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Differential Carnitine/Acylcarnitine Translocase Expression Defines Distinct Metabolic Signatures in Skeletal Muscle Cells

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Import of acylcarnitine into mitochondrial matrix through carnitine/acylcarnitine-translocase (CACT) is fundamental for lipid catabolism. To probe the effect of CACT down-expression on lipid metabolism in muscle, human myocytes were stably transfected with CACT-antisense construct. In presence of low concentration of palmitate, transfected cells showed decreased palmitate oxidation and acetyl-carnitine content, increased palmitoyl-carnitine level, and reduced insulin-dependent decrease of fatty acylcarnitine-to-fatty acyl-CoA ratio. The augmented palmitoyl-carnitine synthesis, also in the presence of insulin, could be related to an altered regulation of carnitine-palmitoyl-transferase 1 (CPT 1) by malonyl-CoA, whose synthesis is dependent by the availability of cytosolic acetyl-groups. Indeed, all the described effects were completely overcome by CACT neo-expression by recombinant adenovirus vector or by addition of acetyl-carnitine to cultures. Acetyl-carnitine effect was related to an increase of malonyl-CoA and was abolished by down-expression, via antisense RNA strategy, of acetyl-CoA carboxylase-β, the mitochondrial membrane enzyme involved in the direct CPT 1 inhibition via malonyl-CoA synthesis. Thus, in our experimental model the modulation of CACT expression has consequences for CPT 1 activity, while the biologic effects of acetyl-carnitine are not associated with a generic supply of energy compounds but to the anaplerotic property of the molecule. J. Cell. Physiol. 203: 439–446, 2005.
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Skeletal muscles satisfy a large portion of their energy needs by mitochondrial oxidation of fatty acids and are consequently important for lipid metabolism throughout the body (Giordano et al., 2003). Oxidization of fatty acids is reduced in the skeletal muscle of obese individuals both before and after weight loss (Kelley et al., 1999). The reduction of muscle lipid oxidation may promote lipid accretion and increase intramuscular lipid content. Lipid accumulation can affect negatively muscle insulin sensitivity, thereby amplifying the metabolic alterations (Kelley and Goodpaster, 2001). Indeed, in obese and type 2 diabetic patients there is a strong correlation between intramuscular levels of lipid and insulin resistance, irrespective of the degree of obesity (Manco et al., 2000).

The cellular mechanisms responsible for the decrease in muscle lipid oxidation with obesity have yet to be elucidated. It is known that long chain fatty acids are transported into the mitochondrial matrix by a complicated mechanism that depends on the availability of carnitine (Kerner and Hoppel, 2000). Free fatty acids are first transformed into their CoA derivatives by acyl-CoA synthase, an enzyme located on the outer most part of the mitochondrial membrane. The long-chain fatty acid-CoA derivative is then converted into the carnitine derivative by carnitine/palmitoyltransferase-1 (CPT 1), an integral protein located on the outer mitochondrial membrane of the contact sites. Acylcarnitines are imported into mitochondria through carnitine/acylcarnitine translocase (CACT). This protein catalyzes a mole-to-mole exchange of carnitines and acylcarnitines so that the fatty acid moieties can be translocated into the mitochondrial matrix. In the mitochondrial matrix, long-chain acylcarnitines are reconverted to the respective long-chain acyl-CoAs by carnitine/palmitoyl-transferase-2 (CPT 2), an enzyme associated with the inner face of the inner mitochondrial membrane.

A factor that could account for decreased lipid oxidation is reduced CPT 1 activity. A hypothesis that might explain reduced CPT 1 activity in obesity involves malonyl-CoA, a molecule that directly inhibits CPT 1 activity and acylcarnitine formation, thereby reducing long-chain fatty acid oxidation (Ruderman et al., 1999; Rasmussen et al., 2002).

Although a decrease in CPT 1 may contribute to reduced lipid oxidation, recent data suggest that other facets of muscle lipid metabolism are altered in the mitochondria of obese individuals. For example, long-chain acylcarnitine oxidation, thought to be oxidized independently of CPT 1, was found to be markedly reduced in obese human skeletal muscle (Jong-Yeon et al., 2000). This finding indicates that post-CPT 1 mechanisms are also responsible for decreased muscle lipid oxidation in obese subjects.

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Recently, we hypothesized that in obesity-related insulin resistance (IR), fatty acid (FA) oxidation may be decreased in skeletal muscle because of decreased CACT activity (Peluso et al., 2002). To test this hypothesis we evaluated CACT at transcriptional and protein levels in patients with phenotypic IR and in control subjects. We also analyzed the transport of acylcarnitine in isolated mitochondria obtained from skeletal muscle tissue of the same subjects to determine whether carrier activity was decreased. We found a significant decrease in CACT, in terms of protein and activity, in IR muscle (Peluso et al., 2002).

The aim of this study was to analyze the metabolic effects of CACT down-expression in human myocyte cells stably transfected with CACT antisense cDNA. Using these CACT-deficient cell clones we were able to examine the functions of CACT as regards both acylcarnitine transport into the mitochondrial matrix, and maintenance of the physiological ratio between acyl- and acetyl-carnitine outside and inside mitochondria. This study reveals a complex metabolic situation determined by insulin and palmitate in CACT-deficient cells, and provides insight into the functional interactions between CACT, CPT 1, malonyl-CoA, and acetyl-carnitine.

