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Mitochondrial function and superoxide generation from submitochondrial particles of aged rat hearts

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A decrease in heart function with ageing might be related to an impairment of mitochondrial function, since these organelles produce the greatest fraction of ATP in the myocyte. Mitochondria extracted from Wistar rat hearts at 3, 14, 18 and 24 months of age were employed to evaluate the changes of the respiratory activity during lifetime. A slight decrease of the respiratory rate (QO_2) was observed in the 14 month group with respect to the 3 month group when succinate was used as substrate, whereas the respiratory control index (RCI) in the presence of glutamate or succinate increased in the 24 month group. The latter result may be related to a condition of moderate hypertrophy that generally occurs in the ageing heart. Submitochondrial particles (SMP) were also prepared to study the superoxide radicals (O_2^-) production at the level of rotenone or antimycin-inhibited regions of the respiratory chain. A strong elevation in the O_2^- generation was observed in the antimycin-inhibited region at 14 months of age; on the contrary, the rate of O_2^- production remained unchanged in the 24 month group in comparison to the youngest group. These observations correlate well with the enhanced tissue level of oxidized glutathione that was observed at 14 and 18 months of age. The products of lipid peroxidation (TBARS) did not change in the rat heart at any of the ages measured, whereas the levels of fluorescent substances progressively increased beginning from 18 months of age, with a greater extent in the mitochondrial compartment. The present study suggests that age does not substantially affect mitochondrial respiration and energy output in the rat heart, while a greater production by cardiac mitochondria of superoxide anions in the adult rats (14 months) might accelerate the fluorescent pigment formation.

Introduction

Many studies have shown that as the heart reaches senescence it undergoes a decline in function that may be related to several physiological, biochemical and morphological alterations [1–3]. Mitochondrial dysfunction is often considered a major cause of modifications that occur in the ageing heart, since in aerobic conditions the greatest fraction of ATP is produced in these organelles. Mitochondrial respiratory function of senescent hearts has been widely studied by many workers but, unfortunately, the results are controversial, due to the variability of experimental conditions and animal species used as the source of cardiac mitochondria. For example, Chen and co-workers [4] have observed a distinct decline in the oxidation of glutamate or glutamate plus malate in mitochondria from old Fischer rats, but not with succinate, ascorbate or pyruvate plus malate. Nohl et al. [5] found a significant decrease in the ADP-stimulated (state 3) respiration and in the ADP/O ratio by heart mitochondria of old rats supported with succinate or 3-hydroxybutyrate, while glutamate plus malate oxidation were unaffected by ageing. On the other hand, Mazelmann and Harmon in a recent study [6], found no differences in heart mitochondrial succinate or malate plus glutamate oxidation between 3-, 12- and 28-month-old Fischer rats. Moreover, according to the free-radical theory of ageing, mitochondria may have a role in the ageing process because they are the principal site of oxygen consumption [7,8]. In fact, superoxide anions (O_2^-) and other reactive substances such as hydroxyl radicals or hydrogen peroxide are generated during mitochondrial respi-

Abbreviations: TBARS, thiobarbituric acid reactive substances; SMP, submitochondrial particles; RCI, respiratory control index; BSA, bovine serum albumin.

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ration [9,10]. These compounds appear to be the principal cause of the peroxidative damage that occurs at the cellular level in several diseases [11,12] and in ageing [13]. In fact, it is known that polyunsaturated fatty acids are primary targets of oxygen radicals and their peroxidation leads to alterations of structural integrity and function of cellular membranes [14]. The aim of this study is to investigate if ageing produces changes in mitochondrial respiratory function and if there is a correlation between mitochondrial superoxide formation, lipid peroxidation and the ageing process.

Materials and Methods

Male Wistar rats at 3, 14, 18 and 24 months of age were used for the experiments. In our colony of ageing rats, approx. 50% survive to 24 months of age. Rats with evident signs of diseases such as ascites, cervical tumefactions and neoplasms were excluded.

Mitochondrial isolation

Mitochondria were isolated from ventricles following the procedure of Williams and Barrie [15]. The heart muscle was homogenized gently in 180 mM KCl, 10 mM EDTA and 0.5% bovine serum albumin (BSA) (pH 7.2), using an Ultra Turrax homogenizer. The homogenate was centrifugated at $1500 \times g_{max}$ for 5 min, filtered and then recentrifuged at $8000 \times g_{max}$ for 10 min. The mitochondrial pellet was washed twice with a medium containing 180 mM KCl, 2 mM EDTA, 0.5% BSA and finally resuspended in 180 mM KCl, 0.5% BSA (pH 7.2).

