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Age-related changes in the activity of the pyruvate carrier and in the lipid composition in rat-heart mitochondria

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The effect of aging on the activity of the pyruvate translocator and on the lipid composition in rat-heart mitochondria has been investigated. It has been found that the rate of pyruvate transport in mitochondria from aged rats (28 months old) is markedly reduced (38%) as compared with that obtained with mitochondria from young adults rats (4 months old). Kinetic analysis of the pyruvate transport shows that only the V_{max} of this process is decreased, while there is no change in the K_m values. The age-related decrement in the activity of the pyruvate carrier is not due to a decrease in the transmembrane ΔpH value, neither does it depend on a decrease in the total number of the pyruvate carrier molecules, titrated with radioactive α-cyanocinnamate. The lower activity of the pyruvate translocator in mitochondria from aged rats is associated to a parallel decrement of the rate of pyruvate-dependent oxygen uptake. There is, however no appreciable difference in either the respiratory control ratios or in the ADP/O ratios between these two types of mitochondrion. The Arrhenius plot characteristics differ for pyruvate transport activity in mitochondria from aged rats as compared with young rats in that the break point of the biphasic plot is shifted to a higher temperature. The heart mitochondrial lipid composition is significantly altered in aged rats. The total cholesterol increases (43%), the phospholipids decrease (15%) and the cholesterol/phospholipid molar ratio increases (68%). Among phospholipids, cardiolipin shows the greatest alteration (28% decrease in aged rats). The lower activity of the pyruvate carrier in mitochondria from aged rats may be ascribed to changes in the lipid domain surrounding the carrier molecule in the membrane.

Introduction

Age-dependent changes in biochemical pathways involved in mitochondrial energy metabolism homeostasis have been reported [1–5]. These changes have been related to molecular and functional changes in the properties of biological membranes. At the cardiac level, aging is known to cause a decline in functional competence. Mitochondria play a key role in the heart metabolism. A crucial point in the regulation of mitochondrial energy metabolism is represented by the transport of metabolites across the mitochondrial membrane. Age-linked decrements in the activity of several transporting systems present in heart mitochondria have been reported [6–8]. These include acylcarnitine-carnitine translocation [6], adenine nucleotide translocation [1,7] and the transport of Ca^{2+} [8].

Pyruvate plays an essential role in heart mitochondrial energy metabolism. The transport of this substrate in mitochondria is mediated by a specific translocator [9,10]. The kinetic properties, substrate specificity and the sensitivity to specific inhibitor of this carrier system have been studied in detail [11–15]. Several molecular aspects of this translocator have been also elucidated [16–18] and recently, purification of the pyruvate carrier molecule has been achieved [19].

The kinetic parameters of the pyruvate carrier have been observed to change in mitochondria isolated from animals under different metabolic conditions such as hormone treatment [20,21] or pathological conditions such as diabetes [22] or in different tumour states [23,24].

The isolation of the pyruvate carrier molecule and the reconstitution of its transporting activity in the liposomes have been shown to be dependent on the presence of phospholipids [19]. In particular, the activity of the pyruvate carrier appears to be cardiolipin-dependent. Very recently, we have reported changes in the activity of the pyruvate carrier in heart mitochondria

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from hyper- and hypothyroid rats [25,26]. These changes were associated to changes in the lipid composition of the mitochondrial membranes. Changes in membrane lipid content and lipid–protein interactions do occur with aging in several tissues and organs, including the heart [27–29].

The above considerations have prompted us to investigate the effect of aging on the activity of the pyruvate carrier and on the lipid composition in rat-heart mitochondria. The results obtained demonstrate an age-dependent decrement in the activity of the pyruvate carrier which appears to be related to modification of lipid composition of the mitochondrial membrane.

Materials and Methods

Chemicals

The radioactive [2-^{14}C]pyruvate was obtained from Amersham International, U.K. Radioactive pyruvate was dissolved in water, divided into 5 μCi samples, freeze-dried and stored in sealed tubes at $-20\,^{\circ}$C. α-Cyanocarboxyl[^{14}C]cinnamic acid was synthesized at Amersham International. Its specific activity was 19.2 mCi and its purity was 98%. 5,5-Dimethyl[^{14}C]oxazolidine-2,4-dione was obtained from New England Nuclear. All other reagents were of reagent grade purity and were purchased from Sigma.