MATERIALS AND METHODS

Cell culture

A human skeletal muscle cell line was obtained from Clonetics (San Diego, CA) and cultured in SkBM, supplemented with SkGM Singlequots. Cell fusion and differentiation into multinucleated myotubes was monitored visually by phase-contrast microscopy. The content of CACT was assayed by a modified ELISA using cellular protein (25 ng) adsorbed on 96-well microtiter plates, affinity-purified anti-CACT antibodies, and peroxidase-conjugated anti-rabbit IgG as secondary antibody. Results were determined by reference to a standard curve (0–0.2 ng of CACT). Biochemical analysis of myotubes was performed when >50% of nuclei were found within multinucleated cells (myotubes). After 48 h of differentiation, myotubes were harvested for RNA isolation, or used for the different tests. Creatine kinase activity assay was used as a measure of myogenic differentiation (Benders et al., 1991). The vitality of cells was evaluated by Trypan bleu exclusion test, while the apoptosis was studied by TUNEL assay (Vescovo et al., 2002).

Myoblast transfection and isolation of clones

For myoblast transfection, cells were plated at a density of 6×10^3 cells/cm^2 in 100 mm dishes in growth medium. Twenty-four hours later the cells were transfected with the mammalian expression plasmid pCMVneo containing a cDNA insert which in turn contained the full-length CACT sequence, in the antisense orientation, kindly donated by Prof. C. Indiveri (Bari University, Italy). The complete insert and a pCMVneo plasmid were digested with *Eco*RI to generate the pCMVneo-CACT recombinant plasmid; positive clones were selected by ampicillin resistance, and the orientation of the insert was determined by polymerase chain reaction; the antisense orientation was confirmed by DNA sequencing. Control cells were transfected with pCMVneo containing no insert. For transfections, 40 mg of plasmid DNA and 30 mg of LipofectAMINE in a total volume of 600 ml of Opti-MEM I were used according to the manufacturer's instructions. After 6 h at 37°C, FCS and chicken embryo extract were added to final concentrations of 10% and 0.5%, respectively, and the cells were incubated overnight. The next day the cells were rinsed twice with Hanks' balanced salt solution and cultured in normal growth medium. Medium was supplemented with G-418 (400 mg/ml) after 3 days. After 2–3 weeks colonies were selected using cloning rings.

Transient transfection

Human acetyl-CoA carboxylase-β (ACC-β) primers were constructed, which would amplify two small regions within the ACC-β sequence coding for the 215 amino acids at the N-terminal region of ACC-β (Ha et al., 1996). The ACC-β sequences were derived from the cDNA from human ACC-β (accession no. M001093). Additionally, the primers were designed to contain restriction endonuclease sites so that the fragments could be inserted into a retroviral expression system vector to express antisense RNA. The primers sequences are: 5′AS1: 5′-AGATCTCTCGAG- ATGGTCTTGCTTCT-3′; 3′AS1: 5′-GGAATTGAATTC-GGTTTGCTTCTGAT-3′; 5′ AS2: 5′-AGATCTCTCGAG-CCTCCAAAGAAGAC-3′; 3′ AS2: 5′-GGAATTGAATTC-GGGTAGACTCACGA-3′. Underlined sequences are directed against ACC-β with 5′ AS1 and 5′ AS2 in the positive strand orientation and 3′ AS1 and 3′ AS2 in the negative strand orientation. Sequences not underlined are viral vector derived. CTCGAG denotes the X*ho*I restriction endonuclease site and GAATTC denotes the *Eco*RI site, used for the proper expression orientation in the vector. The Retroviral Gene Transfer and Expression System (BD Bioscience Clontech Labs, Inc., Palo Alto, CA) was used for these experiments. Finally, the infected cells were collected and screened for inhibition of ACC-β protein expression after 2 days growth.

Infection of myotubes with CACT adenoviral constructs or insert-free adenoviral preparations were also performed (Schönherr et al., 1999). Cells (6×10^3 cells/cm^2), were treated with 7.5×10^7 plaque-forming units of adenovirus containing the CACT sequence or with control virus. Subsequently, the medium was replaced by fresh medium, and the cells were grown for RNA analysis for 2 days. Experiments were performed 48 h after infection.

RNA isolation and Northern blot analysis

Total RNA was isolated from cell cultures using Trizol. RNA samples were electrophoresed in 1.2% agarose/formaldehyde gels, transferred to Nytran membranes, and hybridized with probes for actin, and CACT, as described previously (Peluso et al., 2002). Blots were hybridized with random primed labeled probes in a hybridization buffer at 65°C. Hybridized membranes were washed twice at 65°C and exposed to Kodak X-ray film. For quantitative determination of the mRNA levels the intensity of the hybridization signals were measured by densitometric scanning (BioRad densitometer, Milan, Italy).

Immunoblot analysis

For detection of CPT 1 and CACT proteins, control or transfected cells were collected and dissolved in SDS sample buffer. Proteins were subjected to SDS/PAGE (10% gels) and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h with Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% (w/v) non-fat dried milk. The membrane was washed three times in TBS-Tween at 22°C. Membranes were probed with the CACT specific rabbit antibody (Peluso et al., 2002) by overnight incubation followed by HRP-conjugated goat anti-rabbit (1:5,000; Amersham Pharmacia, Milan, Italy). The blots were developed with enhanced chemiluminescence reagents (Amersham Pharmacia).

Preparation of mitochondria

Cells (~60–70 million) were resuspended in approximately 5 ml of 0.25M mannitol/20 mM HEPES, pH 7.3/2 mM EDTA and homogenized with four strokes in a tightly fitting Potter-Elvehjem homogenizer. Cells debris was pelleted at 600*g* for 5 min. Microscopy showed that the resulting homogenates contained no detectable whole cells. Mitochondria were prepared from the combined supernatants as described previously (Bergseth et al., 1986). The supernatants were centrifuged at 9,000*g* for 10 min. The mitochondria were washed once in mannitol/HEPES/EDTA medium and the centrifugation was repeated. The pellet was resuspended in 1 ml of medium, to a concentration ~15–20 mg/protein/ml. Polarographic studies using the mitochondrial preparations showed a respiratory control ratio of 3.5 with glutamate as substrate.

