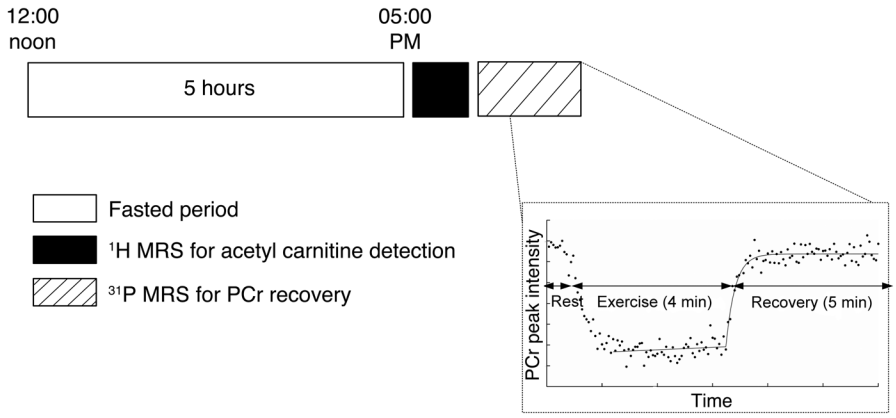

Figure 8. Outline of MR measurements performed. Subjects were fasted for 5 hours before measuring the acetylcarnitine concentration at rest, using our long-TE ^{1}H-MRS protocol. After the acetylcarnitine measurement, an ergometer was placed in the scanner to perform exercise during ^{31}P-MRS. The time course of the ^{31}P-MRS measurement is given in more detail. Subjects performed exercise for 4 minutes in the scanner, after which the PCr peak recovery was monitored for 5 minutes.

Furthermore, we showed that insulin sensitivity strongly associates with skeletal muscle acetylcarnitine concentrations. In the first instance, however, our finding of low acetylcarnitine concentrations in T2DM patients may seem to be in contrast with findings of a previous publication in which a high acetylcarnitine plasma concentration was associated with T2DM patients (20). High plasma concentrations of acetylcarnitine may be caused by an increased efflux of acetylcarnitine from muscle tissue; however, acetylcarnitine is also formed by other tissues, such as liver or heart (21, 22). Moreover, high concentrations of acetylcarnitine in plasma could potentially lead to a loss of carnitine from the body when the (acetyl)carnitine is secreted via urine. Without a concomitant increase in the biosynthesis or dietary intake of carnitine, this may lead to a lower carnitine status in skeletal muscle or in the total body. The fact that plasma acetylcarnitine concentrations are not in agreement with skeletal muscle concentrations illustrates that the investigation of plasma concentrations is not sufficient and emphasizes the need for tools to quantify this metabolite directly in tissue.

The transformation of excessive acetyl-CoA into acetylcarnitine has recently been suggested to be important to maintain metabolic flexibility and insulin sensitivity under conditions of excessive lipid supply to the mitochondria (4, 5). It is well known that in T2DM patients, excessive accumulation of fat in skeletal muscle is associated with the development of insulin resistance (1, 2). Low acetylcarnitine levels are consistent with the hypothesis that excessive fat availability in muscle of T2DM patients may lead to the development of insulin resistance and metabolic inflexibility due to a decreased conversion of excessive acetyl-CoA into acetylcarnitine, in turn leading to an inhibition of the PDH complex. It has indeed been reported that patients with T2DM are characterized by lower PDH activity (23). Conversely, dietary L-carnitine supplementation administered to prediabetic subjects increased muscle PDH activity (5). Interestingly, endurance-trained athletes also have high amounts of fat in skeletal muscle, yet they are very insulin sensitive, a phenomenon called the athletes' paradox (3). Our finding of higher acetylcarnitine concentrations in endurance-trained athletes fits with the concept that the conversion of acetyl-CoA into acetylcarnitine may be important in maintaining insulin sensitivity when muscle fat content is high, further underscored by the significant correlation between acetylcarnitine and insulin sensitivity.

