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Differential inhibitory effect of long-chain acyl-CoA esters on succinate and glutamate transport into rat liver mitochondria and its possible implications for long-chain fatty acid oxidation defects

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Abstract

Long-chain fatty acid β-oxidation defects are associated with a series of clinical and biochemical abnormalities, including accumulation of long-chain acyl-CoA esters which have been shown to inhibit several enzymes and transport systems that may disturb energy metabolism. Using isolated rat liver mitochondria incubated under state 3 conditions, we observed that long-chain acyl-CoA esters and their β-oxidation intermediates inhibit ATP synthesis and oxygen consumption, both with succinate (plus rotenone) and L-glutamate as respiratory substrates. When an uncoupler (2,4-dinitrophenol) was used instead of ADP, to stimulate respiration maximally, the various CoA esters showed differential effects on the oxidation of succinate and L-glutamate, respectively. With succinate as substrate, there was a strong inhibition of oxygen consumption by palmitoyl-CoA, 2,3-unsaturated, 3-hydroxy, and 3-keto-palmitoyl-CoA, in coupled as well as uncoupled mitochondria. On the other hand, with L-glutamate as substrate, inhibition was only observed under coupled conditions. The finding that acyl-CoA esters inhibit the uncoupler-induced respiration with succinate as substrate but not with glutamate, indicates that the observed inhibitory effect is most probably at the level of the transport of succinate across the mitochondrial membrane as mediated by the mitochondrial dicarboxylate carrier. This conclusion was substantiated by mitochondrial swelling studies, which showed inhibition of succinate transport by the different CoA esters whereas no effect was observed on the phosphate/hydroxyl and glutamate/hydroxyl carriers. Furthermore, long-chain acyl-CoA esters were found to potentiate the inhibitory effect of *N*-butylmalonate, a known inhibitor of the dicarboxylate carrier, upon oxygen consumption driven by succinate (plus rotenone). We conclude that the inhibitory effects of long-chain acyl-CoA esters on oxidative phosphorylation are dependent on the type of substrate used with the ATP/ADP carrier and the dicarboxylate carrier as targets for inhibition.

Keywords: Dicarboxylate carrier; Glutamate carrier; Long-chain acyl-CoA esters; Mitochondrial fatty acid β-oxidation; Mitochondrial long-chain fatty acid β-oxidation deficiencies; ATP/ADP carrier; Lactic acidemia; Hypoglycemia; Oxidative phosphorylation

Introduction

Long-chain fatty acids constitute a main source of energy in man, especially under conditions of stress or prolonged fasting [1,2]. Oxidation of these fatty acids via mitochondrial β-oxidation involves the participation of a number of enzymes with the production of reducing equivalents ($FADH_2$ and NADH) that are then reoxidized in the respiratory chain [1,2]. Accordingly, mitochondrial β-oxidation and oxidative phosphorylation are tightly coupled processes.

In the past few years, an increasing number of inherited metabolic diseases affecting fatty acid

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metabolism have been reported [2,3]. Patients with a defect in long-chain fatty acid β-oxidation are much more severely affected as compared to patients with a defect in medium-chain fatty acid oxidation as in medium-chain acyl-CoA dehydrogenase [3]. Different organs and tissues such as heart, muscle, and liver, may be acutely affected in their vital functions [4–7] and death may occur even if the patients are diagnosed early in life and treatment initiated promptly [8].

In case of a certain enzyme deficiency in the mitochondrial β-oxidation system, there is accumulation of the fatty acyl-CoA intermediate immediately proximal to the site of inhibition [3,9–11]. Long-chain acyl-CoA esters are well known to inhibit a variety of important enzymes including transport systems such as the mitochondrial ATP/ADP carrier (see [12,13] for review) [14]. In patients with a defect in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD, EC 1.1.1.211, OMIM 600890) and mitochondrial trifunctional protein (MTP, OMIM 143450), lactic acid acidemia is a frequent finding, with levels as high as 28 mmol/L (normal values <2.2 mmol/L) [4,15–28]. The study of the interaction between acyl-CoAs and the mitochondrial oxidative phosphorylation system is therefore of utmost importance in order to elucidate the underlying mechanisms responsible for the pathogenesis of this group of disorders.

Using selectively permeabilized fibroblasts and succinate as a respiratory substrate, it was shown by us that palmitoyl-CoA, as well as 3-hydroxy-palmitoyl-CoA and 3-keto-palmitoyl-CoA, inhibit ATP synthesis [29,30]. The data obtained suggested that either the phosphorylation system, including the ATP/ADP carrier, F_1F_0-ATP synthase, and phosphate/hydroxyl carrier, or the succinate oxidation system per se, were affected in the presence of those long-chain acyl-CoA esters.

To clarify this issue, the present study was carried out using rat liver mitochondria, as experimental material since rat liver mitochondria enable more reliable measurements of both oxygen consumption and oxidative ATP-synthesis. The results described in the present study are in agreement with the idea that long-chain acyl-CoAs exert a combined inhibitory effect at the oxidative phosphorylation pathway. Moreover, the conducted studies allowed us to conclude that these compounds only affect the dicarboxylate carrier and not the glutamate and phosphate carriers. Taken together, our data may provide an explanation for the apparent association between the mitochondrial β-oxidation system and oxidative phosphorylation as observed in patients with a defect in long-chain fatty acid oxidation. Indeed, in these patients pyruvate is preferentially converted into lactate, rather than being oxidatively degraded to CO_2 and H_2O.