Citrate synthase was measured by standard procedures to evaluate the integrity of mitochondria (Shepherd and Garland, 1969). We also confirmed that the mitochondrial fraction did not contain significant amounts of peroxisomes, since there was no measurable flux through β-oxidation in the presence of the respiratory chain inhibitors cyanide, rotenone and myxothiazol.

CPT and CACT assays in isolated mitochondria

To measure CPT 1 activity the mitochondria were kept intact, and incubations were carried out with and without 100 μM malonyl-CoA as already described (Baillet et al., 2000). Under these conditions, the assay monitors the external malonyl-CoA-sensitive CPT 1 activity. CACT activity was measured in intact mitochondria incubated without external CoASH. Under these conditions, CPT 1 cannot operate, but palmitoyl-[^{14}C]-carnitine is formed because of the combined activity of CACT and internal CPT 2.

In vitro quantitative acylcarnitine and acyl-CoA assays

Acylcarnitines were quantitated with a tandem mass spectrometer (Sim et al., 2002). Briefly, cells were washed twice with PBS and the medium was replaced by freshly prepared bicarbonate-buffered MEM, without FCS, containing 2 mM L-glutamine, 1% (v/v) antibiotics, and 0.1 mM [U-^{13}C]palmitic acid bound to BSA. Incubation of the cells was performed at 37°C in humidified 5% CO_2/95% air. Insulin (100 ng/ml) was added when requested. At the end of incubation period the medium was collected and the cells, after washing twice with PBS, were harvested by trypsinization. Cells and media were combined and processed further. Acylcarnitines were measured, based on their butyl esters, with a Micromass Quattro LC electrospray ionization tandem mass spectrometer (Micromass, Manchester, UK). Acylcarnitine concentrations were measured using Neolynx software, which calculates the intensities of analyte/internal standard ratio and concentration data from externally calibrated analytes. Total protein was measured with the Pierce bicinchoninic acid method using bovine serum albumin in saline as standard and a Cobas BIO analyzer.

Long-chain fatty acyl-CoAs were quantified in clones grown in media containing palmitate (0.1 mM) by negative chemical ionization mass spectrometry; (6-D_3)palmitoyl-CoA was added as internal standard (Tamvakopoulos et al., 1995).

Quantitation of short chain acyl-CoA in cell extracts

We measured the concentration and mass isotopomer distribution of malonyl-CoA with an assay based on two methods (Reszko et al., 2001; Liu et al., 2003). The assay entails five steps: perchloric acid extraction of cells pretreated with 0.5 mM [1,2-$^{13}C_2$]acetyl-carnitine for different periods of time, spiking the extract with [U-$^{13}C_3$]malonyl-CoA internal standard, isolation of short-chain acyl-CoA fraction by capillary electrophoresis in a 100 mM NaH_2PO_4 running buffer containing 0.1% β-cyclodextrin, alkaline hydrolysis to malonate, trimethylsilyl derivatization, and analysis of the mass isotopomer distribution of malonate. Detection limits were in the picomole range.

Measurement of palmitate oxidation

Fatty acid oxidation was measured as already described with some modifications (Antinozzi et al., 1998). Briefly, cells were preincubated for 30 min with the following buffer: 14 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose. After preincubation the assay buffer, containing 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, and 0.1 mM palmitate (containing 10 μCi of [1-^{14}C]palmitate; 1 Ci = 37 GBq) bound to albumin with or without 0.5 mM acetyl-carnitine, was added and the cells were incubated at 37°C for 2 h. After the incubation, 400 μl of benzethonium hydrochloride was added to the center well to collect released $^{14}CO_2$. The reaction was stopped adding 500 μl of 7% perchloric acid to the cells. The cells were then incubated for 2 h at 37°C, after which the benzethonium hydrochloride was removed and the radioactivity was quantitated. Blanks were prepared by adding 500 μl of 7% perchloric acid to the cells before the incubation with the assay buffer for 2 h.

Determination of ACC-β protein and enzymatic activity

The activity of acetyl-CoA carboxylase from mitochondrial fractions of cell clone lysates was determined by measuring the production of malonyl-CoA at 5 min intervals for 30 min, using reverse phase HPLC. The rate of conversion of acetyl-CoA to malonyl-CoA was found to be linear for 20 min. The reaction mixture contained 50 mM Tris, pH 7.5, 6 μM acetyl-CoA, 2 mM ATP, 7 mM KHCO3, 8 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mg/ml bovine serum albumin. Mitochondrial fractions were preincubated (30 min, 25°C) with bovine serum albumin (2 mg/ml) and potassium citrate (10 mM). Reactions were initiated by transferring 50 μl of preincubated mitochondrial fractions to the reaction mixture (final volume 200 μl) and incubated for 5–20 min at 25°C. Reactions were terminated by addition of 50 μl 10% perchloric acid. Following termination of the reaction the samples were centrifuged (3 min, 10,000*g*) and analyzed by HPLC. A mobile phase of 10 mM KH_2PO4, pH 6.7 (solvent A), and MeOH (solvent B) was used. The flow rate was 1.0 ml/min, and the gradient was as follows: hold at 100% solvent A for 1 min followed by a linear gradient to 30% solvent B over the next 5 min, then hold at 30% solvent B for 5 min. Western blot analysis was performed using the same procedure before described using a polyclonal antibody obtained as elsewhere described (Abu-Elheiga et al., 1997).