Animals

Male Fisher rats of 4 months or 28 months (25% survivorship) were used throughout these studies. They were fed ad libitum, until killed, with a basal diet consisting of 25% protein, 4.3% lipid, 59.7% carbohydrate (of which 7.1% cellulose) and a salt and vitamin mixture.

Rat heart mitochondria were prepared as described in Ref. 16. Mitochondria were resuspended in 0.25 M sucrose/5 mM Tris-HCl (pH 6.8) and stored in ice.

Protein concentration was measured by the usual biuret method using serum albumin as standard.

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

Pyruvate transport

The initial rate of pyruvate transport by mitochondria was measured at $10\,^{\circ}$C by the Halestrap inhibitor stop method, using α-cyanocinnamate as inhibitor [10]. The reactions were conducted in plastic centrifuge tubes (1.5 ml capacity). Each reaction mixture contained in 1 ml of the reaction medium described above: 0.5 mM sodium arsenite, 5 μg/ml rotenone, 0.5 μg/ml antimycin, 3 mM ascorbic acid, 0.05 mM TMPD and 0.9–1.1 mg of mitochondrial protein. After 3 min of preincubation of mitochondria, radiolabelled pyruvate was added and, at appropriate times described in the figure legends, the reaction was stopped by the addition of 1 mM α-cyanocinnamate. The tubes were rapidly centrifuged at $18\,000 \times g$ for 1 min. The pellets were washed with 0.25 mM sucrose and dissolved in $HClO_4$ (15%, w/v). The centrifugation of the mitochondrial pellets and all the subsequent operations of washing of the pellets were done at $4\,^{\circ}$C to prevent pyruvate metabolism. The radioactive and enzymic assays of pyruvate gave similar results. The vials were then recentrifuged at $15\,000 \times g$ for 2 min in a refrigerated microcentrifuge. Solubilized mitochondria were transferred to 10 ml scintillation counter. The amount of radiolabelled pyruvate, expressed as nmol per mg of 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 amount of [^{14}C]pyruvate present in the fluid outside the matrix or absorbed to the mitochondria was estimated in reactions in which α-cyanocinnamate was added before [^{14}C]pyruvate.

pH measurments

The external pH was determind potentiometrically on the supernatant obtained after centrifugation of the mitochondrial suspension. The intramitochondrial pH (matrix space) was calculated on the basis of the distribution of [^{14}C]DMO between the matrix space and the medium by the equation of Addanki and al. (Ref. 30; see also Ref. 9).

Measurements of binding

The binding of α-cyanocinnamate to mitochondria was assayed as follows. Mitochondria (0.9–1.1 mg of protein/ml) were preincubated in the standard reaction medium at pH 7.0 and $25\,^{\circ}$C. After 3 min of preincubation increasing concentrations of labelled α-cyanocinnamate were added and 3 min later mitochondria were separated from the medium by rapid centrifugation. The amount of α-cyanocinnamate bound to the mitochondria was determined as described in Ref. 16.

High-pressure liquid chromatography (HPLC) analysis of lipids

Phospholipids, fatty acids and cholesterol were analyzed by HPLC, using a Beckman 344 gradient liquid chromatograph. Extraction and analysis of phospholipids, fatty acids and cholesterol were carried out essentially as described in Ref. 31.

Results

Pyruvate translocator kinetic

Fig. 1 illustrates the results of five separate experiments of the time-course of pyruvate uptake by heart

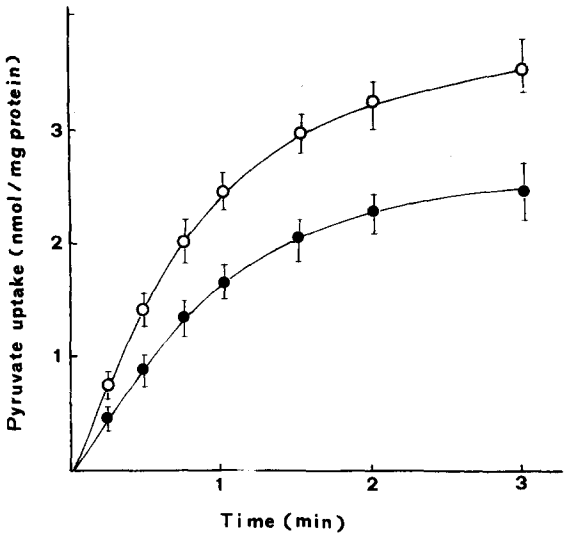

Fig. 1. Time-course of [^{14}C]pyruvate uptake by mitochondria from young and aged rats. The rate of pyruvate uptake by mitochondria was measured as described in Materials and Methods. Mitochondria (1 mg of protein/ml) were preincubated in the standard reaction medium at pH 6.8 and 10 °C. After 3 min of preincubation 250 μM [^{14}C]pyruvate was added. 1 mM α-cyanocinnamate was added, at the times indicated, to stop the reaction. ○, Mitochondria from young rats; ●, mitochondria from aged rats. Values are expressed as the means ± S.E. for three determinations.