Materials and methods

Materials

Ethyleneglycol-bis (β-aminoethyl ether)-*N*,*N*,*N*,*N*′-tetraacetic acid (EGTA), 2-[2 -*N*-morpholino]ethanesulphonic acid (Mes), 3-(*N*-morpholino)propane sulphonic acid (Mops), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), bicinchoninic acid (BCA), bovine serum albumin (BSA) (essentially fatty acid free), oxaloacetic acid, acetyl-CoA, succinic acid, L-glutamic acid, rotenone, antimycine, *N*-ethylmaleimide (NEM), 2,4-dinitrophenol (DNP), mersalyl, and *N*-butylmalonic acid were all obtained from Sigma (St. Louis, USA). Palmitoyl-coenzyme A, adenosine 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP) were obtained from Boehringer (Mannheim, Germany). The 2,3-unsaturated palmitoyl-coenzyme A, 3-hydroxy and 3-keto-palmitoyl-coenzyme A were synthesized enzymatically and purified by semipreparative HPLC using a C18-Reverse phase column, as previously described [31]. All other chemicals were of analytical grade from commercial sources.

Isolation of mitochondria

Rat liver mitochondria were isolated from overnight fasted adult male Wistar rats (250–350 g). After decapitation, the livers were immediately removed and rinsed in ice-cold isolation medium containing: 250 mM mannitol, 5 mM Tris–HCl, and 0.5 mM EGTA (final pH 7.4). The livers were then minced and thoroughly washed before being carefully homogenized with the same isolation medium in a Teflon pestle glass homogenizer maintained in an ice-bath during the whole procedure. The homogenates obtained were centrifuged at 600*g* (10 min, 4 °C). The first supernatant was further centrifuged at 3600*g* (10 min, 4 °C). The resulting pellet was gently resuspended in the isolation medium and recentrifuged at 2700*g* (10 min, 4 °C). The final pellet was taken up in the isolation medium and kept on ice. Mitochondrial protein content was determined by the bicinchoninic acid assay [32] using BSA as a standard. The studies presented in this study were performed only with freshly isolated and intact mitochondria.

Citrate synthase latency measurements in isolated rat liver mitochondria

All the experiments described here were carried out after checking the integrity of the mitochondrial membrane. This was done by measuring the activity of citrate synthase in the absence and presence of Triton X-100 using the DTNB-based assay described in literature [33]. In a 1 cm^3 glass cuvette, isolated rat liver mitochondria (final protein concentration 2–4 μg/ml) were incubated in isolation medium (described above) supplemented with

0.1 mM DTNB (stock solution in 1 M Tris–HCl, pH 8.0), 0.2 mM oxaloacetic acid, and 0.1 mM acetyl-CoA. The oxaloacetate-dependent increase in absorbance at 412 nm (A_{412}) due to the formation of the DTNB–CoASH adduct was followed for 3 min from which the citrate synthase activity could be calculated (value *A*). Triton X-100 at a concentration of 0.1% (w/v) was added to disrupt the inner mitochondrial membrane thereby eliciting full activity of citrate synthase and the increase in A_{412} was followed as above. This value was taken as 100% citrate synthase activity (value *B*). The two values for citrate synthase were taken to calculate latency as follows: $[(B-A)/B] \times 100\%$. Only mitochondria with a citrate synthase latency of 90% or more were used for the experiments described.

Measurement of ATP synthesis in isolated rat liver mitochondria

ATP synthesis was measured in isolated rat liver mitochondria essentially as described before [34]. To this end, mitochondria were incubated at a final concentration of 0.4 mg/ml in a standard medium containing isolation medium (described above), 10 mM potassium phosphate buffer (pH 7.4), 25 mM Tris–HCl (pH 7.4), 2 mM EDTA (pH 7.4), 0.1% BSA, and 10 mM succinate (plus 20 μg/ml rotenone) or 10 mM L-glutamate as respiratory substrates. Palmitoyl-CoA, 2,3-unsaturated, 3-hydroxy, and 3-keto-palmitoyl-CoA were added to the reaction medium from stock solutions prepared in 20 mM Mes (pH 6.0) at final concentrations ranging 5–100 μM. Controls (absence of acyl-CoA esters) were also included in the experiments' protocol.

Reactions were started by the addition of 5 mM ADP, allowed to proceed for 20 min at 25 °C and terminated by adding perchloric acid (PCA, final concentration 0.6 N). Samples were kept on ice for at least 15 min, centrifuged at 10,000*g* (6 min, 4 °C) and the supernatants neutralized to pH 6–7 with 2 M KOH/0.6 M Mops. The ATP produced was measured fluorimetrically, in the deproteinized neutralized samples, according to standard procedures [35].

Oxygen consumption studies using isolated rat liver mitochondria

To check the effect of palmitoyl-CoA, 2,3-unsaturated, 3-hydroxy, and 3-keto-palmitoyl-CoA upon substrate oxidation per se, isolated rat liver mitochondria (0.4–0.6 mg protein/ml) were incubated in a 500 μl vessel equipped with a Clark oxygen electrode, at 30 °C, in a standard medium containing isolation medium (described above), 10 mM potassium phosphate buffer (pH 7.4), 0.1% BSA, and 10 mM succinate (plus 1 μg/ml rotenone) or 10 mM L-glutamate as respiratory substrates. After an equilibration period in the presence of the substrate, maximal respiration was induced either by adding an excess of ADP (1 mM) or DNP (75 μM). Palmitoyl-CoA, 2,3-unsaturated, 3-hydroxy, and 3-keto-palmitoyl-CoA were added to the oxygraph vessel at a final concentration range of 5–100 μM, from stock solutions in 20 mM Mes (pH 6.0) and oxygen consumption was followed continuously. Controls (absence of acyl-CoA esters) were also included in the experiments' protocol. At the beginning of all the experiments, state 3, state 4 and respiratory control ratio (RCR) were evaluated, essentially as described by Estabrook [36], and only mitochondria with a RCR above 3 were used.