Acetylcarnitine is formed by CRAT, a freely reversible enzyme that resides principally in the mitochondrial matrix and that is undetectable in the cytoplasm. Unlike acetyl-CoA, acetylcarnitine is membrane permeant and readily exported from the mitochondrial lumen to other cellular and extracellular compartments. Thus, episodes of increased acetylcarnitine production (e.g., during a meal) could lead to increased efflux and extramitochondrial accumulation of the metabolite (4, 5). We found that muscle CRAT activity correlated positively with MRS-derived acetylcarnitine concentrations and that CRAT activity was similarly disparate among the 4 groups, with the highest activity measured in exercise-trained subjects and the lowest activity found in those with T2DM. Together, these data suggest that the formation and extramitochondrial storage of acetylcarnitine is influenced by CRAT activity and is different between endurance-trained athletes and T2DM patients, which may in turn be related to insulin sensitivity.

In summary, to date, the determinants of acetylcarnitine concentrations in skeletal muscle have not been investigated in detail and it is yet unclear whether acetylcarnitine levels are reflective of a mitochondrial imbalance and/or are indicative of the development of metabolic disease and decreased mitochondrial flexibility (24). With the successful application of a long-TE strategy, we showed that there is an association among the acetylcarnitine concentration, CRAT activity, and insulin sensitivity. The ^{1}H-MRS protocol can easily be applied on any 3T MR scanner, and the long-TE strategy provides a unique tool to further unravel the role of acetylcarnitine levels in relation to metabolic health and insulin resistance.

Methods

Subjects

Resting acetylcarnitine levels were measured in 38 subjects, which were each categorized in 1 of 4 different groups. These groups purposely covered a wide range in whole-body and skeletal muscle insulin sensitivity: endurance-trained athletes (12 subjects), lean sedentary subjects (9 subjects), obese normoglycemic sedentary subjects (8 subjects) and T2DM patients (9 subjects). None of the subjects had a history of cardiovascular disease or had lost more than 3 kg body mass during the 6 months preceding the study.

Endurance-trained and lean sedentary subjects all had BMIs of less than 25 kg/m^2. Subjects were considered endurance trained when their VO_2 max exceeded 55 ml min^{-1}kg^{-1}, had been active in competi-

tive endurance-exercise activities at least 3 times a week during the last 2 years, and had a stable level of training for the last 6 months. For the lean sedentary group, subjects were included when their VO_2 max was below 45 ml $min^{-1}kg^{-1}$ and when they exhibited a sedentary lifestyle in which they did not participate in physical activity for more than 1 hour per week for at least the past 2 years.

Endurance-trained athletes and lean sedentary subjects were age and BMI matched (Table 1). Patients with T2DM were diagnosed at least 1 year prior to beginning the study, were non–insulin dependent, had well-controlled diabetes for at least 6 months (HbA1c < 7.8%), and had no diabetes-related comorbidities. All T2DM patients were treated for diabetes with metformin. Obese control subjects had no family history of diabetes and did not take any medication.

In an additional study, 7 lean subjects (2 male/5 female, age 28 ± 6 years, BMI 20.3 ± 0.7 kg/m^2, VO_2 max 45.4 ± 8.2 ml/min/kg) and 7 obese subjects (7 male subjects, age 57 ± 7 years, BMI 31.5 ± 1.0 kg/m^2, VO_2 max 27.7 ± 1.6 ml/min/kg) were included to determine in vivo T_2 of t-Cr and acetylcarnitine in the human vastus lateralis muscle, which were used in this study to calculate absolute concentrations.

Experimental design

On a separate day, before the other experiments, VO_2 max and the maximal workload (W max) of all subjects were determined by a routine incremental cycling test on a stationary bike. Furthermore, body composition was determined by a dual-energy x-ray absorptiometry scan.