RESULTS

Primary human skeletal muscle cells as a model

In primary human skeletal muscle cells, the concentration of CACT increased from myoblast stage (31 ± 2 pmol/mg of protein; n = 5) to myotube stage, i.e., after 48 h of culture in differentiation medium (47 ± 9 pmol/mg of protein; n = 5). No additional induction of CACT was detected at mRNA or protein level on addition of various concentrations of palmitate, insulin, and/or glucose to the culture medium of myoblasts and myotubes for 24 h.

Stable transfection of myoblasts with antisense CACT cDNA

Because CACT plays an important role in controlling the rate of fatty acid β-oxidation, we reasoned that down-regulation of CACT expression would affect skeletal muscle lipid metabolism by modulating the recruitment of acyl-carnitine into mitochondria. To test this hypothesis, we infected human primary myoblasts with a plasmid containing full-length CACT cDNA in the antisense orientation or with the control vector. CACT mRNA was clearly down-expressed in clones transfected with the antisense CACT cDNA compared with the control vector-transfected clones (Fig. 1A,B). There was no change in the CACT mRNA signal in the antisense clones infected with CACT adenoviral constructs, which confirms the validity of the antisense approach (Fig. 1A,B).

We next evaluated the specificity of the effect on CACT synthesis. Western blots showed that, in contrast to wild-type cells, nine clones synthesized only trace amounts of CACT protein. Three of these clones, in which mitochondrial CACT activity was ≤20% versus wild-type cells, were selected for more detailed studies after myotube differentiation. Thus, the capacity to transport acylcarnitines of the in vitro cell model paralleled that of the skeletal muscle of obese subjects. As shown in Figure 2, the antisense clones (A1, A6, and A7) synthesized significantly less CACT than did

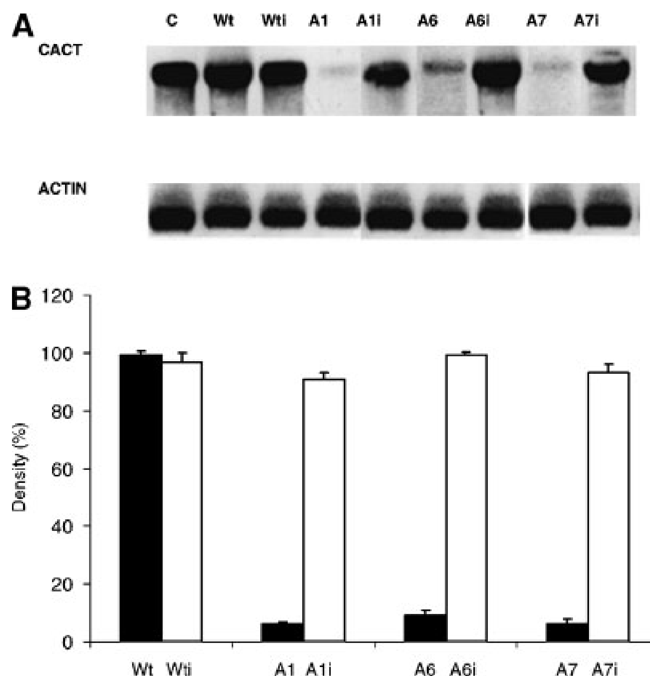

Fig. 1. Northern blots of carnitine/acylcarnitine-translocase (CACT) mRNA levels in control and transfected cell clones. **A**: CACT expression in control cells (C), in transfected (WT), and infected (WTi) control cells, in antisense CACT-transfected cells (A1, A6, and A7), and in transfected cells infected with an adenovirus containing the full-length sequence for human CACT (A1i, A6i, and A7i) was evaluated by Northern blot analysis. **B**: CACT mRNA levels measured by densitometry were significantly lower in A1, A6, and A7 than in the other cell cultures ($*P < 0.005$). For each clone, densities of lanes (adjusted for actin mRNA) were expressed as percentage control density. Vertical axis indicates the mean of these percentages and reflects relative cellular CACT mRNA abundance.

wild-type cells, whereas CPT 1 synthesis was the same as wild-type cells. The synthesis of CPT 2, and cytosolic and mitochondrial proteins was similar in antisense clones and wild-type cells (data not shown). After infection with the adenovirus containing the CACT sequence, antisense cells expressed normal levels of CACT as evaluated with specific antibodies (Fig. 2). Again, viral infection did not affect the overall synthesis of proteins. These results demonstrate that transfection with antisense CACT cDNA resulted in stable clones with greatly reduced CACT expression.

Morphological and growth characteristics of myoblast and myotube cultures

In the proliferative stage, all transfected clones were morphologically similar to non-transfected myoblasts.

Fig. 2. Decreased expression of CACT protein in control and transfected cell clones. Synthesis of CACT, and carnitine-palmitoyl-transferase 1 (CPT 1) was evaluated in control cells (lane 1), in transfected and infected control cells (WT and WTi; lanes 2 and 3), in stably antisense CACT-transfected cells (A1, A6, and A7; lanes 4–6), and in transfected cells infected with an adenovirus containing the full-length sequence for human CACT (A1i, A6i and A7i; lanes 7–9). The cell extracts were subjected to immunoblotting using specific polyclonal antibodies against CACT and CPT 1.

[^{3}H]-Thymidine incorporation studies did not reveal significant differences in the proliferation rate of antisense CACT cDNA-transfected myoblasts compared with wild-type and control vector-transfected cells. After the growth medium had been changed to differentiation medium, morphological differences became visible in all cell cultures. In differentiation medium, myotube formation was normal in control and antisense-transfected cells.

The CK activity of antisense cDNA-transfected cells in the myotube-forming clones was comparable to that in the wild-type and control vector-transfected cells.