Mitochondrial swelling studies

Isolated rat liver mitochondria (0.25 mg/ml) were suspended (final volume 1 ml) in a standard medium containing 125 mM of the different ammonium salts plus 20 mM Tris–HCl (pH 7.4), 1 mM EGTA (pH 7.4) (to avoid spontaneous swelling), 0.1% BSA plus 1 μg/ml rotenone and 4 μg/ml antimycine (to block respiratory chain activity). The different ammonium salts used included: ammonium succinate, ammonium phosphate, and ammonium glutamate. In case of swelling studies in ammonium succinate, 4 mM ammonium phosphate was added as well. The swelling of mitochondria was monitored for 5 min by following the decrease in absorbance at 540 nm, at room temperature. Palmitoyl-CoA, 2,3-unsaturated, 3-hydroxy, and 3-keto-palmitoyl-CoA were added to the standard media at a final concentration of 30 μM from stock solutions in 20 mM Mes (pH 6.0). Controls (absence of acyl-CoA esters) were also included in the experiments' protocol.

The effect of the CoA esters on the carriers was compared with the effect of known inhibitors of these transport systems: 300 μM NEM (inhibitor of the dicarboxylate, phosphate/hydroxyl, and glutamate/hydroxyl carriers), 100 μM mersalyl (inhibitor of the dicarboxylate and phosphate/hydroxyl carriers), and 400 μM *N*-butylmalonate (specific competitive inhibitor of the dicarboxylate carrier) [37,38].

Study of the inhibitory effect of N-butylmalonate on the dicarboxylate carrier in the absence and presence of palmitoyl-CoA and β-oxidation intermediates

The inhibitory effect of *N*-butylmalonate and the different acyl-CoA esters on the dicarboxylate carrier was also studied polarographically. To this end, rat liver mitochondria (0.4 mg protein/ml) were incubated in a 500 μl vessel equipped with a Clark oxygen electrode at 25 °C in the standard medium containing isolation medium (described above), 10 mM potassium phosphate buffer (pH 7.4), 50 mM Tris–HCl (pH 7.4), 100 mM KCl, 1 mM EGTA (pH 7.4), 0.1% BSA, and 10 mM succinate plus 2 μg/ml

rotenone. After an equilibration period, the different acyl-CoA esters were added followed by DNP (60 μM) in a concentration sufficient to achieve 80% of maximal respiration (see legend of Fig. 4). In control experiments (absence of acyl-CoA esters), 40 μM DNP was sufficient to attain 80% of maximal respiration. Finally, and after reaching again equilibrium, *N*-butylmalonate was added to the oxygraph in a final concentration range of 50–1200 μM.

Results

Effect of acyl-CoA esters on ATP synthesis and oxygen uptake in rat liver mitochondria

The effect of palmitoyl-CoA, 2,3-unsaturated, 3-hydroxy, and 3-keto-palmitoyl-CoA, at a final concentration range of 5–100 μM, on the synthesis of ATP from ADP and phosphate, in isolated rat liver mitochondria, is displayed in Figs. 1A–D, respectively. The results show an identical pattern of inhibition of ATP synthesis driven by succinate (in the presence of rotenone) as earlier observed by us using digitonin-permeabilized human fibroblasts [29,30]. When succinate (plus rotenone) was replaced by L-glutamate, as a respiratory substrate (Figs. 1A–D), a different pattern was found with significantly less inhibition with L-glutamate at concentrations of the acyl-CoA esters studied above 15–30 μM.

Since isolated rat liver mitochondria enable us to study the oxidation process per se by measuring oxygen consumption, oxygen consumption studies were performed using either succinate (with rotenone) or L-glutamate (Figs. 2A and B) as substrates. A similar effect was exerted by all the different long-chain acyl-CoA esters studied displaying Figs. 2A and B a typical profile obtained with 3-hydroxy-palmitoyl-CoA. In the first set of experiments, the effect of the different acyl-CoA esters was tested under state 3 conditions, when mitochondria synthesize ATP at the highest rate. Fig. 2A shows that oxygen consumption is progressively inhibited by the acyl-CoA ester independent whether succinate (plus rotenone) or L-glutamate were used as respiratory substrates.

However, when the same set of experiments was done under uncoupled conditions using 75 μM DNP rather than

