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Inhibition of adenine nucleotide transport in rat liver mitochondria by long-chain acyl-coenzyme A β-oxidation intermediates

Fátima V. Ventura [a,b,*], Isabel Tavares de Almeida [a], Ronald J.A. Wanders [b,c]

[a] *Centro de Patogénese Molecular – Unidade de Biologia Molecular e Biopatologia Experimental, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal*

[b] *Laboratory of Genetic Metabolic Diseases, Department of Clinical Chemistry, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands*

[c] *Department of Pediatrics (G8-205), Emma Children's Hospital AMC, Academic Medical Centre, Amsterdam, The Netherlands*

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Abstract

Long-chain acyl-coenzyme A esters (LCAC), which may accumulate under different pathological conditions and especially in patients with a mitochondrial fatty acid β-oxidation defect, have long been known as potent inhibitors of several enzymes in multiple metabolic pathways, particularly the oxidative phosphorylation system (OXPHOS). To shed more light on the inhibitory mechanisms of acyl-CoA esters upon energy metabolism, the effect of palmitoyl-CoA and its β-oxidation intermediates on OXPHOS was studied. We have recently shown that, using rat liver mitochondria, LCAC inhibit L-glutamate driven oxygen consumption in the presence of ADP whereas no effect is found when an uncoupler is used to stimulate respiration maximally. A similar inhibitory effect of these compounds is now reported upon the distribution of ATP for intra- and extra-mitochondrial utilization. Taken together these data strongly suggest that the inhibition of ADP-induced respiration with L-glutamate as substrate by LCAC is primarily due to inhibition of the mitochondrial ADP/ATP carrier.

Keywords: Long-chain acyl-CoA esters; Oxidative phosphorylation; ADP/ATP carrier; Fatty acids β-oxidation; Mitochondrial long-chain fatty acids β-oxidation disorders; Inborn errors of metabolism

Mitochondrial fatty acid β-oxidation (FAO) is a crucial metabolic pathway in response to fasting and represents the primary source of energy for high-energy demanding tissues such as the heart. Among the inherited metabolic diseases described over the past few years, the ones affecting the oxidation of long-chain fatty acids have been recognized as severe disorders that may seriously compromise life even when early diagnosed and treated [1,2].

The consequences of a defect in mitochondrial FAO are diverse. First, there is impaired formation of ketone bodies which represent, especially upon fasting, an important origin of energy for several tissues including brain. Second, a block in FAO causes the intramitochondrial accumulation of different forms of acyl-CoA [1]. This will lead not only to a depletion of free coenzyme A, a critical metabolite, but also to a disturbance in multiple metabolic pathways due to the inhibition of one or more enzymes, particularly when there is accumulation of long-chain acyl-CoA esters (LCAC). These compounds have been shown to inhibit the OXPHOS system, which may explain the hyperlacticidemia [3–5] found in patients with a defect in long-chain FAO, suggesting a secondary involvement of pyruvate metabolism [6].

Oxidative phosphorylation driven by a respiratory substrate is a complex process composed of (i) substrate transport and product export, (ii) substrate dehydrogenation, (iii) electron transport through the respiratory chain, and (iv) phosphorylation of ADP and phosphate to ATP,

* Corresponding author. Fax: +351 21 7946491.
E-mail address: fatima.ventura@ff.ul.pt (F.V. Ventura).

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followed by export of ATP in exchange for ADP. In a previous study, using digitonin permeabilized human fibroblasts, our group [7,8] have shown that ATP synthesis driven by succinate (plus rotenone) is progressively inhibited by increasing concentrations of not only palmitoyl-CoA but also its β-oxidation derivatives—2,3-unsaturated-, 3-hydroxy-, and 3-keto-palmitoyl-CoA. Further studies were necessary to elucidate the nature of the inhibitory effect of these compounds. Using isolated rat liver mitochondria (a more preferable model for oxidation studies than fibroblasts due to the low mitochondrial content of these cells), incubated under state 3 conditions, we have recently [9] shown that LCAC and their β-oxidation intermediates inhibit ATP synthesis and oxygen consumption, not only with succinate (plus rotenone) but also with L-glutamate as respiratory substrates. Nevertheless, when an uncoupler was used instead of ADP, to stimulate respiration maximally, LCAC inhibited the uncoupler-induced respiration with succinate but not with glutamate, indicating that the observed inhibitory effect is most probably at the level of the transport of succinate across the mitochondrial membrane through the dicarboxylate carrier. The results obtained with L-glutamate lead us to the work presented here whereby with the same model and through an experimental system based on the use of intra- and extra-mitochondrial ATP utilizing reactions (Fig. 1) we have now found that with this substrate, the acyl-CoA esters primarily exert their effect by inhibiting the ADP/ATP carrier. The specificity of this effect is compared to that of atractyloside, a plant toxin characterized as an amphiphilic ligand and a well-known competitive, non-penetrant, inhibitor of the ADP/ATP carrier [10].