Finally, we did not show any increase of cell death via apoptosis or necrosis after incubation of the different clones with palmitate, also when their ability to allow acylcarnitines to enter the mitochondria was impaired.

CPT and CACT activities and β-oxidation analysis

To analyze further the decreased CACT synthesis in antisense-transfected cells, we evaluated acylcarnitine translocation in the mitochondrial matrix of transfected, infected, and wild-type cells. As expected, CACT activity was decreased only in antisense transfected cells (Fig. 3A). We also analyzed the enzymatic reactions catalyzed by CPT 1 and 2 to verify whether CACT down-regulation altered their activity. As depicted in Figure 3B, CPT 1 activity was unaltered in all the tested

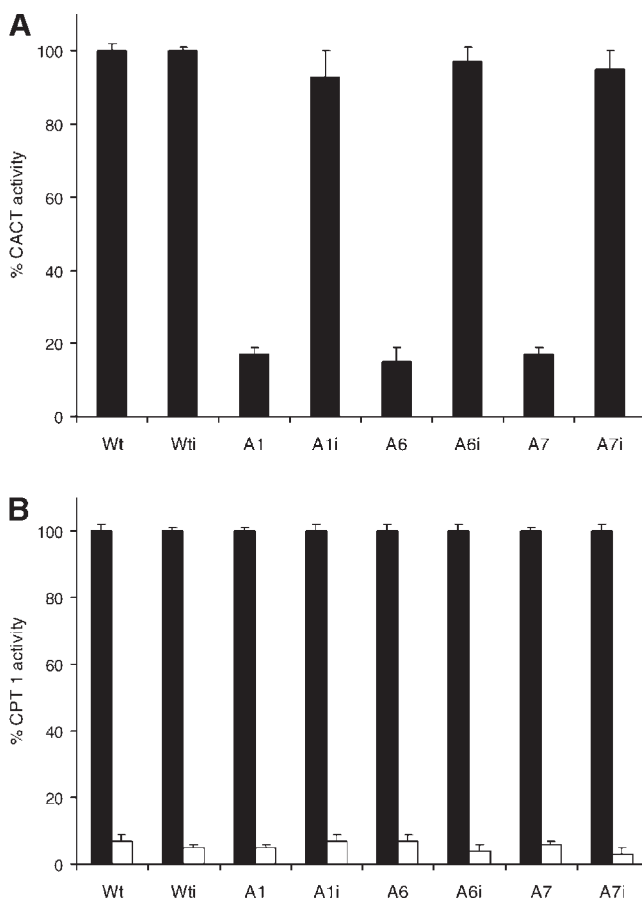

Fig. 3. CACT and CPT 1 activity in control and transfected cell clones. **A**: CACT activity was evaluated in mitochondrial-enriched cell fractions. Results are the mean ± SD of three experiments performed in duplicate. **B**: CPT 1 activity was measured in mitochondrial-enriched cell fractions, before (■) and after (□) treatment with Etomoxir (200 μM). Results are the mean ± SD of three experiments performed in duplicate.

cell clones. Similarly, CPT 2 activity was unchanged in cell clones versus wild-type cells.

Since decreased CACT activity may result in increased palmitoyl-carnitine availability, we assessed the effect of different amounts of palmitoyl-carnitine on CPT and CACT activity. None of the palmitoyl-carnitine concentrations tested significantly affected CPT 1 activity or suppressed CPT 2 activity in mitochondria from control and transfected cells.

In sharp contrast to its lack of effect in CPT 1 and 2 assays, the exchange of ^{14}C between carnitine and palmitoyl-carnitine in the CACT assay was greatly suppressed in transfected cells, with inhibition reaching 83% at a palmitoyl-carnitine concentration of 25 μM. Palmitoyl-carnitine affected CACT activity in a dose-dependent manner in all the cells tested, and this effect was more pronounced in antisense transfected cells that had an IC_{50} of 10 μM. The observation that neither CPT 1 nor CPT 2 was affected by the palmitoyl-carnitine concentrations examined here, shows that palmitoyl-carnitine acts only on the CACT component of the mitochondrial carnitine system. Finally, fatty acid oxidation, measured as $^{14}CO_2$ production, after exposure to palmitate (0.1 mM) was significantly depressed in antisense transfected myocytes (Fig. 4).

Impact of CACT down-regulation on cell palmitoyl-carnitine and acetyl-carnitine cell content in the absence and presence of insulin

The mean acetyl-carnitine level in control myotubes incubated with palmitate (0.10 mM) for 30, 60, and 90 min was 0.6, 1.0, and 2.3 nmol/mg protein (SD ± 0.1, 0.3, and 0.1 nmol/mg protein), respectively. Acetyl-carnitine production was lower in the antisense clones, i.e., a mean of 0.3 nmol/mg protein (SD ± 0.1) after 30 min incubation with palmitate, which decreased to <0.1 at 60 and 90 min of incubation. Moreover, a large amount of palmitoyl-carnitine (16.5 ± 1.2 vs. 1.9 ± 0.3 nmol/mg protein) remained in CACT-deficient cell lines.