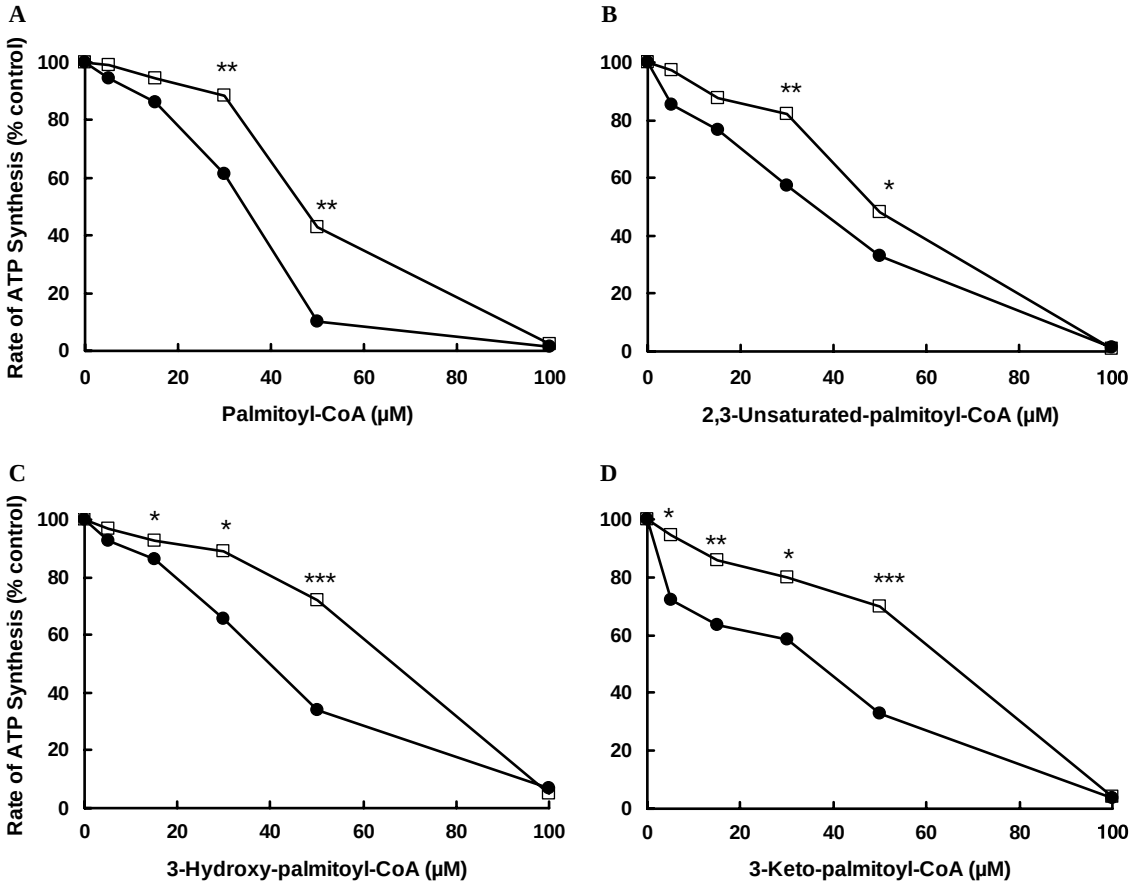

Fig. 1. ATP synthesis in isolated rat liver mitochondria in the presence of long-chain acyl-CoA esters. ATP synthesis in isolated rat liver mitochondria (0.4 mg/ml) was studied in the presence of palmitoyl-CoA (A), 2,3-unsaturated palmitoyl-CoA (B), 3-hydroxy-palmitoyl-CoA (C), and 3-keto-palmitoyl-CoA (D) using, as respiratory substrates, 10 mM succinate (plus 20 μg/ml rotenone) (-●-) ($n = 2$) or 10 mM L-glutamate (-□-) ($n = 3$). Reactions were allowed to proceed at 25 °C for 20 min in a standard medium as described in Materials and methods followed by measurement of ATP in the neutralized, protein-free supernatants. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

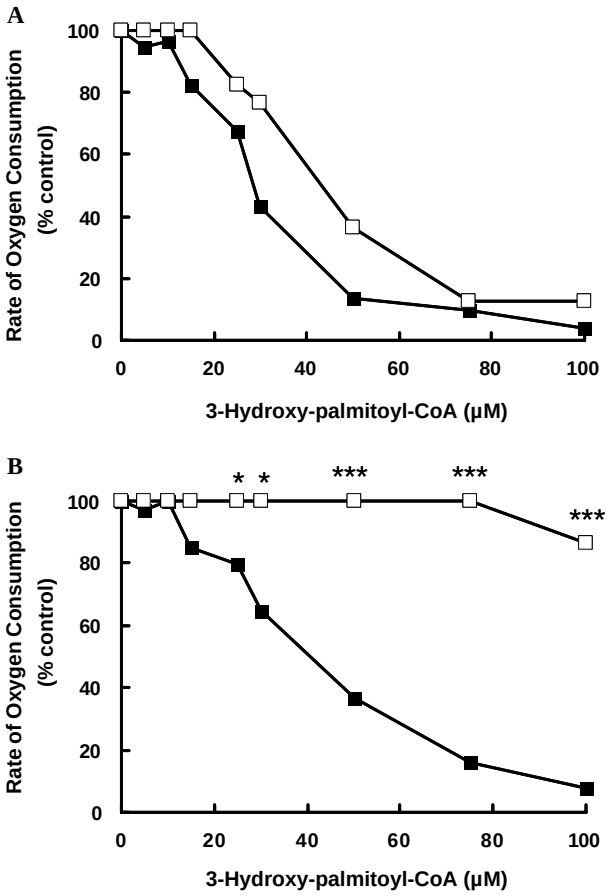

Fig. 2. Oxygen consumption driven by succinate and glutamate in isolated rat liver mitochondria in the presence of 3-hydroxy-palmitoyl-CoA. Effect of 3-hydroxy-palmitoyl-CoA on the rate of oxygen consumption in isolated rat liver mitochondria (0.4–0.6 mg/ml) using 10 mM succinate (plus 1 μg/ml rotenone) (-■-) or 10 mM glutamate (-□-) as respiratory substrates. Two set of experiments ($n = 2$) were performed in which either 1 mM ADP (A) or 75 μM DNP (B) were added to achieve maximal respiration. Mitochondrial oxygen consumption was followed continuously using a Clark-type electrode (see Materials and methods). A similar effect was exerted by palmitoyl-CoA, 2,3-unsaturated, and 3-ketopalmitoyl-CoA. $^{*}P < 0.05$; $^{***}P < 0.001$.

ADP, to stimulate respiration maximally, a completely different profile was originated (Fig. 2B). Indeed, with succinate (plus rotenone) the different acyl-CoA esters were found to inhibit respiration. With this substrate the acyl-CoA concentrations required to achieve 50% inhibition were found to be very close to those observed in Figs. 1A–D. Remarkably, with L-glutamate as substrate there was hardly any effect of the acyl-CoA esters on oxygen consumption up to concentrations of 75 μM.

Swelling studies

The finding that the acyl-CoA esters inhibit oxygen consumption under uncoupled conditions with succinate as substrate but not with L-glutamate, suggested an effect on the uptake of succinate into the mitochondria, which occurs via the dicarboxylate carrier. This was tested by means of swelling experiments, a classical technique which contributed greatly to the identification of the mitochondrial transport systems (see [37] for review). To this end, isolated rat liver mitochondria were incubated at room temperature in isoosmotic solutions of ammonium succinate (125 mM) plus ammonium phosphate (4 mM). In such a medium, mitochondria swell due to the efflux of succinate in exchange for phosphate, which permeates via the phosphate/hydroxyl carrier followed by the influx of ammonia (NH_3) [38]. Swelling of mitochondria was monitored by following the absorbance at 540 nm.