Submitochondrial particle isolation

Submitochondrial particles (SMP) were prepared by sonicating the mitochondria (10 mg/ml) in 2 mM EDTA, (pH 8.5), at 40 W, four times for 15 s with a 15 s interval at 4°C in a Labsonic Sonifier cell disruptor [9]. The sonicated mitochondrial suspension was centrifuged at $10000 \times g_{max}$ for 10 min and the resulting supernatant recentrifuged at $105000 \times g_{max}$ for 30 min.

The SMP pellet was washed twice with 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), by centrifugation at $105000 \times g_{max}$ for 30 min. The washed particles were essentially free from superoxide dismutase, as shown by activity measurements according to Guarnieri et al. [16].

Biochemical measurements

Mitochondrial respiratory control index (RCI), state 3 oxygen consumption (QO_2) and the ADP/O ratio were calculated at 25°C from the O_2 partial pressure measured by a Clark electrode fitted in a closed water jacket (Gilson Instruments, France). The assay medium consisted of 3 mM glutamate, succinate or ascorbate + 0.5 mM *N,N,N,N'*-tetramethyl-*p*-phenylene-diamine, 250 mM sucrose, 0.5 mM EDTA, 3 mM KH_2PO_4 (pH 7.4). ADP (250 μM) was added to the incubation mixture to initiate state 3-mitochondrial respiration.

Production of O_2^- by SMP was measured by the superoxide dismutase-sensitive oxidation of adrenaline to adrenochrome [17]. The assay system included 250 mM sucrose, 50 mM Hepes (pH 7.5), 1 mM adrenaline and 200–300 μg SMP protein. Absorbance changes were monitored at 25°C in a Perkin-Elmer double-beam spectrophotometer at 480 nm adding 0.125 mM NADH ($E = 4.02\ mM^{-1} \cdot cm^{-1}$). These measurements were carried out during the initial linear phase (1 min) of the adrenochrome formation which is representative of the true rate of O_2^- generation.

The levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) in the heart tissue were determined as described by Tietze [18] and slightly modified according to Guarnieri et al. [19]. The hearts were immediately denatured by 6 M PCA containing 2 mM EDTA (for GSH + GSSG determinations) by using an Ultra Turrax homogenizer. For GSSG assay, the above acid solution also contained 50 mM *N*-ethylmaleimide. After centrifugation, the supernatants were neutralized with 6 M K_2CO_3/0.3 M Mops and analyzed for glutathione content. The assay mixture contained: 100 mM potassium phosphate buffer (pH 7.4); 1 mM EDTA; 0.1 mM 5',5-dithiobis(2-nitrobenzoic acid); 0.15 mM NADPH; 6 units/ml glutathione reductase and an appropriate volume of neutralized sample. For GSSG measurements, the neutralized samples were extracted with diethyl ether in order to remove the excess of *N*-ethylmaleimide. After 1 min, the increase in absorbance at 412 nm was measured for 3 min using a double-beam Perkin-Elmer spectrometer.

The products of lipid peroxidation (TBARS) were measured following the procedure of Jackson et al. [20] which employs 0.8% thiobarbituric acid as reactant. In order to evaluate the amount of TBARS present in the tissue rather than that generated during the incubation procedure, the determination was carried out in the presence of 2 mM EDTA [20].

Tissue levels of fluorescent products were determined according to Dillard and Tappel [21]. Chloroform/methanol, 2:1 (v/v), in a volume-to-weight ratio of 20:1, was added to 0.2 g of freshly excised tissue which was homogenized for 1.5 min in a 25°C water bath. After homogenization an equal volume of water was added, the contents were vortexed and centrifugated at about 3000 rpm for 1–2 min. The fluorescence emission spectra of the chloroform-rich layer was recorded in the region 420–470 nm with an excitation wavelength of 360 nm. Mitochondrial levels of fluorescent products were determined as described above by extracting 2.5 mg of mitochondrial protein into 3 ml of chloroform/methanol, 2:1 (v/v). The standard used was 1 μg of quinine sulphate/ml of 0.05 M H_2SO_4, which gave 100 relative fluorescence units (100%).

The protein concentrations were estimated by the method of Bradford [22] using bovine serum albumin as a standard.