In addition to these screening test days, subjects reported to the university on 2 different occasions. On a first day, the acetylcarnitine and PCr recovery measurements were successively performed in the afternoon. All subjects consumed a light lunch at 12:00 noon and remained fasted and refrained from heavy exercise for the following 5 hours. Subjects were positioned in the MR scanner at 5:00 pm, and a long-TE ^{1}H-MR spectrum was acquired to measure acetylcarnitine. Directly after the acetylcarnitine measurement, ^{31}P-MRS was performed to measure the PCr recovery rate, which is a measure of in vivo mitochondrial function (25). The ^{31}P measurement was performed after the acetylcarnitine measurement to make sure that acetylcarnitine concentrations were not influenced by exercise (Figure 8). On a separate day, after an overnight fast, a hyperinsulinemic-euglycemic clamp was performed to measure insulin sensitivity. Prior to the start of the clamp, a muscle biopsy was taken from the m. vastus lateralis.

VO_2 max and body composition

A routine incremental cycling test on a stationary bike was used to determine maximal exercise capacity as described previously (26). Body composition was determined by DXA (DXA, discovery A; Hologic).

Hyperinsulinemic-euglycemic clamp

All participants underwent a 2-step, 6-hour hyperinsulinemic-euglycemic clamp (10 and 40 mU m^2/min) (27). After an overnight fast, participants remained resting on a bed for 2 hours. Insulin infusion was started with a low concentration (10 mU /m^2/min) for 2 hours until a steady state was reached, after which blood sampling was performed during 30 minutes. Thereafter, high insulin concentration infusion was started (40 mU/m^2/min) for 2 hours, after which steady state was reached and blood was again sampled for 30 minutes. Here, we report the GIR (μmol/kg/min) determined during high insulin concentration infusion, as a measure of peripheral insulin sensitivity.

Muscle biopsy

The muscle biopsies were taken from the vastus lateralis muscle under local anesthesia (2% lidocaine), as previously described (28). The muscle tissue was directly frozen in melting isopentane and stored at –80°C until further processed.

Tissue samples were processed and analyzed by the Sarah W. Stedman Nutrition and Metabolism metabolomics/biomarker core laboratory. Acylcarnitine measurements were made using flow injection tandem MS and sample preparation methods previously described (29, 30). Whole-tissue homogenates were prepared from biopsy samples by diluting 20-fold (mass:vol) in CelLytic MT (Sigma-Aldrich). After homogenization, samples were centrifuged at 18,000 *g* to precipitate insoluble proteins and CRAT activity was assessed using 0.01 mg of soluble protein lysate in 50 mM Tris, pH 7.4, 1 mM EDTA, 0.1 M DTNB, 1.0 mM acetyl-CoA, and 5 mM L-carnitine at 25°C.

^{31}P-MRS (PCr recovery)

All MR experiments were performed on a 3T whole-body MRI scanner (Achieve 3T-X; Philips Healthcare). PCr recovery was measured in all participants using ^{31}P-MRS to determine in vivo mitochondrial function as previously described (31). A knee-extension protocol was performed on a custom-built MR-compatible ergometer with a pulley system in the MR scanner. The knee-extension exercise was performed for 4 minutes with weight corresponding to 50% to 60% of the subject's predetermined maximal knee-extension capacity. A 6-cm surface coil was placed over the middle of the vastus lateralis muscle. MR images were acquired using the body coil to confirm the position of the coil and to determine the shimming volume.

A series of 150 unlocalized ^{31}P spectra was acquired with parameters: repetition time (TR), 4000 ms; spectral bandwidth, 1500 Hz; offset frequency, –1.9 ppm. An adiabatic excitation pulse was used, and shimming was performed with a FASTMAP-based shimming routine (32). The 10 minutes of acquisition time were divided into 3 parts: 1 minute of rest, 4 minutes of knee-extension exercise, and 5 minutes of recovery.

^{1}H-MRS (acetylcarnitine)

Subjects were positioned supine in the magnet bore, with the left leg parallel to the main magnetic field and with the foot constrained by 2 sandbags. A 2-element flexible surface coil was placed over the vastus lateralis muscle.

T_2-weighted turbo spin echo images were acquired, consisting of 3 transversal slices and FOV equal to 250 × 210 mm, slice thickness equal to 0.9 mm, TR/TE equal to 2000/100 ms, and turbo factor 20.