The effect of palmitoyl-CoA and its β-oxidation intermediates on the dicarboxylate carrier was compared with that of specific inhibitors of this transporter, including: (a) *N*-butylmalonate, a competitive inhibitor of the dicarboxylate carrier, (b) mersalyl, which inhibits both the dicarboxylate and phosphate/hydroxyl carriers, and (c) NEM, with inhibitory effects on the transport of phosphate either via the phosphate/hydroxyl or the dicarboxylate carriers [37,38]. The results summarized in Fig. 3A show that all four acyl-CoA esters tested at a concentration of 30 μM were inhibitory. The inhibition observed was only partial when compared with *N*-butylmalonate. In contrast, NEM and mersalyl produced complete inhibition.

To establish whether the effects observed in Fig. 3A were specific for the dicarboxylate carrier, we repeated the experiment with 125 mM ammonium phosphate (Fig. 3B) and 125 mM ammonium glutamate (Fig. 3C) as suspending media. Mitochondrial swelling in these media reflects the activity of the phosphate/hydroxyl and glutamate/hydroxyl carriers, respectively. Figs. 3B and C show that palmitoyl-CoA, 2,3-unsaturated, 3-hydroxy, and 3-keto-palmitoyl-CoA at a concentration of 30 μM had no effect on the rate of mitochondrial swelling under these conditions. NEM and mersalyl (Figs. 3B and C) were found to be inhibitory, in accordance with previously published data [37,38].

Acyl-CoA esters potentiate the inhibitory effect of N-butylmalonate on the dicarboxylate carrier

If the different acyl-CoA esters indeed exert a direct inhibitory effect on the dicarboxylate carrier, one would expect that these acyl-CoA esters potentiate the inhibitory action of *N*-butylmalonate on the transport of succinate. This was investigated by performing oxygen consumption studies (Fig. 4), whereby isolated rat liver mitochondria were incubated under uncoupled conditions in the absence (control) and presence of each of the different acyl-CoA esters added at different concentrations to obtain, at the starting point, similar inhibitory effect (80% inhibition) (see legend to Fig. 4). Subsequently, *N*-butylmalonate was added at increasing concentrations. The results of Fig. 4 show a much more

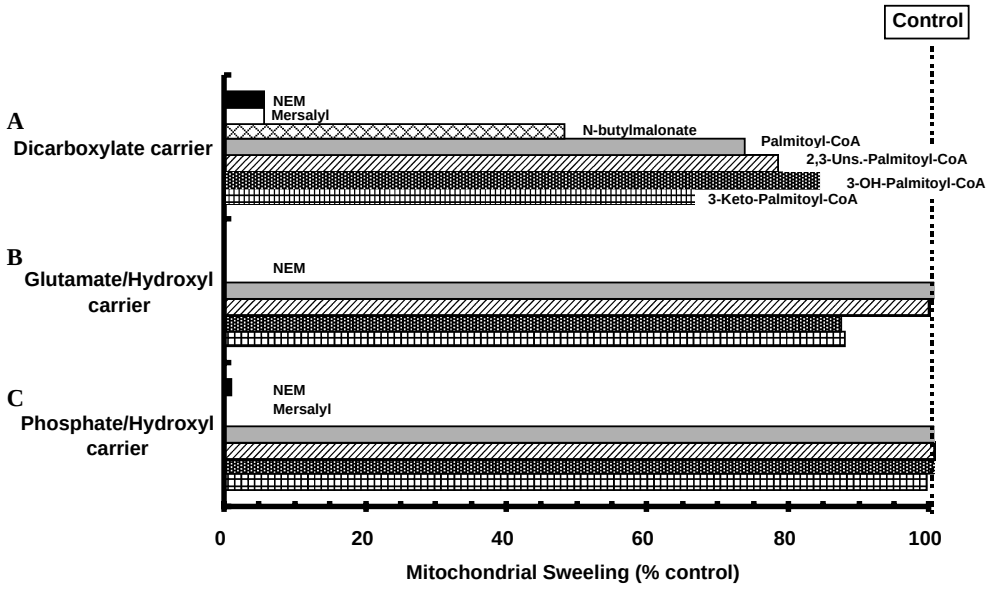

Fig. 3. Study of the dicarboxylate (A), phosphate/hydroxyl (B), and glutamate/hydroxyl (C) carriers. The study was performed by following the decrease in absorbance at room temperature for 5 min at 540 nm corresponding to the swelling of mitochondria (0.25 mg protein/ml) suspended in a standard medium (see Materials and methods for details) plus 125 mM ammonium succinate and 4 mM ammonium phosphate (A), 125 mM ammonium phosphate (B), and 125 mM ammonium glutamate (C). Final volume was 1 ml. The effect of 30 μM palmitoyl-CoA, 2,3-unsaturated palmitoyl-CoA, 3-hydroxy-palmitoyl-CoA, and 3-keto-palmitoyl-CoA on mitochondrial swelling was studied and compared with 300 μM NEM (A–C), 100 μM mersalyl (A and C), and 400 μM *N*-butylmalonate (A), inhibitors of the mitochondrial dicarboxylate (NEM, mersalyl, *N*-butylmalonate), phosphate/hydroxyl (NEM, mersalyl), and glutamate/hydroxyl (NEM) transporters. The data shown here represent a typical experiment. Similar results were obtained in another experiment.

pronounced inhibitory effect of *N*-butylmalonate in the presence of the different acyl-CoA esters.

Discussion

The results reported in this study, using isolated rat liver mitochondria, show that long-chain acyl-CoA esters exert the same inhibitory effect on ATP synthesis with succinate as substrate as previously observed in permeabilized fibroblasts [29,30]. Importantly, oxygen consumption was also inhibited by acyl-CoA esters in the same fashion. When an uncoupler (DNP) rather than ADP was used to induce maximal rates of respiration, the different long-chain acyl-CoA esters showed a similar inhibitory behavior, suggesting that the effect of the acyl-CoA esters was primarily at the level of the oxidation system including succinate transport, succinate dehydrogenase, and the rest of the respiratory chain (complexes III and IV) with much less effect on the phosphorylation part.

