The effect of aging and acetyl-L-carnitine on the pyruvate transport and oxidation in rat heart mitochondria

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Abstract The effect of aging and acute treatment with acetyl-L-carnitine on the pyruvate transport and oxidation in rat heart mitochondria was studied. The activity of the pyruvate carrier as well as the rates of pyruvate-supported respiration were both depressed (around 40%) in heart mitochondria from aged rats, the major decrease occurring during the second year of life. Administration of acetyl-L-carnitine to aged rats almost completely restored the rates of these metabolic functions to the level of young control rats. This effect of acetyl-L-carnitine was not due to changes in the content of pyruvate carrier molecules. The heart mitochondrial content of cardiolipin, a key phospholipid necessary for mitochondrial substrate transport, was markedly reduced (approximately 40%) in aged rats. Treatment of aged rats with acetyl-L-carnitine reversed the age-associated decline in cardiolipin content. As the changes in cardiolipin content were correlated with changes in rates of pyruvate transport and oxidation, it is suggested that acetyl-L-carnitine reverses the age-related decrement in the mitochondrial pyruvate metabolism by restoring the normal cardiolipin content.

Key words: Aging; Pyruvate metabolism; Acetyl-L-carnitine; Cardiolipin; Rat heart mitochondrion

1. Introduction

Aging is known to be associated with alterations of various aspects of the cell function. At the cardiac level, aging causes a decline in functional competence. The molecular basis of this phenomenon is still not well understood. Mitochondria are considered to be a likely subcellular locus of this decline due to their central role in cardiac cell bioenergetics [1]. A crucial point in regulation of the mitochondrial energy metabolism is represented by the transport of metabolites across the mitochondrial inner membrane. Age-linked decrements in the activity of several anion transporting systems present in heart mitochondria have been reported [2–5].

Pyruvate plays an essential role in the heart mitochondrial energy metabolism. The transport of this substrate into mitochondria is carrier-mediated [6,7]. More recently, the pyruvate carrier has been isolated and its activity reconstituted in liposomes. A requirement of cardiolipin for mitochondrial pyruvate translocase activity has been demonstrated [8].

A growing body of evidence suggests that the age-related deficit in the mitochondrial function can be slowed or reversed by acetyl-L-carnitine (ALCAR), a normal component of the mitochondrial membrane [9–12]. We have recently reported that the activity of several anion carrier proteins such as the phosphate [13], ADP/ATP [14] and carnitine carriers [5] as well as that of cytochrome oxidase [14] is depressed in cardiac mitochondria from aged rats and that treatment of aged rats with ALCAR restored the activity of these membrane-bound proteins to the level of young control rats. Changes in the mitochondrial cardiolipin content have been suggested to be responsible for this effect of ALCAR. As the activity of the pyruvate carrier appears to be cardiolipin-dependent [8], we have examined the effect of aging and ALCAR treatment on the pyruvate translocase activity, on the pyruvate oxidation and on the cardiolipin content in rat heart mitochondria.

2. Materials and methods

Male Fisher rats of 5 months (young), 12 months (mature) and 28 months (aged) were used for these studies. In each experiment, two rats of each group were injected intraperitoneally with 300 mg/kg body weight ALCAR in NaCl (0.9%) [13]. In the same way, control animals received only the solvent for the same period. 3 h after the injection, the rats were killed by decapitation.

Rat heart mitochondria were prepared by differential centrifugation of heart homogenates as described previously [3].

Rat heart mitoplasts were prepared according to [15].

The mitochondrial protein concentration was determined by the usual biuret method using serum albumin as standard.

The standard medium used in the measurements of pyruvate transport, respiratory activity and binding experiments usually contained 100 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 1 mM $MgCl_2$ and 0.5 mM EDTA.

The transport of pyruvate in mitochondria was measured by the inhibitor stop method, using α-cyanocinnamate as inhibitor, essentially as described in [3]. Briefly, mitochondria (1–1.5 mg of protein) were pre-incubated in plastic centrifuge tubes at 10°C in a reaction medium described above in the presence of 0.5 μM sodium arsenite, 5 μg/ml rotenone, 0.5 μg/ml antimycin, 3 mM ascorbic acid, 0.05 mM TMPD, final pH 7.4. After 3 min of pre-incubation, radiolabelled pyruvate was added and 30 s later, the reaction was stopped by the addition of 1 mM α-cyanocinnamate. The tubes were rapidly centrifuged and the mitochondrial pellet was solubilized and transferred to a scintillation counter. The amount of radiolabelled pyruvate, expressed as nmol/mg mitochondrial protein, associated with the mitochondria was calculated from the amount of radioactivity in the mitochondrial pellet and the specific activity of the [^{14}C]pyruvate.

The content of pyruvate translocator was determined by titrating the amount of α-cyano[^{14}C]cinnamate bound to mitochondria as described in [16].