Statistics

The results are expressed as means ± S.D. A one-way ANOVA followed by Duncan's multiple range test was performed on all the data. Differences were considered significant when $P \leq 0.05$. The survival curve of our ageing colony was obtained using a regression polynomial analysis (Energraphic, Enertronics Research, 1983).

Results

The survival curve for a typical group of 100 male Wistar rats in our ageing colony is shown in Fig. 1. The mean life-span was 24.2 months. Table I shows that the mitochondrial respiratory function of the rat heart did not substantially change during ageing. Only the RCI of the 24 month group, in the presence of glutamate or succinate, increased with respect to the RCI of the 3 month group. These increases were caused by a lower state 4 respiration in the 24 month group compared to the 3 month group (8.78 ± 0.93 v.s 6.25 ± 0.88 and 45.0 ± 2.15 vs. 39.41 ± 1.27 for glutamate and succinate, respectively). On the contrary, no differences were found in the state 3 respiratory between the young and the oldest group (113.2 ± 7.0 vs. 99.0 ± 10.3 and 152.3 ± 7.3 vs. 160.8 ± 25.4 for glutamate and succinate, respectively). In addition, in the presence of succinate, a significant slowing of the state 3 respiration was observed in the 14-month-old rats (124.5 ± 8.8) compared to the 3-month-old rats (152.3 ± 7.3) ($P < 0.05$), while the ADP/O ratio showed no change in all groups tested. Fig. 2 shows that in the 14 month group, the O_2^- production by SMP at the level of the antimycin-inhibited region of the respiratory chain was strongly enhanced with respect to the 3 month group. This augmentation was less pronounced in the 18 month group with respect to the youngest group, whereas the O_2^- production in the 24 month group remained unchanged. Fig. 3 shows a significant increase of myocardial oxidized glutathione (GSSG) level at 14 and 18 months of age and no variation at 24 months of age with respect to the youngest group. A parallel decrease of reduced glutathione (GSH) content was observed only in the 14- and 18-month-old rats. Mitochondrial

TABLE I

Mitochondrial respiratory function (RCI) of rat hearts at 3, 14, 18 and 24 months of age

RCI, respiratory control index, calculated as ratio of oxygen consumed in the presence of ADP to that taken up after phosphorylation of ADP. The values represent the means ± S.D. of six separate experiments.

Age (months)	Glutamate	Succinate	Ascorbate
3	12.9 ± 0.9	3.38 ± 0.21	1.27 ± 0.06
14	12.6 ± 1.1	3.27 ± 0.54	1.19 ± 0.20
18	13.4 ± 0.8	3.88 ± 0.38	1.30 ± 0.09
24	15.9 ± 1.0 *	4.08 ± 0.42 *	1.41 ± 0.12

* Significantly different from the 3-month-old group mean.

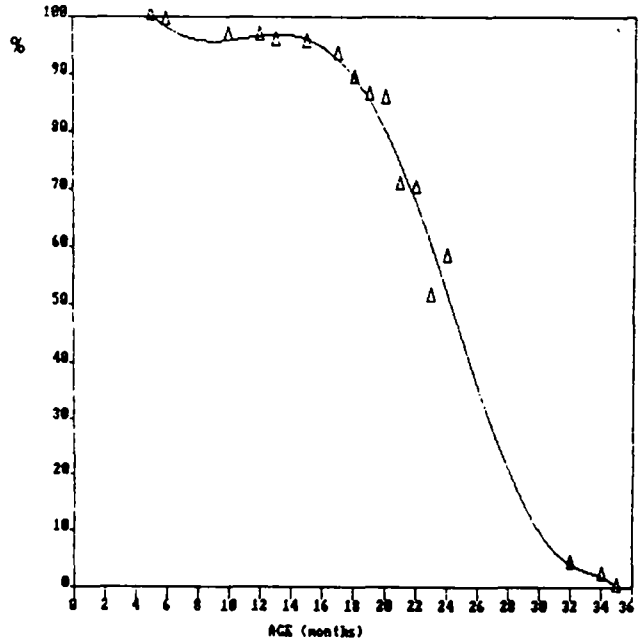

Fig. 1. Survivorship curve of the animal ageing colony. The rat survivorship data are expressed as percentage survival of a population of 100 male Wistar rats.