Since all the experiments were performed in the absence of L-carnitine, thereby prohibiting acyl-CoA ester transport across the mitochondrial membrane, the most likely site of inhibition would be the dicarboxylate carrier. This hypothesis was substantiated by the swelling experiments of Fig. 3A and the *N*-butylmalonate experiments of Fig. 4. The results found by us are therefore not in agreement with the work of Ciapaite et al. [14] who concluded that palmitoyl-CoA does not affect

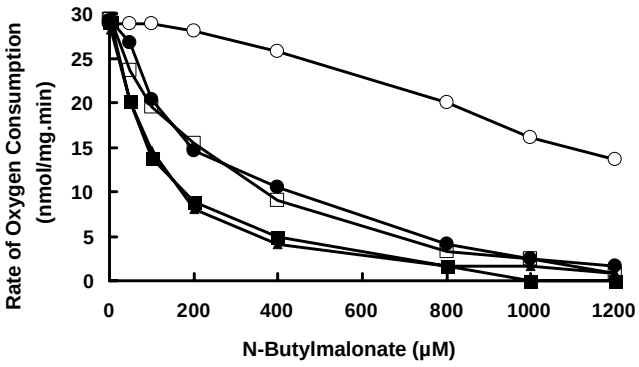

Fig. 4. Inhibition of the dicarboxylate carrier by its competitive inhibitor *N*-butylmalonate. The study was performed in the absence (control) (-○-) and presence of 15.0 μM palmitoyl-CoA (-●-), 16.2 μM 2,3-unsaturated palmitoyl-CoA (-▲-), 17.5 μM 3-hydroxy-palmitoyl-CoA (-■-), and 28.8 μM 3-keto-palmitoyl-CoA (-□-). Reactions ($n = 2$) were allowed to proceed in a Clark electrode vessel (500 μl) at 25 °C using the standard medium (described in Materials and methods) plus 10 mM succinate and 2 μg/ml rotenone. In all experiments, respiration was adjusted to 80% of maximal rate by either adding DNP alone (40 μM; control) or by adding each of the acyl-CoA esters at the concentrations given above plus DNP (60 μM).

ATP synthesis, the respiratory chain and the transport of succinate.

With L-glutamate as substrate, different results were found (Figs. 1A–D, 2A and B, and 3C). Indeed, with this substrate the different acyl-CoA esters did inhibit ADP-stimulated respiration and ATP synthesis whereas

respiration was almost not affected when an uncoupler was used to stimulate oxygen consumption. These data suggest that under conditions of L-glutamate oxidation the oxidation system per se which includes the glutamate/aspartate translocator, mitochondrial glutamate oxaloacetate aminotransferase, 2-ketoglutarate dehydrogenase, succinyl-CoA thiokinase, succinate dehydrogenase, fumarase, malate dehydrogenase, and complexes I–IV of the respiratory chain, are not affected by the different acyl-CoA esters.

The data described in this study strongly suggest that the inhibition of ATP synthesis observed with L-glutamate as substrate is due to the inhibitory action of these acyl-CoAs on the phosphorylation system with the ATP/ADP carrier as the most likely site of inhibitory action, as already suggested for straight long-chain acyl-CoA esters [12–14,39].

The observed inhibitory effects cannot be attributed to detergent properties of the different acyl-CoA esters since we have previously shown in human fibroblasts that at palmitoyl-CoA concentration necessary to attain half maximal inhibition of the oxidative phosphorylation (30–40 μM) the inner mitochondrial membrane is still intact [29]. Morel et al. [39] suggested that the effect of long-chain acyl-CoAs on mitochondrial carriers is probably due to the hydrophobic interaction between the acyl moiety of the acyl-CoAs and the carrier, since free CoA has been shown not to have an inhibitory effect on substrate transport [40]. The characteristic hydrophobicity of each carrier would thus determine, at least in part, the efficiency of its interaction with the acyl-CoA [39]. One can speculate that this may be the reason for the differential effect observed by the acyl-CoA esters studied on succinate and glutamate transport.

The significance of the inhibition by acyl-CoA esters of the dicarboxylate carrier under in vivo conditions remains to be established, notably in patients with a defect in the oxidation of long-chain fatty acids.

The dicarboxylate carrier is an inner mitochondrial membrane carrier that is responsible for the electroneutral exchange of dicarboxylates, such as malate and succinate, inorganic phosphate, and inorganic sulphur-containing compounds (e.g., sulphite, sulphate, and thiosulphate). Besides being involved in urea synthesis and in sulphur metabolism, the dicarboxylate carrier also plays an essential role in gluconeogenesis as derived for instance from the amino acid alanine [41]. In this case, pyruvate is converted into oxaloacetate within the mitochondrial matrix and further into malate, which is then exported by the dicarboxylate carrier to the cytosol where it is involved in the reduction of NAD^+ and reconverted to oxaloacetate, which then generates phosphoenolpyruvate (PEP) that will be used in the synthesis of glucose. In case of a reduced flux through the dicarboxylate carrier by inhibition by long-chain acyl-CoA intermediates, such as shown by us in the present study, the gluconeogenic pathway may well be compromised. This hypothesis may contribute to explain, at least in part, the hypoglycemia, which occurs as one of the first signs in a fatty acid β-oxidation disorder. Nevertheless, hypoglycemia is also a characteristic feature of MCAD-deficiency in which case there is no reason to believe that flux through the dicarboxylate carrier would be inhibited. It is clear that much remains to be learned about the consequences of an inhibition of the dicarboxylate carrier by acyl-CoAs.