Mitochondrial respiratory parameters were measured at 25°C with approximately 0.8–1.2 mg protein in a 1.5 water-jacketed cell and oxygen consumption was measured with a Rank oxygen electrode.

Phospholipids were analyzed by high-pressure liquid chromatography, using a Beckman 344 gradient liquid chromatograph. Extraction and analysis of phospholipids were carried out essentially as described in [17].

Results are expressed as mean ± S.E.M. Statistical significance was determined by Student's *t*-test.

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Abbreviation: ALCAR, acetyl-L-carnitine

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Table 1
The effect of aging and ALCAR treatment on the kinetic parameters of the pyruvate carrier in rat heart mitochondria

Animals	K_m (μM)	V_{max} (nmol/min/mg protein)	Change %
Young	215 ± 21	6.8 ± 0.7	
Young+ALCAR	218 ± 24	7.0 ± 0.8	
Mature	222 ± 19	6.5 ± 0.9	
Mature+ALCAR	230 ± 28	6.7 ± 0.8	
Aged	231 ± 23	4.1 ± 0.5[b]	40
Aged+ALCAR	209 ± 28	6.6 ± 0.8[a]	3

The K_m and V_{max} values for pyruvate transport were calculated from double reciprocal plots of the rates of pyruvate uptake versus pyruvate concentrations (ranging from 0.05 to 1 mM) essentially as described in Section 2. Each value represents the mean ± S.E.M. for three separate experiments with two rats for each group.
[a]Control versus treated, $P < 0.01$.
[b]Aged versus young, $P < 0.05$.

3. Results

The kinetic parameters of the pyruvate carrier in mitochondria isolated from young, mature and aged rats, pre-treated or not with ALCAR, are presented in Table 1. While the affinity of the carrier for pyruvate was practically the same in all these mitochondrial preparations, the maximal activity of this carrier system was significantly depressed (40%) in mitochondria from aged rats when compared with that obtained in mitochondria from young control rats. No appreciable change was observed in mitochondria isolated from mature rats. Treatment of aged rats with ALCAR almost completely restored the activity of the pyruvate carrier to the value of young rats. This compound had practically no effect on the activity of the pyruvate carrier in mitochondria isolated from young and mature rats.

It has been demonstrated that the transport of pyruvate in heart mitochondria is the rate-limiting step for pyruvate oxidation [18]. Thus, changes in the activity of pyruvate transport may be associated with parallel changes in the rates of pyruvate-dependent oxygen utilization. Results of experiments on pyruvate oxidation in heart mitochondria isolated from young, mature and aged rats, pre-treated or not with ALCAR, are presented in Table 2. Rates of phosphorylating oxygen consumption with pyruvate were significantly depressed (38%) in mitochondria from aged rats, whereas they were the same in mitochondria isolated from mature rats when compared to the values of young control rats. Treatment of aged rats with ALCAR restored the rates of pyruvate oxygen consumption to the level of young rats.

Table 2
The effect of aging and ALCAR treatment on the rates of pyruvate-supported respiration in rat heart mitochondria

Animals	Rates of oxygen consumption (ng atoms O/min/mg protein)		
	State 3	State 4	RCR
Young	282 ± 27	35.5 ± 3.5	7.94
Young+ALCAR	286 ± 28	36.2 ± 3.7	7.90
Mature	272 ± 24	32.4 ± 3.1	8.39
Mature+ALCAR	281 ± 26	34.7 ± 2.9	8.11
Aged	176 ± 21	28.2 ± 3.1[b]	6.24
Aged+ALCAR	278 ± 25	34.8 ± 3.8[a]	7.98

The pyruvate-dependent oxygen uptake was measured as described in Section 2. Mitochondria (0.8–1.2 mg protein/ml) were pre-incubated in the standard reaction medium described above. Final pH 7.2, 25°C. When a steady state of oxygen consumption was obtained, 0.5 mM pyruvate and 2 mM malate were added. 1 min later, respiration was stimulated by the addition of 2 mM ADP. Each value represents the mean ± S.E.M. for three separate experiments with two rats for each group.
[a]Control versus treated, $P < 0.01$.
[b]Aged versus young, $P < 0.05$.

The ability of ALCAR to reverse the altered pyruvate carrier activity in mitochondria from aged rats could be due to an increase in the pyruvate carrier level. To test this possibility, the pyruvate carrier molecules were titrated by following the binding of radiolabelled α-cyanocinnamate to mitochondria. The total number of α-cyanocinnamate binding sites to mitochondria can be taken as quantitative expression of the number of pyruvate carrier molecules [16]. No change was found in the level of pyruvate carrier molecules in mitochondria isolated from young and aged rats pre-treated or not with ALCAR (a value around 48 pmol/mg protein of α-cyanocinnamate binding sites was obtained for all these preparations of mitochondria).