A point resolved spectroscopy (PRESS) (33) sequence was used for volume selection, and outer volume suppression was applied to eliminate residual signals of subcutaneous adipose tissue. The spectra were acquired with the following acquisition parameters: TR/TE equal to 6000/350 ms, spectral bandwidth equal to 2 kHz, number of acquired data points equal to 2048, number of averages (NSA) equal to 20, phase cycling steps equal to 4. Shimming was again performed with FASTMAP-based shimming. When compared with conventional short-TE spectroscopy, the voxel size was increased from ca. 2 ml to 48 ml (40 mm × 20 mm × 60 mm) in order to compensate for the signal loss as a result of larger T_2 relaxation. The voxel was placed in the vastus lateralis muscle, but unavoidably, a small bit of surrounding muscle tissue was included as well. At this long TE, water suppression was redundant.

In the endurance-trained and lean sedentary subjects, an additional water spectrum was acquired directly after the acetylcarnitine measurement using a TE of 38 ms. All other parameters were identical. These spectra were used to compare the use of water and t-Cr as internal references.

Determination of T_2

As the T_2 of acetylcarnitine at 3T was unknown, a study was performed in 7 lean and 7 obese subjects to measure in vivo T_2 values of acetylcarnitine in the vastus lateralis muscle. These measurements were also used to measure the T_2 of t-Cr. The T_2 values were required to calculate acetylcarnitine concentrations from the acetylcarnitine to t-Cr ratio. Subjects also consumed a light lunch at 12:00 noon and remained fasted and refrained from exercise for the following 5 hours. At 5:00 pm, the subjects were positioned in the MR scanner and the MR measurement was started.

To be able to determine the T_2, a series of 6 spectra with variable TE (300, 325, 350, 400, 450, and 500 ms) was acquired. NSA was adapted from 12 to 76 throughout the protocol to maintain a fairly constant SNR. Other acquisition parameters were identical to the measurement with a fixed TE. All 6 spectra were used in the T_2 determination of the t-Cr peak, while for the acetylcarnitine peak only the last 4 spectra (TE above 350 ms) were used to prevent contamination from lipid resonances at shorter TE. The determination of T_2 took 35 minutes, which is a time frame in which acetylcarnitine concentration can change. To distinguish between signal changes due to T_2 relaxation and changes due to a variation in concentration over time, spectra with a fixed TE of 350 ms (NSA 20) were measured before and after each spectrum of the series.

Phantom experiment

To evaluate our long-TE MR protocol, 5 different phantoms were prepared. Each of the phantoms (500 ml flask) contained an aqueous solution (0.9 % NaCl) with 30 mM of creatine to mimic our in vivo measurements. The phantoms contained 0, 0.5, 1.0, 2.0, and 5.0 mM of acetylcarnitine, respectively. Both creatine and acetylcarnitine were commercially available (Sigma-Aldrich). The exact same volume (48 ml) and same sequence parameters (PRESS sequence, TR/TE = 6000/350 ms) were used to determine the acetylcarnitine concentration with MR spectroscopy. The phantom with the highest acetylcarnitine concentration, 5.0 mM, was used to determine T_1 (inversion recovery, TR = 10000 ms, inversion time [TI] = 350–2500 ms), and T_2 (TR = 6000 ms, TE = 38–488 ms) relaxation times of creatine and acetylcarnitine in the phantom.

Spectral analysis

All spectra were analyzed and fitted in the time domain by using the non linear least-squares AMARES algorithm in the jMRUI software package (34).

^{31}P-MRS (PCr recovery). All spectra from the time series were apodized (15 Hz Lorentzian shape). In total, the signal intensities of 5 peaks were determined; the Pi and 3 ATP peaks were fitted with a Gaussian lineshape and the PCr peak was fitted with a Lorentzian lineshape. The pH was determined prior to and at the end of the exercise protocol by measuring the frequency shift between the PCr and Pi peak. The exercise protocol was designed to avoid a drop in pH below 6.9 in all cases. The pH after exercise was similar in the groups.