Apart from its inhibitory action on the dicarboxylate carrier, acyl-CoAs also inhibit the ATP/ADP carrier, thereby blocking electron flow through the respiratory chain. This may well block oxidative degradation of pyruvate to CO_2 and H_2O, which shifts pyruvate metabolism away from oxidation to formation of lactate. Such a mechanism may well explain the lactic acidaemia observed in mitochondrial long-chain fatty acid β-oxidation defects, as described previously, both in vitro [42] and in vivo [4,15–28].

In conclusion, in case of a mitochondrial long-chain fatty acid β-oxidation deficiency, the accumulation of long-chain acyl-CoA esters and their subsequent inhibitory effect upon the dicarboxylate carrier and the ATP/ADP carrier may add to the exacerbation of the already compromised energy metabolism, due to the defect in the fatty acid oxidation, mainly via the impairment of the gluconeogenesis and consequent occurrence of severe hypoglycemia and lactic acidosis. This new finding may shed some light on the severe and life-threatening pathogenesis of this group of metabolic disorders.

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References

[1] S. Eaton, Control of mitochondrial beta-oxidation flux, Prog. Lipid Res. 41 (2002) 197–239.
[2] R.J. Wanders, P. Vreken, M.E. den Boer, F.A. Wijburg, A.H. van Gennip, L. IJlst, Disorders of mitochondrial fatty acyl-CoA beta-oxidation, J. Inherit. Metab. Dis. 22 (1999) 442–487.
[3] C. Roe, J. Ding, Mitochondrial fatty acid oxidation disorders, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, eighth ed., McGraw-Hill, New York, 2001, pp. 2297–2326.
[4] R. Parini, B. Garavaglia, J.M. Saudubray, P. Bardelli, D. Melotti, G. Zecca, S. Di Donato, Clinical diagnosis of long-chain acyl-coenzyme A-dehydrogenase deficiency: use of stress and fat-loading tests, J. Pediatr. 119 (1991) 77–80.
[5] J.M. Saudubray, G. Mitchell, J.P. Bonnefont, G. Schwartz, C. Nuttin, A. Munnich, M. Brivet, A. Vassault, F. Demaugre, D. Rabier, Approach to the patient with a fatty acid oxidation disorder, Prog. Clin. Biol. Res. 375 (1992) 271–288.