Materials and methods

Materials. Ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*,*N*′-tetraacetic acid (EGTA), 2-[2-*N*-morpholino]ethanesulfonic acid (MES), 3-(*N*-morpholino)propane sulfonic acid (MOPS), bicinchoninic acid (BCA), bovine serum albumin (BSA) (essentially fatty acid free), atractyloside, L-ornithine, L-citrulline, L-glutamic acid, and L-malate, were all obtained from Sigma (St. Louis, USA). Palmitoyl-coenzyme A, hexokinase, adenosine 5′-diphosphate (ADP), and adenosine 5′-triphosphate (ATP) were obtained from Boehringer (Mannheim, Germany). The 2,3-unsaturated-, 3-hydroxy-, and 3-ketopalmitoyl-coenzyme A were synthesized enzymatically and purified by semi-preparative HPLC using a C18-Reverse phase column [11]. All other chemicals were of analytical grade from commercial sources.

Isolation of mitochondria. Rat liver mitochondria were isolated from male Wistar rats (250–350 g) by a method adapted from the work of de Duve [12] using 250 mM mannitol, 5 mM Tris–HCl, and 0.5 mM EGTA (final pH 7.4) as the isolation medium. After homogenization of the liver, the homogenate was centrifuged at 600*g* (10 min, 4 °C) and the supernatant further centrifuged at 3600*g* (10 min, 4 °C). The pellet obtained was carefully 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 until further use. In order to achieve an increased intramitochondrial concentration of *N*-acetylglutamate [13], the essential activator of carbamoyl-phosphate synthetase necessary for citrulline synthesis, the rats used in this work were fed with high-protein diet (60% w/w) for three days, followed by an overnight period of fasting prior to sacrifice.

Protein determination. Protein was determined by the bicinchoninic acid assay [14] using bovine serum albumin (BSA) as a standard.

Testing viability and membrane integrity of isolated rat liver mitochondria. All the experiments described in this paper were performed after checking the viability and membrane integrity of the isolated rat liver mitochondria through measurement of oxygen consumption. Oxygen uptake rates and the coupling state of isolated rat liver mitochondria were measured in a 2 ml Clark oxygen electrode vessel at 25 °C, containing 0.8–1.2 mg protein/ml of mitochondria in a standard medium containing isolation medium (described above), 10 mM potassium phosphate buffer (pH 7.4), 10 mM L-glutamate, and 5 mM L-malate. After an equilibration period, in the presence of substrate, state 3 respiration was initiated by the addition of a limited amount of ADP (0.25 mM). State 4 respiration was calculated from the oxygraph trace after return of the mitochondria to the resting state, which is an indicator for the degree of coupling of the mitochondria and the integrity of the inner membrane. The respiratory control was determined by the ratio between state 3 and state 4 and only mitochondria with a ratio above three were included in the studies described in this paper.

Determination of hexokinase concentration necessary to attain maximum respiration rate. The amount of hexokinase generating maximum respiration rate, in the conditions of the experiments further described, was determined by oxygen consumption studies. Isolated rat liver mitochondria (final concentration 1.5 mg/ml) were incubated, using a 2 ml Clark oxygen electrode vessel, at 25 °C in a standard medium containing: isolation medium (described above), 15 mM KCl, 73.75 mM Tris–HCl (pH 7.4), 20 mM potassium phosphate buffer (pH 7.4), 0.875 mM EGTA, 0.1 mM ATP, 10 mM NH_4Cl, 30 mM glucose, 10 mM ornithine, 16.6 mM $KHCO_3$, 0.1% BSA, 30 mM $MgCl_2$, and 20 mM L-glutamate. After an equilibration period in the presence of the substrates increasing concentrations of hexokinase were added from a stock solution at an approximate concentration of 30 U/ml in 10 mM potassium phosphate buffer (pH 7.4). This hexokinase stock solution was obtained after an overnight period of dialysis at 4 °C in 10 mM potassium phosphate buffer (pH 7.4). Oxygen consumption was followed continuously during hexokinase addition until respiration attained maximum rate.

