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Carnitine acetyltransferase: A new player in skeletal muscle insulin resistance?

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ABSTRACT

Carnitine acetyltransferase (CRAT) deficiency has previously been shown to result in muscle insulin resistance due to accumulation of long-chain acylcarnitines. However, differences in the acylcarnitine profile and/or changes in gene expression and protein abundance of CRAT in myotubes obtained from obese patients with type 2 diabetes mellitus (T2DM) and glucose-tolerant obese and lean controls remain unclear. The objective of the study was to examine whether myotubes from obese patients with T2DM express differences in gene expression and protein abundance of CRAT and in acylcarnitine species pre-cultured under glucose and insulin concentrations similar to those observed in healthy individuals in the over-night fasted, resting state. Primary myotubes obtained from obese persons with or without T2DM and lean controls (n=9 in each group) were cultivated and harvested for LC-MS-based profiling of acylcarnitines. The mRNA expression and protein abundance of CRAT were determined by qPCR and Western Blotting, respectively. Our results suggest that the mRNA levels and protein abundance of CRAT were similar between groups. Of the 14 different acylcarnitine species measured by LC-MS, the levels of palmitoylcarnitine (C16) and octadecanoylcarnitine (C18) were slightly reduced in myotubes derived from T2DM patients ($p < 0.05$) compared to glucose-tolerant obese and lean controls. This suggests that the CRAT function is not the major contributor to primary insulin resistance in cultured myotubes obtained from obese T2DM patients.

1. Introduction

Carnitine acetyltransferase (CRAT), a mitochondrial matrix enzyme proposed as a potent regulator of metabolic inflexibility, has been found to impact whole-body glucose homeostasis and muscle-specific loss of function results in reduced metabolic control, which resembles the insulin resistant state [1]. A study conducted by Noland and colleagues suggests that CRAT overexpression in primary human skeletal myocytes increased glucose uptake and improved lipid-induced suppression of glucose oxidation [2]. In this regard, CRAT could be involved in the compensatory response to glucose intolerance. However, the potential role of CRAT within skeletal muscle insulin resistance is poorly understood.

Recently, Seiler and colleagues proposed that CRAT activity was decreased in response to genetic diabetes, high-fat diet and lipid exposure [3]. They also stated that reduced CRAT activity was accompanied by accumulation of long chain acylcarnitines in skeletal muscle and a decline in the acetylcarnitine/acetyl coenzyme A (CoA) ratio [3]. Since long chain acylcarnitines have been shown to be associated with insulin resistance, and reflect increased lipid flux in T2DM [4], it is possible that carnitine supplementation is an ideal solution for treating T2DM [1]. However, contradictory results have shown that muscular lipid oxidation is normal or even increased in T2DM patients and mice fed a high-fat diet [5].

Previously, we have shown that myotubes established from T2DM patients express primary insulin resistance at the level of glucose uptake, storage and oxidation [6], however, the molecular background is unclear. In the present study, we took advance of this model to investigate CRAT expression and acylcarnitine distribution under basal conditions, in order to clarify whether changes in mRNA levels and protein abundance of CRAT and/or the present acylcarnitine profile can explain primary (genetic) insulin resistance in these cells.

Abbreviations: CRAT, carnitine acetyltransferase; LC-MS, Liquid Chromatography Mass Spectrometry; PDH, Pyruvate Dehydrogenase; T2DM, type 2 diabetes mellitus; ZDF, Zucker diabetic fatty

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Table 1
Clinical characteristics.

	Control, lean	Control, obese	T2DM
N	9	9	9
Age (years)	50.8 ± 1	48.1 ± 1	50 ± 1
Weight (kg)	71.6 ± 3.0	105.5 ± 6.4*	102.2 ± 4.1*
BMI (kg/m^2)	24.2 ± 0.5	34.0 ± 1.4*	33.5 ± 1.1*
Fasting plasma glucose (mM)	5.7 ± 0.1	5.7 ± 0.2	10.0 ± 0.7#
Fasting serum insulin (pM)	23.9 ± 5.7	52.6 ± 5.0*	96.7 ± 10.1#
Glucose infusion rate (mg/min)	383.3 ± 20.4	254.1 ± 28.3*	117.8 ± 18.6#
HbA$_{1c}$ (%)	5.5 ± 0.1	5.4 ± 0.1	7.5 ± 0.5#
Fasting total cholesterol (mM)	5.29 ± 0.22	5.33 ± 0.41	5.42 ± 0.37
Fasting LDL cholesterol (mM)	2.94 ± 0.22	3.18 ± 0.33	3.20 ± 0.27
Fasting HDL cholesterol (mM)	1.85 ± 0.15	1.54 ± 0.15	1.36 ± 0.03*
Fasting plasma triglyceride (mM)	1.12 ± 0.16	1.34 ± 0.18	1.93 ± 0.40

Data are means ± SEM.* Significant different from the lean controls ($p < 0.05$),# significant different from the lean and obese controls ($p < 0.05$).

2. Methods

2.1. Participants and cell culture

The present study examined myotubes obtained and cultured from muscle biopsies collected in a previously reported study [7]. In brief, 10 obese T2DM patients were matched to 10 obese and 10 lean control participants according to BMI, age and gender. Their clinical characteristics have previously been published [7] (Table 1). All participants gave written, informed consent, and the local ethics committee of Funen and Vejle County approved the study. Human myotubes cultures were established as previously described [6–8] and allowed to differentiate under basal conditions of insulin (25 pmol/l) and glucose (5.5 mmol/l) for 8 days, similar to those observed in healthy individuals in the over-night fasted, resting state. However, one person in each group was lost under sample preparation. Therefore, only nine persons in each group were used.