mitochondria from young and senescent animals. The rate of pyruvate uptake by mitochondria from aged rats was significantly lower than that obtained with mitochondria from young control rats.

The kinetic parameters of the pyruvate transport by mitochondria from young and aged rats were determined by studying the dependence on substrate concentration of the rate of pyruvate uptake. The following results were obtained from five different experiments: K_m 210 ± 15 and 222 ± 23 μM and V_{max} 6.8 ± 0.7 and 4.2 ± 0.5 nmol per min per mg of mitochondrial protein in mitochondria from young control and aged rats, respectively.

The uptake of pyruvate by mitochondria is very sensitive to the transmembrane ΔpH [9,10]. Thus, the reduced activity of the pyruvate carrier in mitochondria from aged rats may, in principle, be due to a decrease in the mitochondrial pH gradient. This possibility was explored by measuring the transmembrane ΔpH in both mitochondria from young and senescent rats. The results obtained indicate that there was no change in the transmembrane ΔpH values in mitochondria from young and aged rats, the values being respectively 0.84 ± 0.8 and 0.85 ± 0.9 (mean ± S.E. for four experiments).

Titration of α-cyanocinnamate binding sites

The reduced activity of the pyruvate translocator in mitochondria from aged rats may reflect a decrease in the number of the pyruvate carrier molecules in the mitochondrial membrane. To test this possibility, the pyruvate carrier molecules were titrated by following the binding of radiolabelled α-cyanocinnamate to mitochondria [16,17]. The binding curves of α-cyanocinnamate were practically similar in these two types of mitochondrion. Schatchard plots of these binding data, obtained from five different experiments, gave the same total number of α-cyanocinnamate binding sites (48 ± 5 pmol per mg of mitochondrial protein) and the same value for the apparent dissociation constant (K_d 0.105 ± 0.011 μM).

Temperature dependence of the pyruvate transport

The uptake of pyruvate in heart mitochondria from young and aged rats was measured at different temperatures. The Arrhenius plots of a representative experiments of the pyruvate uptake in mitochondria from young and aged rats is reported in Fig. 2. The rate of pyruvate uptake in heart mitonchondria from young animals exhibited a biphasic plot, i.e., two linear portions with different slopes intersecting at 16.4 ± 1.4 °C (mean ± S.E. for four experiments). With heart mitochondria from aged rats, the Arrhenius plot of the pyruvate uptake was also biphasic, but the position of the break was shifted to a higher temperature 20.8 ± 1.7 °C (mean ± S.E. for four experiments). The values for the activation energies (kJ/mol) of the pyruvate transport process below and above the transition temperature were 76.5 ± 7.9 and 28.8 ± 3.3 in mitochondria from young rats and 75.6 ± 7.9 and 24.2 ± 2.9 in those from aged rats, respectively.

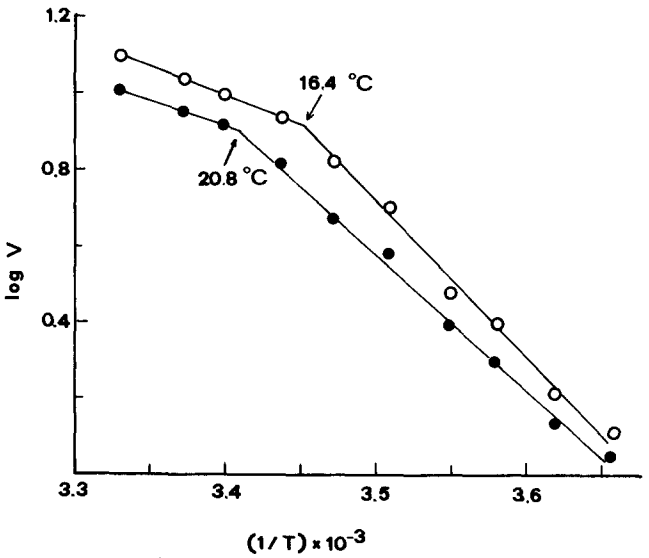

Fig. 2. Arrhenius plots of the temperature dependence of the rate of pyruvate uptake in heart mitochondria from young and aged rats. Experimental conditions as in Fig. 1. Mitochondrial protein was 1.1 mg/ml. The rates of pyruvate uptake were calculated from the amount of pyruvate taken up within the initial time period during which pyruvate uptake was linear. V is expressed in nmol/min per mg protein. ○, Mitochondria from young rats; ●, mitochondria from aged rats.