Study of the effect of palmitoyl-CoA and its β-oxidation derivatives upon ADP/ATP carrier activity. Comparison with atractyloside. To investigate the effect of long-chain acyl-CoA β-oxidation intermediates upon ADP/ATP carrier we devised a system adapted from our earlier work [15] illustrated in Fig. 1. The activity of the ADP/ATP carrier was studied through the analysis of the products of two different ATP-utilizing reactions, an extra-mitochondrial reaction—glucose-6-phosphate formation—and an intramitochondrial reaction—citrulline synthesis. Mitochondria were incubated at a final concentration of 1.5 mg/ml in a standard medium containing: isolation medium (described above), 15 mM KCl, 73.75 mM Tris–HCl (pH 7.4), 20 mM potassium phosphate buffer (pH 7.4), 0.875 mM EGTA, 20 mM L-glutamate, 0.1 mM ATP, 30 mM glucose, 10 mM ornithine, 16.6 mM $KHCO_3$, 0.1% BSA, and finally 30 mM $MgCl_2$. 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 the concentrations indicated in Fig. 3A and B. In this study, the observed effect of the CoAs was compared with that of atractyloside (see Fig. 2 for more details). Reactions were started by the addition of 10 mM of NH_4Cl and 10 s later of hexokinase from a stock solution in 50 mM Tris–HCl (pH 7.4) and allowed to proceed for 30 min at 25 °C. The amount of hexokinase, added to induce maximum activity, corresponded to five times the concentration minimally required to induce respiration maximally (see above). ATP was added to the system at a low concentration (0.1 mM) in order to avoid corrections for adenine nucleotides present in the mitochondrial intermembrane space [15]. Reactions were terminated by adding perchloric acid (final concentration 0.6 N). Samples were stored at 4 °C for at least 15 min, further 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.

Determination of metabolites. Glucose-6-phosphate was measured in the deproteinized and neutralized samples, fluorimetrically, according to standard procedures [16]. Citrulline was measured in the deproteinized and neutralized samples using an aminoacid autosampler analyzer