[6] S. Yamaguchi, Y. Indo, P.M. Coates, T. Hashimoto, K. Tanaka, Identification of very-long-chain acyl-CoA dehydrogenase deficiency in three patients previously diagnosed with long-chain acyl-CoA dehydrogenase deficiency, Pediatr. Res. 34 (1993) 111–113.
[7] C. Bertrand, C. Largilliere, M.T. Zabot, M. Mathieu, C. Vianey-Saban, Very long chain acyl-CoA dehydrogenase deficiency: identification of a new inborn error of mitochondrial fatty acid oxidation in fibroblasts, Biochim. Biophys. Acta 1180 (1993) 327–329.
[8] R.J. Pollitt, Disorders of mitochondrial beta-oxidation: prenatal and early postnatal diagnosis and their relevance to Reye's syndrome and sudden infant death, J. Inherit. Metab. Dis. 12 (1989) 215–230.
[9] S. Jackson, R.S. Kler, K. Bartlett, H. Briggs, L.A. Bindoff, M. Pourfarzam, D. Gardner-Medwin, D.M. Turnbull, Combined enzyme defect of mitochondrial fatty acid oxidation, J. Clin. Invest. 90 (1992) 1219–1225.
[10] M. Pourfarzam, J. Schaefer, D.M. Turnbull, K. Bartlett, Analysis of fatty acid oxidation intermediates in cultured fibroblasts to detect mitochondrial oxidation disorders, Clin. Chem. 40 (1994) 2267–2275.
[11] R.J. Pollitt, Disorders of mitochondrial long-chain fatty acid oxidation, J. Inherit. Metab. Dis. 18 (1995) 473–490.
[12] P.V. Vignais, Molecular and physiological aspects of adenine nucleotide transport in mitochondria, Biochim. Biophys. Acta 456 (1976) 1–38.
[13] L. Wojtczak, Effect of long-chain fatty acids and acyl-CoA on mitochondrial permeability, transport, and energy-coupling processes, J. Bioenerg. Biomembr. 8 (1976) 293–311.
[14] J. Ciapaite, G. van Eikenhorst, K. Krab, Application of modular control analysis to inhibition of the adenine nucleotide translocator by palmitoyl-CoA, Mol. Biol. Rep. 29 (2002) 13–16.
[15] E. Bertini, C. Dionisi-Vici, B. Garavaglia, A.B. Burlina, M. Sabatelli, M. Rimoldi, A. Bartuli, G. Sabetta, S. DiDonato, Peripheral sensory-motor polyneuropathy, pigmentary retinopathy, and fatal cardiomyopathy in long-chain 3-hydroxyacylCoA dehydrogenase deficiency, Eur. J. Pediatr. 51 (1992) 121–126.
[16] D.E. Hale, M.J. Bennett, Fatty acid oxidation disorders: a new class of metabolic diseases, J. Pediatr. 121 (1992) 1–11.
[17] M. Duran, L. Bruinvis, D. Ketting, L. Dorland, Diagnosis of mitochondrial fatty acid oxidation defects, Padiatr. Padol. 28 (1993) 19–25.
[18] R. Moore, J.F. Glasgow, M.A. Bingham, J.A. Dodge, R.J. Pollitt, S.E. Olpin, B. Middleton, K. Carpenter, Long-chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency-diagnosis, plasma carnitine fractions and management in a further patient, Eur. J. Pediatr. 152 (1993) 433–436.
[19] W.R. Treem, P. Rinaldo, D.E. Hale, C.A. Stanley, D.S. Millington, J.S. Hyams, S. Jackson, D.M. Turnbull, Acute fatty liver of pregnancy and long-chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency, Hepatology 19 (1994) 339–345.
[20] T. Tyni, A. Majander, H. Kalimo, J. Rapola, H. Pihko, Pathology of skeletal muscle and impaired respiratory chain function in long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency with the G1528C mutation, Neuromuscul. Disord. 6 (1996) 327–337.
[21] T. Tyni, A. Palotie, L. Viinikka, L. Valanne, M.K. Salo, U. von Dobeln, S. Jackson, R. Wanders, N. Venizelos, H. Pihko, Long-chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency with the G1528C mutation: clinical presentation of thirteen patients, J. Pediatr. 130 (1997) 67–76.
[22] J.A. Ibdah, M.J. Dasouki, A.W. Strauss, Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency: variable expressivity of maternal illness during pregnancy and unusual presentation with infantile cholestasis and hypocalcaemia, J. Inherit. Metab. Dis. 22 (1999) 811–814.
[23] J.A. Ibdah, M.J. Bennett, P. Rinaldo, Y. Zhao, B. Gibson, H.F. Sims, A.W. Strauss, A fetal fatty-acid oxidation disorder as a cause of liver disease in pregnant women, N. Engl. J. Med. 340 (1999) 1723–1731.
[24] A. Chakrapani, S. Olpin, M. Cleary, J.H. Walter, J.E. Wraith, G.T. Besley, Trifunctional protein deficiency: three families with significant maternal hepatic dysfunction in pregnancy not associated with E474Q mutation, J. Inherit. Metab. Dis. 23 (2000) 826–834.
[25] U. Spiekerkoetter, B. Sun, Z. Khuchua, M.J. Bennett, A.W. Strauss, Molecular and phenotypic heterogeneity in mitochondrial trifunctional protein deficiency due to beta-subunit mutations, Hum. Mutat. 21 (2003) 598–607.
[26] M.E. den Boer, C. Dionisi-Vici, A. Chakrapani, A.O. van Thuijl, R.J. Wanders, F.A. Wijburg, Mitochondrial trifunctional protein deficiency: a severe fatty acid oxidation disorder with cardiac and neurologic involvement, J. Pediatr. 142 (2003) 684–689.
[27] J.E. Lee, H.R. Yoon, K.H. Paik, S.J. Hwang, J.W. Shim, Y.S. Chang, W.S. Park, A.W. Strauss, D.K. Jin, A case of mitochondrial trifunctional protein deficiency diagnosed by acylcarnitine profile and DNA analysis in a dried blood spot of a 4-day-old boy, J. Inherit. Metab. Dis. 26 (2003) 403–406.
[28] J.E. Ireland, B.A. Heese, A.W. Strauss, V.V. Michels, D. Whiteman, S.H. Hahn, P. Rinaldo, D. Matern, Severe lactic acidosis in the absence of dicarboxylic aciduria in a newborn with mitochondrial trifunctional protein deficiency (MTP), J. Inherit. Metab. Dis. 27 (2004) 107.
[29] F.V. Ventura, J.P.N. Ruiter, L. IJlst, I.T. Almeida, R.J. Wanders, Inhibition of oxidative phosphorylation by palmitoyl-CoA in digitonin permeabilized fibroblasts: implications for long-chain fatty acid beta-oxidation disorders, Biochim. Biophys. Acta 1272 (1995) 14–20.
[30] F.V. Ventura, J.P.N. Ruiter, L. IJlst, I.T. de Almeida, R.J. Wanders, Inhibitory effect of 3-hydroxyacyl-CoAs and other long-chain fatty acid beta-oxidation intermediates on mitochondrial oxidative phosphorylation, J. Inherit. Metab. Dis. 19 (1996) 161–164.
[31] W. Seubert, I. Lamberts, R. Kramer, B. Ohly, On the mechanism of malonyl-CoA-independent fatty acid synthesis. I. The mechanism of elongation of long-chain fatty acids by acetyl-CoA, Biochim. Biophys. Acta 164 (1968) 98–517.
[32] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[33] P.A. Srere, Citrate synthase, Methods Enzymol. 13 (1969) 3–11.
[34] R.J. Wanders, J.P. Ruiter, F.A. Wijburg, Studies on mitochondrial oxidative phosphorylation in permeabilized human skin fibroblasts: application to mitochondrial encephalomyopathies, Biochim. Biophys. Acta 1181 (1993) 219–222.
[35] J.R. Williamson, B.E. Corkey, Assay of citric acid cycle intermediates and related compounds—update with tissue metabolite levels and intracellular distribution, Methods Enzymol. 55 (1979) 200–222.
[36] R.W. Estabrook, Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios, Methods Enzymol. 10 (1967) 41–47.
[37] J.B. Chappell, K.M. Haardoff, The penetration of the mitochondrial membrane by anions and cations, in: E.C. Slater, Z. Kaniuga, L. Wojtczak (Eds.), Biochemistry of Mitochondria, Academic Press/Pollish publishers, London and New York/Warsaw, 1967, pp. 75–91.
[38] K.F. LaNoue, A.C. Schoolwerth, Metabolite transport in mitochondria, Ann. Rev. Biochem. 48 (1979) 871–922.
[39] F. Morel, G. Lauquin, J. Lunardi, J. Duszynski, P.V. Vignais, An appraisal of the functional significance of the inhibitory effect of long chain acyl-CoAs on mitochondrial transports, FEBS Lett. 39 (1974) 133–138.

[40] S.C. Halle-Smith, A.G. Murray, M.J. Selwyn, Palmitoyl-CoA inhibits the mitochondrial inner membrane anion-conducting channel, FEBS Lett. 236 (1988) 155–158.
[41] G. Fiermonte, V. Dolce, R. Arrigoni, M.J. Runswick, J.E. Walker, F. Palmieri, Organization and sequence of the gene for the human mitochondrial dicarboxylate carrier: evolution of the carrier family, Biochem. J. 344 (1999) 953–960.
[42] F.V. Ventura, J.P. Ruiter, L. IJlst, I.T. de Almeida, R.J. Wanders, Lactic acidosis in long-chain fatty acid beta-oxidation disorders, J. Inherit. Metab. Dis. 21 (1998) 645–654.