2.2. Metabolic profiling of acylcarnitines

The human myotubes were harvested in cold PBS and sonicated for 10–15 min. Protein concentrations were determined using the Pierce BCA protein assay kit. Internal standards were added (5pmol Hexadecanoyl-L-carnitine chloride (71–1732-5 Larodan), 5 pmol Octadecanoyl (18,18,18-D3)-L-carnitine chloride (71–1748 Larodan), 250 pmol Acetyl (D3)-L-carnitine chloride (71–1746-5 Larodan), 20 pmol Butyryl (4,4,4-D3)-carnitine chloride (71–1734 Larodan)). The cell lysate was extracted in 3:1 ACN: MeOH, vortexed and spun down for 10 min, 10.000 rpm, 4 °C. The sample was freeze-dried and re-dissolved in 50 µl 0.1% formic acid. Sample (10 µl) was injected into the HPLC-MS system. Samples were analysed on an Agilent 1290 HPLC coupled to an Agilent 6530 mass spectrometer. Analyte separation was achieved using a Agilent ZORBAX RRHD Eclipse Plus C18 column (2.1×150 mm^2, 1.8 µm) with a Agilent ZORBAX Eclipse Plus C18 guard column (2.1 mm, 1.8 µm) both heated to 40 °C, 300 µl/min flow of mobile phase 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B) with the following gradient: 3% B for 5 min, to 15% B over 3 min, to 30% B over 2 min, to 97% B over 9 min and 97% B for 3 min. Reference ions were infused with a flow of 10 µl/min. The mass spectrometer was operated in positive ion mode with the following settings: 2 GHz extended dynamic range from 100 to 1500 *m/z*, gas temperature: 300 °C, drying gas flow (nitrogen): 10 l/min, nebulizer: 35 psi, sheath gas temperature: 350 °C, Sheath gas flow:11 L/min, capillary voltage: 3500 V, fragmentor: 125 V, acquisition rate: 3 spectra/s, reference correction two points at *m/z* 121.050873 and *m/z* 922.009798. MassHunter Profinder, Agilent Technologies was used to detect and quantify 14 different acylcarnitine species. These were C0, C2, C3, C4, C6, C8, C10, C12, C14, C16:1, C16, C18:2, C18:1, C18:0.

2.3. qPCR

Total RNA was purified and cDNA was synthesized as previously described [9]. qPCR was performed on The LightCycler® 480 Real-Time PCR system (Roche) using commercially available SYBR Green Jumpstart Taq Ready Mix (Sigma) according to the manufacturer's instructions. The following conditions were used: 2 min at 95 °C (hot start), 40 cycles of 15 s at 95 °C, 45 s at 60 °C, and 45 s at 72 °C. The *CRAT* expression was normalized against the expression of the housekeeping gene *TBP*. The following primers were used: *CRAT* fwd: 5′-GACACAGTCAGCAACTTCAGC, *CRAT* rev: 5′ GCTGCACAAA GATCTGATCCG; *TBP* fwd: 5′-GCCCGAAACGCCGAATAT, *TBP* rev: 5′CCTCATGATTACCGCAGCAAA.

2.4. Western Blot

Proteins were extracted and analysed as previously described [9]. Protein concentration was determined using the Pierce BCA protein assay kit and equal amounts of cell protein (10 µg) were loaded in each well. Rabbit Anti-CRAT (1:250) (Sigma HPA022815) and Rabbit Anti-VDAC1 (1:1000) were used as primary antibodies. VDAC1 was used as loading control in order to normalize the protein abundance of CRAT to the mitochondrial content. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) (Promega W401B) was used as secondary antibody.

2.5. Statistics

Statistical analysis was calculated as One-way ANOVA with Dunnett's multiple comparisons test using GraphPad Prism version 5.01. $p < 0.05$ was considered statistically significant; n used for analysis is the total number of individuals. Mean ± SEM is shown.

3. Results

The clinical characteristics show that fasting glucose, serum insulin and HbA1c levels were significantly higher, whereas the fasting HDL cholesterol level was significantly lower in T2DM patients compared to lean controls, (Table 1). Serum insulin level was higher in obese compared to lean controls, and the glucose infusion rate (GIR) measured during the hyperinsulinemic euglycemic clamp was significantly lower in obese with and without T2DM compared to lean controls (Table 1).

The protein and mRNA levels of CRAT were determined in cultured myotubes from obese with and without T2DM and lean persons (Fig. 1 A and B). Our results show no significant difference between the investigated groups. By using LC-MS, no significant difference was found in the total acylcarnitine level (166.32 ± 8.37, lean; 170 ± 7.1, obese; 130 ± 15.5, T2D, Mean ± SEM, pmol/mg protein, $p < 0.05$) (data not shown) or among individual short- and medium-chain acylcarnitines between groups (Mean ± SEM, pmol/mg protein, and $p < 0.05$) (Fig. 1C). Muscular levels of C16 and C18 carnitine (Mean ± SEM, pmol/mg protein, $p < 0.05$) were significantly reduced 36% and 53% in T2DM patients compared to lean controls (Fig. 1C). No significant differences were observed among other long-chain acylcarnitines (Mean ± SEM, pmol/mg protein, $p > 0.05$) (Fig. 1C).