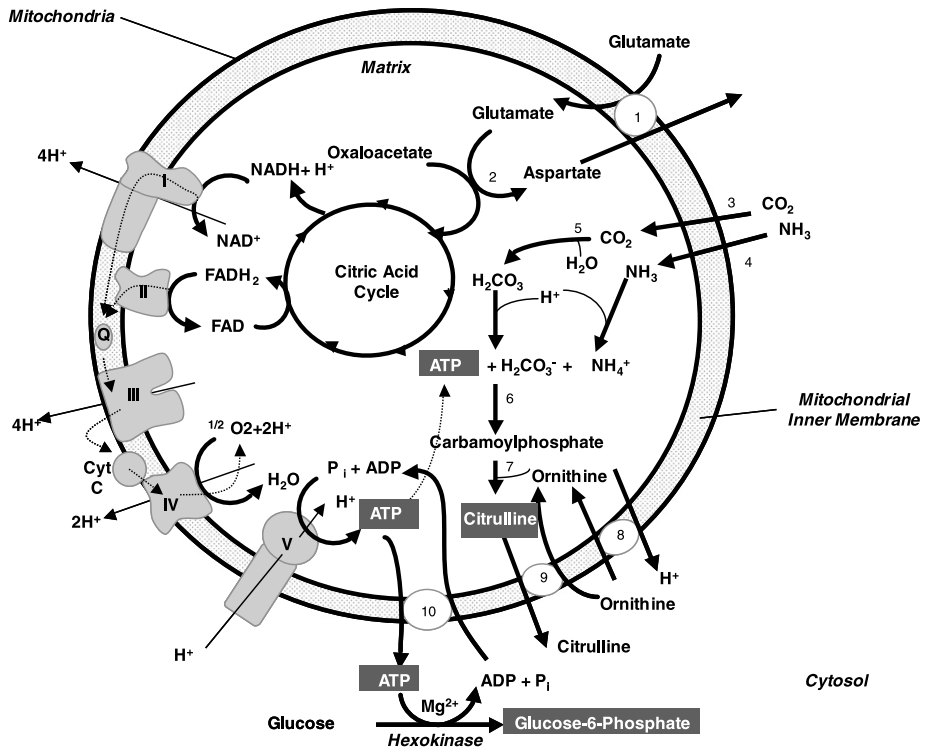

Fig. 1. Schematic representation of the reactions involved in the synthesis of citrulline and glucose 6-phosphate in rat liver mitochondria. Intramitochondrial (citrulline synthesis), extra-mitochondrial (glucose-6-phosphate synthesis) ATP-utilizing reactions and mechanisms associated. (1) Glutamate/aspartate carrier; (2) aspartate aminotransferase; (3) CO_2 transport; (4) ammonia transport; (5) carbonic anhydrase; (6) carbamoyl-phosphate synthetase; (7) ornithine transcarbamoylase; (8) ornithine/H^+ carrier; (9) ornithine/citrulline carrier; and (10) ADP/ATP carrier.

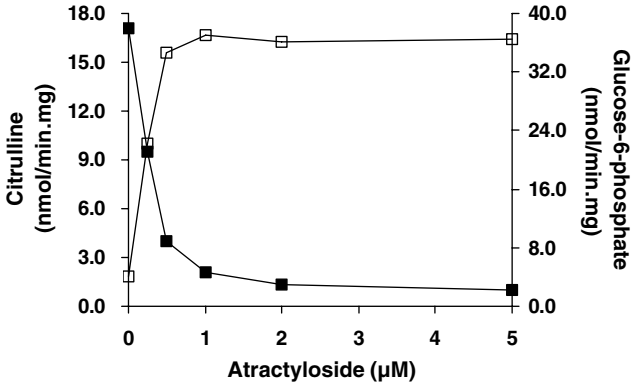

Fig. 2. Citrulline synthesis and glucose-6-phosphate formation as intra- and extra-mitochondrial ATP-utilizing reactions, respectively, with L-glutamate as respiratory substrate in the presence of atractyloside. The data shown here represent a typical experiment. Similar results were obtained in three other experiments. For more details see Materials and methods. Citrulline synthesis (—□—) and glucose-6-phosphate formation (—■—).

according to the conditions for aminoacid analysis routinely performed in our laboratory.

Results

Effect of atractyloside upon ADP/ATP carrier activity

In search for an experimental system to study the effect of long-chain acyl-CoAs (LCAC) upon ADP/ATP carrier, we decided to investigate the effect of these compounds on the distribution of ATP generated by OXPHOS between an intra- and an extra-mitochondrial ATP utilizing reaction. Citrulline synthesis from added ornithine, ammonia, and bicarbonate was selected as intramitochondrial ATP-utilizing reaction whereas formation of glucose-6-phosphate from glucose in the presence of added hexokinase was used as extra-mitochondrial ATP-utilizing reaction. Fig. 1 illustrates the developed system, where the studied reactions, glucose-6-phosphate and citrulline formation, depend on the availability of ATP at the side of the mitochondrial membrane where they occur. To show that this system is indeed appropriate to study the effect of palmitoyl-CoA and its β-oxidation intermediates on the phosphorylating system, we first performed some studies using atractyloside, a selective inhibitor of the ADP/ATP carrier [10,15]. To this end, mitochondria were incubated under state 3 conditions (excess hexokinase) in which the ADP/ATP carrier is working at its maximum activity. As shown in Fig. 2, addition of increasing amounts of atractyloside to the system led to a progressive decrease in the formation of glucose-6-phosphate. In contrast, citrulline formation increased progressively since ATP is now available for the synthesis of citrulline within the mitochondrial matrix. These results show that inhibition of the ADP/ATP carrier leads to a shift in the ratio glucose-6-phosphate/citrulline and are in line with earlier studies which showed that citrulline