A MATLAB (The Mathworks Inc.) script generated in-house was used for further calculations of the PCr recovery rate. Peak area intensities of the PCr peak from jMRUI were used as input in the script. The time courses of the last 2 minutes of the exercise protocol (steady state) were fitted linearly, without restricting the direction of the slope of the line. The level at the end of the steady state period was used as a starting value in the fit of the recovery period. A mono-exponential increase of the PCr peak after exercise is assumed:

$$PCr\,(t) = PCr\,(t_0) + C\,(1 - e^{-t/\tau}) \quad \text{(Equation 1)}$$

with *PCr* [AU] being the intensity level of the PCr peak, *C* [AU] a constant, τ [s] the rate constant for PCr recovery, and *t* [s] time, starting at the end of the exercise protocol (t_0).

The end of the exercise protocol (t_0) was constrained to an interval of 1 second before and 4 seconds after the time point at which the subject was instructed to stop the exercise. In vivo mitochondrial function is expressed as the PCr recovery rate τ [s], with a lower τ indicating a faster recovery and thus a better mitochondrial function.

^{1}H-MRS. A 3-Hz Gaussian apodization filter was applied to reduce noise in the spectra. The ^{1}H chemical shift of all metabolites was referenced to the t-Cr peak, which was set to 3.03 ppm. The first order phase was fixed to 0, while the 0-order phase was optimized automatically in jMRUI. The areas of both the acetylcarnitine and the t-Cr peaks were obtained. The peaks were fitted using a Gaussian line shape. Because of the long TR of 6000 ms, no T_1 correction was applied for any peak. To calculate the acetylcarnitine concentration, the t-Cr peak was used as internal reference, assuming a concentration of t-Cr of 30 mmol/kgww (15). A correction for T_2 relaxation, for both t-Cr and acetylcarnitine, was applied based on the T_2 measurements in the present study. In addition, we applied a fixed factor to correct for the short component decay of t-Cr, estimated to be 30% of the signal, based on the literature (13). The T_2 of t-Cr has been reported before, but was measured with short TE only (35).

Spectral analysis of the phantom experiments was identical to that of the in vivo ^{1}H-MRS measurements, with correction for T_1 and T_2 relaxation that was performed using the derived relaxation rates from the phantom.

To determine T_2, the t-Cr and acetylcarnitine resonances at various TEs were quantified in jMRUI and the results were used in a MATLAB script generated in-house. Peak intensities from the spectra of the TE series were corrected for changing concentration during the relatively long T_2 determination. Changes in concentration were deduced from the time course of the spectra acquired with a fixed TE by taking the mean of the peak intensities of the fixed TE spectra before and after as a reference. After correction and normalization, a semilogarithmic plot of the peak area versus TE was created and a linear function was fitted through the data points:

$$\ln(A) = -(TE/T_2) + C \quad \text{(Equation 2)}$$

with *A* [AU] the mean area under the peak of the metabolite of interest and *C* [AU] a constant, with *TE* and T_2 both in ms (not shown). The calculated T_2 is returned by the fit procedure directly.

The separate water spectrum that was collected in the endurance-trained and lean sedentary subjects was apodized with a 3-Hz Lorentzian filter and was fitted with a Lorentzian lineshape.

Statistics

Statistical analysis was performed with SPSS Statistics 20 (IBM Corp. Released 2011.). Subject characteristics are expressed as mean ± SD, while all other results are expressed as mean ± SEM. To test for differences between the groups, a 1-way ANOVA test was applied. Post hoc tests were performed using Bonferroni's correction. Using the individual data, Pearson's correlation coefficients were calculated to test for associations between parameters (acetylcarnitine, age, BMI, fat percentage, VO_2 max, PCr recovery rate, insulin sensitivity, CRAT activity, CS activity), as well as between the water- and t-Cr reference signals and the MR-derived and LC-derived acetylcarnitine concentrations. An extra stepwise linear regression was performed, to evaluate the association between acetylcarnitine and insulin sensitivity, when corrected for the other measured parameters. We also calculated the correlation coefficient between the acetylcarnitine concentrations and insulin sensitivity for the means of the groups. In all tests, results were considered to be significantly different with a P value below 0.05.

Study approval

If not indicated differently, all measurements were performed at the Maastricht University Medical Center and were approved by the institutional medical ethics committee. Written informed consent was obtained from all subjects prior to inclusion.

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