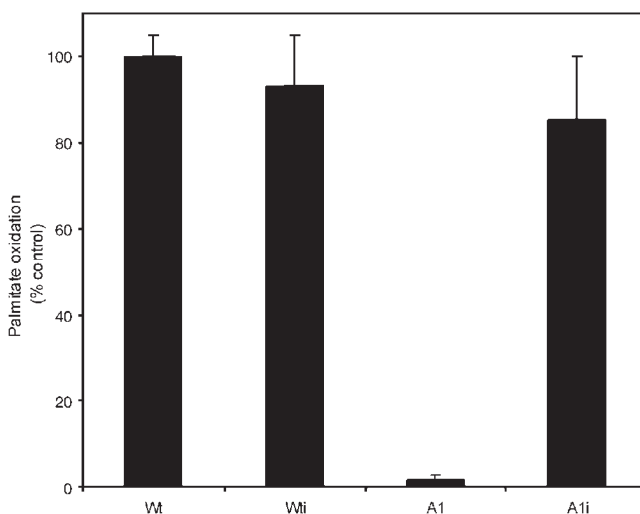

Fig. 4. Long-chain fatty acid oxidation capacity of control and transfected cell clones. Cell clones were cultured in the presence of palmitate for 2 h. Subsequently [1-^{14}C]palmitate oxidation was measured, as $^{14}CO_2$ production, in control cells (C), transfected or infected control cells (Wt and WTi), and transfected cell clones (A1 and A1i). Data are presented as mean ± SD of three independent cell cultures. The 100% value of palmitate oxidation was 3.1 ± 0.3 nmol CO_2/h/mg cellular protein. Similar results were obtained using the cell clones A6 and A7. $P < 0.05$ C, Wt, Wti, A1i cells versus A1.

In palmitate-pretreated (0.10 mM) control cells, insulin (100 ng/ml) practically doubled the long-chain fatty acyl-CoA concentration and significantly decreased the fatty acylcarnitine-to-fatty acyl-CoA ratio (Table 1). These effects might be due to a "metabolic crossover," which implies that the insulin-dependent malonyl-CoA increase inhibited CPT 1. Inhibition of CACT reversed the changes induced by insulin in palmitate-pretreated myotubes. Accordingly, when we increased the palmitate concentration to 0.10 mM, insulin neither increased the level of long-chain fatty acyl-CoA nor decreased the fatty acylcarnitine-to-fatty acyl-CoA ratio (Table 1). Again, this effect could be related to a lack of regulation of acyl-carnitine synthesis by malonyl-CoA.

Effect of acetyl-carnitine on insulin response of CACT antisense transfected myotubes

Addition of acetyl-carnitine to the culture medium dramatically affected the ability of myocytes to respond to insulin treatment.

In presence of 0.5 mM acetyl-carnitine, insulin produced a significant decrease of acylcarnitine-to- acyl-CoA ratio in CACT-antisense transfected cells, while no exacerbation of insulin effect was seen in control cells treated with acetyl-carnitine (Fig. 5).

Because inhibition of CACT would decrease the supply of cytosolic acetyl-CoA deriving from mitochondrial substrate oxidation, and because acetyl-CoA could serve as a precursor for the de novo synthesis of malonyl-CoA, we considered the possibility that addition of acetyl-carnitine to cell cultures might lead to augmented production of malonyl-CoA. We thus monitored the time course of malonyl-CoA accumulation in CACT antisense myocytes after addition of 0.5 mM acetyl-carnitine to the culture medium. Malonyl-CoA levels were significantly increased by acetyl-carnitine treatment (Fig. 6).

Role of acetyl-CoA carboxylase on the modulatory effect of acetyl-carnitine

The correlation observed between addition of acetyl-carnitine to myocytes and malonyl-CoA production suggests the involvement of acetyl-CoA carboxylase (ACC). Since between the two isoforms of ACC, ACC-α and ACC-β, only the second is predominant in muscle cells and seems to be directly involved in the regulation of FA oxidation (Hopkins et al., 2003), we evaluated the effects of down-regulation of ACC-β expression, by an antisense RNA strategy, on the capacity of acetyl-carnitine to modulate the insulin response in CACT deficient cells. To demonstrate the efficiency of antisense ACC-β mRNA in down-regulating ACC-β expression, all the clones infected were initially screened by assaying their ACC activity on mitochondrial fraction and by comparing the results of ACC-β AS infected cells with control infected (with empty viral vector) cells. As shown in Figure 7A, it was possible, by this procedure, to select ACC-β AS clones in which the ACC activity was significantly decrease. Down-regulation of ACC-β protein expression by Western blotting is demonstrated in Figure 7B. Again, the results confirm that the decrease in the activity of ACC was related to the decrease of the ACC-β protein level. To determine whether a specific decrease in ACC-β affected the malonyl-CoA synthesis after acetyl-carnitine supplementation, the short term (30 min) malonyl-CoA production was examined. The malonyl-CoA synthesis in mitochondria from CACT antisense myocyte clones after ACC-β AS infection was diminished significantly in proportion to the decline in

TABLE 1. Amount of palmitoyl-carnitine and palmitoyl-CoA detected in cultured palmitate-fed cells

Cell clone	Addition to incubation mixture	A: Palmitoyl-carnitine (nmol/mg protein)	B: Palmitoyl-CoA (pmol/mgprotein)	Ratio A/B
Control	Palmitate[a]	1.9 ± 0.3	130 ± 12	14.6 ± 5*
	Palmitate + Insulin[a]	0.7 ± 0.5	240 ± 20	2.9 ± 0.7
Wt	Palmitate[a]	2.0 ± 0.5	132 ± 11	14.5 ± 3*
	Palmitate + Insulin[a]	0.9 ± 0.3	240 ± 18	3.7 ± 1
Wti	Palmitate[a]	2.1 ± 0.5	138 ± 11	15.2 ± 1*
	Palmitate + Insulin[a]	0.9 ± 0.2	247 ± 20	3.6 ± 1
A1	Palmitate[a]	16.5 ± 1.2	110 ± 15	150 ± 10
	Palmitate + Insulin[a]	15.7 ± 1.7	107 ± 12	147 ± 14
A1i	Palmitate[a]	2.3 ± 0.7	140 ± 16	16.4 ± 4.5*
	Palmitate + Insulin[a]	1.1 ± 0.5	260 ± 15	4.2 ± 1

[a]Data are averages of triplicate incubations of the clone cell lines with palmitate (0.1 mM) $\pm$ insulin (100 ng/ml).
*$P < 0.05$ when compared with incubation in presence of insulin.