TABLE I

Rates of pyruvate-dependent oxygen uptake in heart mitochondria from young and aged rats

The pyruvate-dependent oxygen uptake was measured with a Clark-type electrode. Mitochondria (0.9–1.1 mg of protein/ml) were preincubated in the standard reaction medium described in Materials and Methods. Final pH, 7.2; Temp., 25 °C. When a steady state of oxygen consumption had been obtained, 0.25 mM pyruvate was added. 1 min later respiration was stimulated by the addition of 2 mM ADP. For the determination of RCR and ADP/O values, pyruvate was added at concentration of 2.5 mM together with 2.5 mM malate. Each value represents the mean ± S.E. obtained for six experiments with four rats each

Animals	Pyruvate oxidation (ngatom O/min per mg protein)	Respiratory control ratio	ADP/O
Young	215 ± 19	9.78 ± 0.95	2.85 ± 0.25
Aged	151 ± 16 [a]	9.65 ± 0.98	2.78 ± 0.31

[a] $P < 0.01$.

Pyruvate oxidation

The transport of pyruvate in heart mitochondria is a rate-limiting step for pyruvate oxidation [32]. Thus, changes in the rate of pyruvate transport can be associated with parallel changes in the rate of pyruvate supported oxygen uptake. The results reported in Table I show that the rate of pyruvate oxidation in mitochondria from aged rats was significantly depressed (30%) as compared with that obtained with mitochondria from young rats. No such decrement was observed when pyruvate was used at higher concentration (2.5 mM) to make the carrier no more limiting for pyruvate oxidation [33]. Neither the respiratory control ratios nor the ADP/O ratios were altered in these two types of mitochondrion. This indicates that aging affects both the pyruvate transport and the pyruvate oxidation without affecting the intactness of the mitochondrial membrane and the efficiency of the mitochondrial respiratory functions.

TABLE II

Effect of aging on cholesterol and phospholipid content in rat heart mitochondria

For cholesterol and phospholipid extraction and characterization, see the Materials and Methods section. Each value represents the mean obtained for six experiments with four rats each ± S.E.. Cholesterol is expressed as nmol/mg protein and phospholipids as nmol lipid P_i/mg protein

	Young	Aged
Cholesterol	10.7 ± 1.4	15.3 ± 2.0 [a]
Phospholipids	282.0 ± 12.1	240.3 ± 13.4 [a]
Ratio cholesterol/ phospholipid	0.038 ± 0.008	0.064 ± 0.01 [a]

[a] $P < 0.01$.

TABLE III

Phospholipid composition in rat heart mitochondria as determined by HPLC

For phospholipid extraction and analysis, see the Materials and Methods section. Each value represents the mean ± S.E. obtained from six different experiments with four rats each

Phospholipid	Distribution (mol%)	
	young	aged
Cardiolipin	13.4 ± 1.0	9.6 ± 1.2 [a]
Phosphatidylethanolamine	35.4 ± 1.8	35.1 ± 1.5
Phosphatidylinositol	1.4 ± 0.3	1.4 ± 0.2
Phosphatidylserine	2.2 ± 0.5	3.0 ± 0.4
Phospatidylcholine	47.6 ± 1.4	50.9 ± 1.7 [b]

[a] $P < 0.01$.
[b] $P < 0.02$.

Lipid composition in the mitochondrial membrane

The effect of aging on rat heart mitochondrial phospholipid and total cholesterol content is shown in Table II. From this table, total cholesterol can be seen to increase by 43% and inversely, phospholipid decrease by 15% in mitochondria from aged rats. Consequently, the inverse relationship between the change in total cholesterol and phospholipid content observed in these organelles caused a 68% increase in the total cholesterol/phospholipid molar ratio.

Table III compares the phospholipid pattern of mitochondrial membranes from aged rats with the young controls. No appreciable variation in mitochondrial phospholipid composition of either of these types of rat occurred, except for the negatively charged phospholipid cardiolipin, the level of which was markedly reduced (28%) in aged animals.