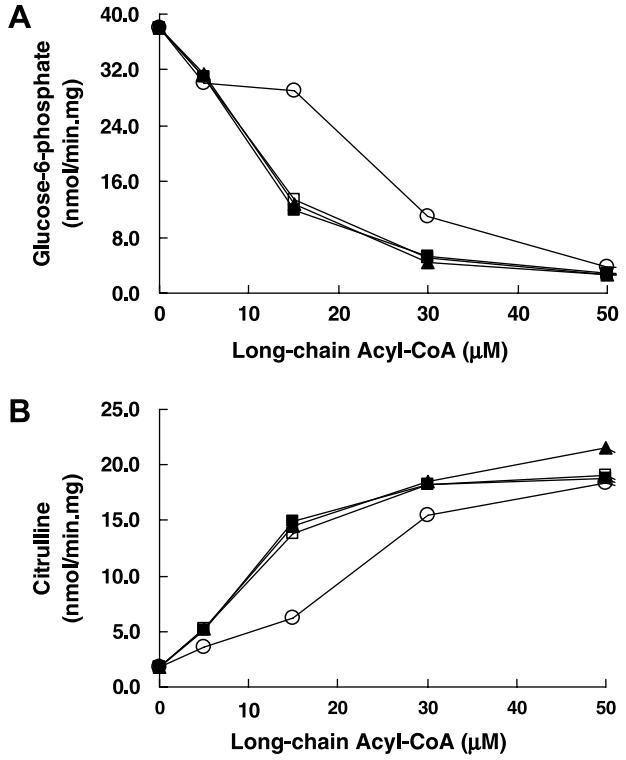

Fig. 3. Glucose-6-phosphate formation (A) as an extra-mitochondrial ATP-utilizing reaction and citrulline production (B) as an intramitochondrial ATP-utilizing reaction with L-glutamate as respiratory substrate in the presence of palmitoyl-CoA (—■—), 2,3-unsaturated palmitoyl-CoA (—□—), 3-hydroxy-palmitoyl-CoA (—▲—), and 3-keto-palmitoyl-CoA (—○—). The data shown here represent a typical experiment. Similar results were obtained in three other experiments. For more details see Materials and methods.

synthesis is much higher under state 4 (−ADP) than under state 3 (+ADP) conditions [15].

Effect of palmitoyl-CoA and its β-oxidation derivatives upon ADP/ATP carrier activity

The inhibition of adenine nucleotides translocation by LCAC and β-oxidation intermediates across mitochondrial membrane was then investigated using the above described system. The addition of increasing concentrations of palmitoyl-CoA, 2,3-unsaturated, 3-hydroxy-, and 3-ketopalmitoyl-CoA strongly inhibit the conversion of glucose into glucose-6-phosphate (Fig. 3A) while the synthesis of citrulline is progressively stimulated (Fig. 3B). The data obtained show that the inhibitory action of the different acyl-CoA esters closely resembles that of atractyloside. Furthermore, the observed effect appears to be equal for all the CoA esters studied with the exception of the intermediate 3-keto, which shows to be less inhibitory.

Discussion

Patients suffering from a FAO disorder show hepoketotic hypoglycemia, regardless of the nature of the enzyme block. In addition, in some mitochondrial FAO deficiencies, notably in long-chain FAO deficiencies [3–5], there is lactic acidosis which points to a compromised pyruvate metabolism. A defect in one of the enzymes of the mitochondrial FAO system is always associated with accumulation of acyl-CoA esters, likely in both the intra- and extra-mitochondrial compartments. The accumulation of long-chain acyl-CoA esters (LCAC) and β-oxidation intermediates [1] is presumably one, if not the major, contributing factor for the severe and life threatening clinical and biochemical picture drawn for long-chain FAO deficient patients, probably correlated with an alteration on the energy metabolism.

Much attention was paid in 1960–70 to the inhibition by palmitoyl-CoA of some cellular enzymes and intramitochondrial transport systems. The particular interest then was the possible role of acyl-CoA-sensitive enzymes in the mechanism regulating lipogenesis [10,17,18]. Our interest today has to do with the pathogenesis of the long-chain FAO disorders [1]. A problem with all these studies is that results have usually been obtained with isolated enzymes under artificial conditions in which the interaction of the enzyme with the rest of the pathway has usually been lost.