It has been reported that cardiolipin, a phospholipid located in the inner mitochondrial membrane, is required for the functioning of the isolated pyruvate carrier reconstituted in liposomes [8]. Thus, changes in the mitochondrial cardiolipin content may be responsible for the observed changes in the pyruvate carrier activity with age. As the pyruvate carrier protein is located at the level of the inner mitochondrial membrane, the phospholipid composition of mitoplasts isolated from young and aged rats, pre-treated or not with ALCAR, was analyzed. The results reported in Table 3 show that the level of cardiolipin was significantly reduced (39%) in mitoplasts from aged rats when compared to the value of young control rats. ALCAR administration to aged rats reversed this loss in cardiolipin concentration to a level not significantly different from young animals. Similar results have recently been reported by Hagen and colleagues with hepatocytes isolated from old rats supplemented with ALCAR [12]. No effect was observed on the concentration of other mitochondrial phospholipids.

4. Discussion

The observations of this study are consistent with a previous report showing that the cardiac mitochondrial pyruvate metabolism is depressed in aged animals [3]. In fact, it is shown here that the activity of the pyruvate carrier and the rates of pyruvate-supported oxygen consumption are both depressed in heart mitochondria from aged rats, the major decrease occurring during the second year of life. Administration of ALCAR to aged rats almost completely restored the rates of these metabolic functions to the level of young control rats.

Table 3
The effect of aging and ALCAR treatment on the phospholipid composition in rat heart mitoplasts

Phospholipid	Distribution (mol %)			
	Young	Young+ALCAR	Aged	Aged+ALCAR
DPG	22.4 ± 1.8	22.8 ± 2.1	13.7 ± 1.1[b]	22.1 ± 1.6[b]
PE	35.6 ± 2.7	35.0 ± 3.1	39.8 ± 2.2	35.9 ± 2.1
PC	40.4 ± 2.9	40.6 ± 3.6	44.4 ± 3.7	40.2 ± 3.1
PI	0.4 ± 0.06	0.4 ± 0.06	0.6 ± 0.08	0.6 ± 0.08
PS	1.2 ± 0.2	1.2 ± 0.1	1.5 ± 0.5	1.2 ± 0.2

For phospholipids extraction and analysis, see Section 2. Each value represents the mean ± S.E.M. of three experiments with two rats of each group. DPG, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine.
[a]Control versus treated, $P < 0.01$.
[b]Aged versus young, $P < 0.05$.

An increase in pyruvate carrier protein levels might represent one mechanism by which ALCAR reverses the aged-related decline in pyruvate carrier activity. However, this possibility appears to be unlikely in that no change was found in the level of pyruvate carrier molecules in mitochondria isolated from aged rats treated with ALCAR.

Restoration of the correct lipid environment of the pyruvate carrier molecules in the inner mitochondrial membrane may represent a plausible mechanism by which ALCAR reverses the altered pyruvate carrier activity with age. In fact, it has been reported that cardiolipin, a phospholipid almost exclusively associated with the inner mitochondrial membrane, is required for the functioning of the isolated pyruvate carrier reconstituted in liposomes [8]. In addition, we have previously reported that the transport of pyruvate in mitochondria is inhibited by doxorubicin [19], an anti-tumoral agent which is known to form a specific complex with cardiolipin [20]. Thus, changes in the mitochondrial cardiolipin level may affect the activity of the pyruvate carrier in vivo. Our results demonstrate that the cardiolipin content is markedly reduced in mitoplasts from aged rats as compared with control young rats. This reduced level of cardiolipin is brought back to the level of young rats by treatment of aged rats with ALCAR. These changes in the cardiolipin level are quantitatively associated with parallel changes in the rates of pyruvate transport and oxidation. Thus, it is conceivable that the restoration of the pyruvate metabolism in heart mitochondria from aged rats by ALCAR may depend upon the ability of this compound to restore the correct level of cardiolipin, which is needed for functioning of the pyruvate carrier. It should be noted that administration of ALCAR to young and mature rats had practically no effect either on the level of cardiolipin or on the activity of the mitochondrial pyruvate carrier and pyruvate oxidation. This suggests that the observed effects of ALCAR are related to changes produced by aging.

The results reported do not provide evidence on the molecular mechanism by which ALCAR restores the normal cardiolipin content in cardiac mitochondria from aged rats. One possibility is that ALCAR may influence the activity of cardiolipin synthase, the enzyme protein responsible for the biosynthesis of cardiolipin, as recently suggested for the effect of thyroxin on the activity of this enzyme in rat liver mitochondria [21,22].

Pyruvate, together with free fatty acids, is the major energy source for the heart tissue. Therefore, the observed effect of ALCAR on the mitochondrial level of cardiolipin and thereby on the activity of pyruvate carrier (present results) as well as on that of other substrate transport proteins [5,13,14] and on the cytochrome oxidase activity [14] may explain, at least in part, the stimulatory effect of this compound on the cardiac energy metabolism of aged animals.

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