TABLE IV

Pattern of fatty acids in rat heart mitochondria as determined by HPLC
Extraction and analysis of fatty acids were carried out as described in the Materials and Methods section. Each value represents the mean ± S.E. obtained from six different experiments with four rats each. The unsaturation index (U.I.) is defined as Σmol% of each fatty acid × number of double bonds of the same fatty acid

Fatty acid	Distribution (mol%)	
	young	aged
16:0	14.6 ±0.8	15.3 ±0.7
16:1	6.9 ±0.6	8.9 ±0.5 [a]
18:0	16.8 ±0.8	15.6 ±0.6
18:1	8.5 ±0.4	7.8 ±0.7
18:2	26.1 ±1.2	22.1 ±1.0 [a]
20:3	2.5 ±0.3	2.3 ±0.2
20:4	23.0 ±1.0	25.9 ±1.3 [a]
22:6	1.6 ±0.2	2.1 ±0.3 [b]
U.I.	176.7 ±2.4	184.0 ±2.0
20:4/18:2	0.88 ± 0.07	1.17 ± 0.09 [a]

[a] $P < 0.01$.
[b] $P < 0.02$.

Chemical changes in the mitochondrial membrane lipid composition were further investigated by analyzing the fatty acids composition in these mitochondrial membranes (see Table IV). Alterations of fatty acids distribution were observed in mitochondrial membrane from aged rats. In particular, a significant increase in both palmitoleic (16 : 1) and docosahexaenoic acid (22 : 6) and a decrease in linoleic acid (18 : 2) occurred with aging. The unsaturation index did not change significantly with aging.

Discussion

The results presented in this paper demonstrate that the rate of pyruvate transport in heart mitochondria from aged rats is significantly decreased as compared to that obtained in mitochondria from young rats. Neither a decrease in the transmembrane ΔpH nor a decrease in the total number of the pyruvate carrier molecules can account for the depressed rate of pyruvate transport in mitochondria from aged rats.

One may envisage the change in the activity of the pyruvate carrier in mitochondria from aged rats as being caused by a change in the turnover number of the carrier due to an altered carrier protein–lipid interaction. There are many examples showing that the carrier-mediated transport processes are sensitive to changes in the composition and in the physicochemical state of the surrounding membrane lipids [25,26,34,35]. Similar mechanism may be responsible for the change in the activity of the pyruvate carrier in mitochondria from aged rats. In fact, the activity of the pyruvate carrier is quite differently affected by changes in the temperature in mitochondria from young and aged rats. There is a shift in the temperature breakpoint of the biphasic Arrhenius plot for pyruvate transport activity in mitochondria isolated from aged rats. The responses of membrane dependence processes to change in temperature depend mainly on the lipid environment of the carrier protein or enzymes [36].

The analysis of the mitochondrial membrane lipids reveals substantial changes in the lipid composition in young and aged rats. Specifically, the total cholesterol and the cholesterol/phospholipids molar ratio were both significantly increased in mitochondrial membrane from aged rats. All these changes are associated with a decrease in membrane fluidity. The shift in the temperature breakpoint of the Arrhenius plot for the pyruvate transport in direction of higher temperature, as observed in mitochondria from aged rats, is consistent with a less fluid membrane in old age.

While cholesterol content and cholesterol/phospholipids molar ratio increased with age, that of cardiolipin decreased markedly. Cardiolipin provides the environment necessary for the activity of the pyruvate carrier. In fact, cardiolipin appears to be specifically required for isolation and reconstitution in the liposomes of the transport activity of the isolated pyruvate carrier [19]. Furthermore, we have recently found that the transport of pyruvate in heart mitochondria is inhibited by doxorubicin [37], an antitumoral agent which is known to form specific complexes with cardiolipin [38]. It can, therefore, be proposed that the reduced activity of the pyruvate carrier in heart mitochondria from aged rats may be ascribed either to a general modification of membrane lipid composition, which decreases the membrane fluidity and thereby the mobility of the carrier molecule in the membrane, or to a more localized change in the lipid microenvironment (specifically cardiolipin content) surrounding the carrier molecule in the mitochondrial membrane.

Pyruvate, together with free fatty acids, is the major energy source in the heart. The reduced activity of the pyruvate carrier in heart mitochondria from aged rats can account, in addition with other factors, for the decline in cardiac functional competence with aging.

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