ACC activity as shown in Figure 6. Furthermore, the recovery of insulin response induced by acetyl-carnitine was significantly decreased in the antisense ACC-β mRNA expressing cells (Fig. 5).

DISCUSSION

CACT is essential for mitochondrial oxidation of exogenous and endogenous long-chain fatty acids because carnitine esters enter the mitochondrial inner space via CACT in exchange for free carnitine (Ramsay et al., 2001). It also catalyzes the export of certain acylcarnitines from the mitochondrion to the cytosol and subsequently to the extracellular space. For example, in the case of mitochondrial β-oxidation enzyme deficiency, such as medium-chain acyl-CoA dehydrogenase deficiency, palmitate can undergo partial oxidation to produce several acyl-CoA derivatives which are then converted to carnitine esters and exported to the cytosol by CACT activity (Ramsay et al., 2001).

Mitochondrial CACT also plays an important role in modulating matrix and cytosolic acetyl-CoA concentrations. Matrix acetyl-groups, produced as a consequence of glucose metabolism or fatty acid β-oxidation, are used for many pathways including oxidation in the tricarboxylic acid cycle, and synthesis of molecular species once exported as acetyl-carnitine from the mitochondrion to the cytosol. Indeed, CACT transports acetyl-carnitine from the mitochondria to the cytosol where it serves as a substrate for acetyl-CoA carboxylase thereby increasing malonyl-CoA production and decreasing fatty acid oxidation (Patel and Roche, 1990; Broderick et al., 1992). Interestingly, not all the acetyl-groups generated within the mitochondrial matrix undergo the same fate. Lysiak et al. (1986) showed that most of the acetyl-coA generated from pyruvate by pyruvate dehydrogenase (PDH) is readily accessible to carnitine acetyl-transferase (the enzyme catalyzing the synthesis of acetyl-carnitine from acetyl-CoA), whereas that generated by β-oxidation is for the tricarboxylic acid cycle. Using a different approach (dichloroacetate PDH stimulation), increased acetyl-coA production by PDH was strongly correlated with increased malonyl-CoA production (Saddik et al., 1993).

In view of the complexity of creating an animal model resembling CACT deficiency, we analyzed the biological effects of CACT down-expression in an in vitro model, i.e., in cultured human primary myoblasts stably transfected with full-length CACT antisense cDNA (Pande and Murthy, 1994; Huizing et al., 1998). Normally CACT, which has a very high affinity for long-chain acylcarnitines, regulates the acylcarnitine concentration within the mitochondrial inter-membrane space. As consequence, in our model characterized by CACT-deficiency and normal CPT 1 activity, there is an

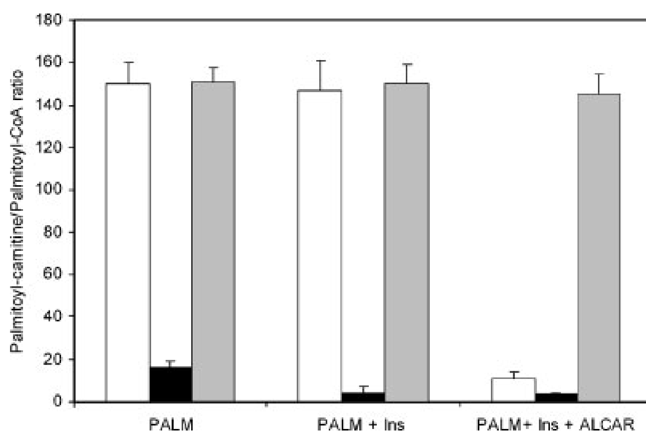

Fig. 5. Effect of acetyl-carnitine on palmitoyl-carnitine/palmitoyl-CoA ratio. A1 (□), A1i (■) and ACC-β AS-infected A1 (▒) cell clones were preincubated with 0.1 mM [U-^{13}C]palmitic acid bound to BSA, in presence or absence of insulin (100 ng/ml) ±acetyl-carnitine (0.5 mM). The concentrations of palmitoyl-carnitine and palmitoyl-CoA were analyzed as described under "Materials and Methods." Data are presented as mean $\pm$ SD of three independent cell cultures. Similar results were obtained using the cell clones A6 and A7.

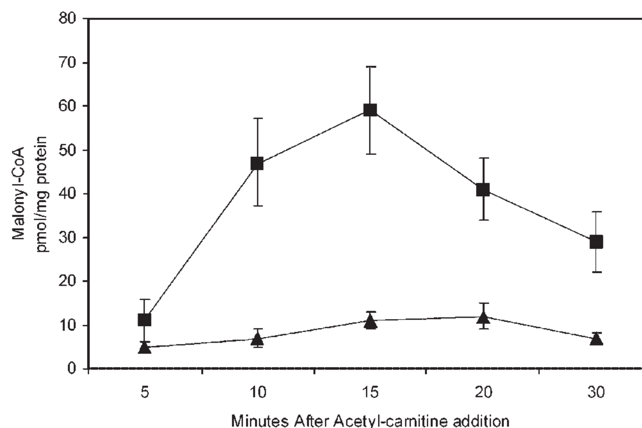

Fig. 6. Time course of acetyl-carnitine induced changes in malonyl-CoA level. Malonyl-CoA content was evaluated using stably antisense CACT-transfected A1 clone (■) and ACC-β AS infected A1 clone (▲). Cell extracts were prepared from myocytes following different periods of treatment with 0.5 mM [1,2-$^{13}C_2$]acetyl-carnitine. A 250 μl aliquot of sample extract, representing approximately 6 mg of cell protein, was analyzed for malonyl-CoA level as described under "Materials and Methods." The results represent the mean $\pm$ standard errors for six separate experiments. Similar results were obtained for clones A6 and A7.