Using digitonin permeabilized human control fibroblasts as a model to elucidate the inhibitory effect of acyl-CoA esters upon energy metabolism, we have previously found [7,8] that not only LCAC but also their β-oxidation intermediates are able to strongly inhibit the OXPHOS process. Moreover, we have recently [9] found that in rat liver mitochondria under ADP-induced state 3 respiration, L-glutamate-driven OXPHOS is progressively inhibited by the different LCAC whereas no effect was found in the case of uncoupler-induced respiration. These data suggested that at least with L-glutamate as substrate, LCAC exert their effect by inhibiting the phosphorylating part of OXPHOS, with no effect on the oxidation part per se including the import of L-glutamate into the mitochondrion.

We further decided to investigate whether it is the ADP/ATP carrier, phosphate/hydroxyl carrier or the F_1F_0-ATPase, which is inhibited by palmitoyl-CoA and its β-oxidation derivatives. Mitochondrial swelling studies [9] previously performed, pointed that phosphate/hydroxyl carrier was like the glutamate transport not affected by the action of the compounds in study. The conformational arrangement of ATPase in the inner mitochondrial membrane structure, with the active site facing the matrix, and the fact that no L-carnitine was added to the reaction media from the studies performed, makes the ADP/ATP carrier a higher potential target for the inhibitory action of these LCAC.

The above findings and considerations lead us to the work reported in this paper, where we tried to find some more evidence for the postulated inhibitory effect on the ADP/ATP carrier by LCAC and mainly their β-oxidation intermediates. ADP/ATP carrier is one of the mitochondrial anion transport systems, located in the inner mitochondrial membrane and catalyzing a mole:mole exchange

between intramitochondrial (ATP) and extra-mitochondrial (ADP) adenine nucleotides. It is through this specific mechanism that ATP, generated by OXPHOS, can be transferred into the cytoplasm of the cell to support energy-requiring reactions [19–21]. With the aim of studying the activity of the ADP/ATP carrier we devised a system based on the work of Wanders and colleagues [15], whereby two distinct reactions both utilizing ATP are studied (Fig. 1). The effect of the studied acyl-CoAs upon the extra-mitochondrial formation of glucose-6-phosphate and the intramitochondrial synthesis of citrulline was compared with that of atractyloside. The results obtained clear point to the ADP/ATP carrier as the primary target of the acyl-CoAs (Fig. 3A and B), at least in rat liver mitochondria.

The influence of long-chain acyl-CoA β-oxidation intermediates on the ADP/ATP carrier as reported here, is substantiated by previous studies in which the ADP/ATP carrier was evaluated in different species and tissues in intact cells and isolated mitochondria [18,22–24]. According to these studies free fatty acids and straight LCAC inhibit adenine nucleotides translocation at least *in vitro*. The inhibitory effect of free fatty acids was suggested to occur either directly [25] or indirectly after conversion to the corresponding acyl-CoA esters in the mitochondria [26]. The results described here show that it is not only the acyl-CoA esters, which are inhibitory, but also the corresponding enoyl, 3-hydroxy, and 3-keto acyl-CoAs. Since CoA alone [18], short-chain [7,27] or medium-chain acyl-CoA esters [7] do not mimic the effect observed with LCAC it appears that the presence of a long hydrocarbon acyl chain is a prerequisite for full inhibitory efficiency of acyl esters upon the ADP/ATP carrier, thereby indicating some degree of Van der Walls interaction between the hydrocarbon tail and non-polar entities of the inner membrane [10,28].

While inhibiting the ADP/ATP carrier, long-chain acyl-CoA β-oxidation intermediates would have a deep effect in the sub-cellular ATP system: the mitochondrial ATP/ADP ratio increases and the cytosolic ratio decreases [26]. Pyruvate metabolism, and in particular pyruvate dehydrogenase (PDH) activity, is regulated by several factors among which the ATP/ADP ratio: an increased intramitochondrial ATP/ADP ratio leads to activation of pyruvate phosphate kinase and further to the phosphorylation of PDH thus inhibiting its activity. This process would result in the accumulation of pyruvate that is subsequently converted into lactate. This could be the explanation for the moderate to severe lactic acidemia found *in vivo* [3–5] and *in vitro* [29] in very long-chain acyl-CoA dehydrogenase, 3-hydroxy long-chain acyl-CoA dehydrogenase or mitochondrial trifunctional protein deficiencies, which by itself contributes to the pathogenesis of these severe inherited metabolic disorders.

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