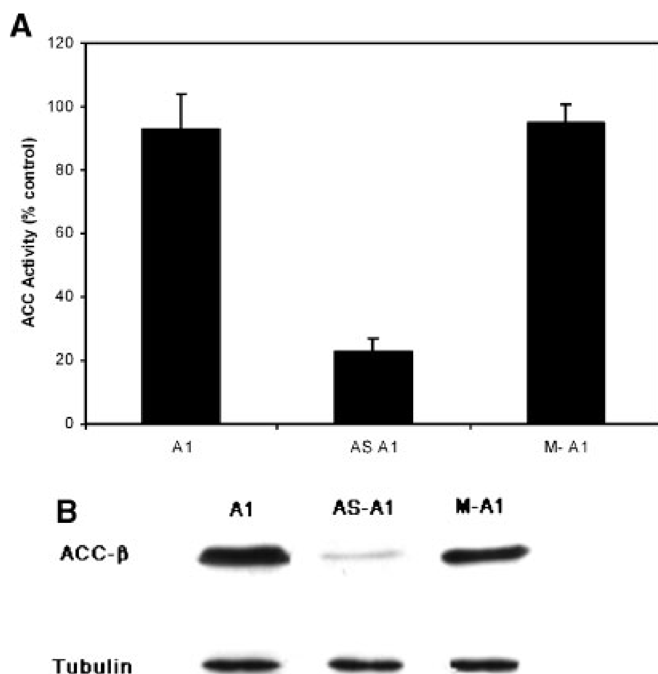

Fig. 7. Acetyl-CoA carboxylase activity in cell extracts. **A**: Mitochondrial fractions were prepared from A1, ACC-β AS A1 and mock-infected A1 clones and acetyl-CoA carboxylase activity was measured by analytical reverse phase HPLC as described under "Materials and Methods." Similar results were obtained using the cell clones A6 and A7. **B**: The mitochondrial fractions used to measure acetyl-CoA carboxylase activity were used to examine acetyl-CoA carboxylase protein levels. One-hundred micrograms of each extract was subjected to immunoblotting using specific polyclonal antibodies against ACC-β, with tubulin used as a control protein species. A1, antisense CACT-transfected clone; A1-AS, ACC-β AS-infected A1 cells; M-A1, mock-infected (with empty viral vector) A1 cells.

increased concentration of long-chain acyl-esters, a condition that we confirm has a deleterious effect on the residual CACT activity (Baillet et al., 2000). It is not clear how acylcarnitines might regulate CACT activity. However, it is well known that acyl moieties are membrane-active molecules that modulate mitochondrial function by means of their detergent properties and/or specific interactions with inner-membrane proteins.

While it is relatively easy to correlate an impaired β-oxidation with CACT down-expression, it is difficult to fathom the other biological effects of CACT deficiency. For example, the effect of insulin seems paradoxical since the increase of palmitoyl-carnitine, shown in our experimental model, ought to be inhibited by malonyl-CoA, the synthesis of which is up-regulated by insulin. Thus, one might expect exacerbation of the insulin effect on fatty acylcarnitine-to-fatty acyl-CoA ratio rather than its depression. But, under conditions of abolished CACT activity, when cytosolic acetyl-CoA levels drop due to translocase inhibition by acylcarnitines, one would expect a parallel decrease in malonyl-CoA levels that would accelerate palmitoyl-carnitine production without any corresponding increase in β-oxidation. In addition, the availability of acetyl groups in the cytoplasm cannot be supported by other organelles. Normally, acyl-carnitine esters are rapidly exported into the mitochondrial and peroxisomal matrices because of the high CACT expression in the matrix-bounding membranes of these organelles. The reduction of CACT also limits peroxisomal β-oxidation, which results in decreased formation of chain-shortened acyl moieties that can be transported into the various cell compartments depending on the identity of the acyl moiety.

Therefore, modulation of CACT activity has consequences for CPT 1 activity since the altered mitochondrial acetyl-carnitine transferase/acetyl-carnitine translocase system results in decreased concentrations of acetyl-CoA within the cytosolic compartment, which is a conditio sine qua non for the increase in malonyl-CoA levels and CPT 1 inhibition.

Our findings that addition of acetyl-carnitine, by increasing the availability of cytosolic acetyl groups, leads to an increase in malonyl-CoA and restores a substantial response to insulin of myocytes, again support the previous suggestion. Moreover, the acetyl-carnitine effect is significantly decreased in cells in which ACC-β is down-expressed. ACC-β, the predominant species of ACC in heart and skeletal muscle tissues, is a mitochondrial membrane enzyme in close physical proximity to CPT 1 and, for this reason, seems to be involved in the direct CPT 1 inhibition via malonyl-CoA synthesis. Thus, the inhibition of ACC-β expression does not allow the synthesis of malonyl-CoA also in the presence of an increased availability of acetyl groups and cannot modulate CPT 1.

Finally, the increase of malonyl-CoA could shunt fatty acyl-CoA into triglyceride and secondarily diminished lipotoxicity of acyl-carnitines that caused mitochondrial dysfunction and CACT inactivation (Listenberger et al., 2003).

In this optics, the biologic effects of acetyl-carnitine are not associated with a generic supply of energy compounds but to the anaplerotic property of the molecule.

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