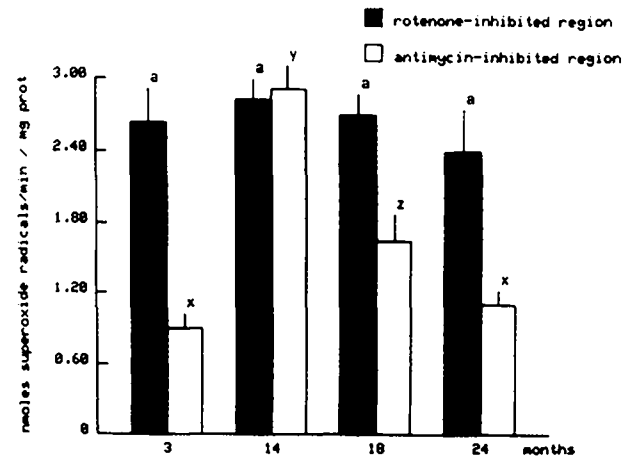

Fig. 2. Production of O_2^- by SMP from rat hearts at different months of age. The production of O_2^- was evaluated as described in Materials and Methods, by incubating the SMP with 1.5 μM rotenone or 2 μM antimycin. The data of the antimycin-inhibited region were calculated by subtracting the O_2^- rate determined in the presence of rotenone from those obtained with antimycin. The values represent the mean ± S.D. of four separate experiments. The SMP of each experiment were extracted from a pool of three hearts. Different letters denote significance between the mean values.

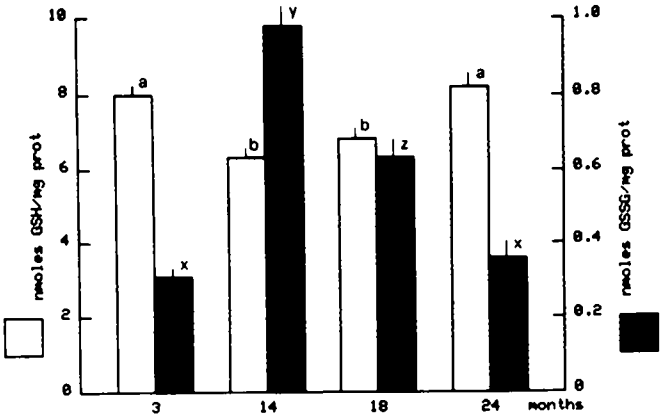

Fig. 3. Glutathione content of rat hearts at different months of age. GSH levels were calculated by subtracting the data of total glutathione from those of GSSG. Different letters denote significance between the mean values ($n = 6$).

and tissue levels of TBARS did not change during lifetime (data not shown), while a progressive accumulation of fluorescent products with age was observed either in the mitochondria or in the myocardial tissue after 14 months (Fig. 4).

Discussion

It has been described that in the aged heart there is an imbalance of the high phosphate charge level, with a decrease of the ATP/ADP ratio [23]. This event could be primary involved in the reduction of cardiac activity, particularly evident in the senescence during stress [1,2]. Nevertheless, our data indicate that the function of cardiac mitochondria isolated from aged rats is not decreased, on the contrary, there is an increased activity at 24 months of age. This result is in contrast with other studies which find that RCI values of cardiac mitochondria are unchanged [6] or decreased [5] in old rats compared with young rats. However, in accordance to most other reports, we observe that state 3 respiration is

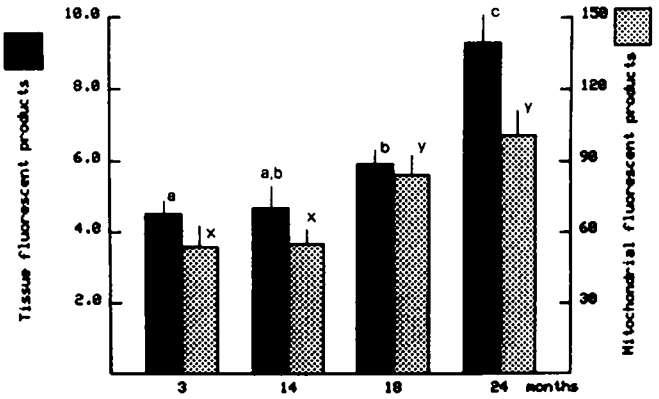

Fig. 4. Changes in fluorescent product levels of the rat heart during ageing (% fluorescence/mg protein). Different letters denote significance between the mean values ($n = 6$).

not increased in the animals aged 24 months [4,24,25] but that the higher RCI values are due to a reduction of state 4 respiration. A similar subtle increase in RCI has been reported in mitochondria isolated from hearts during the development of experimental cardiac hypertrophy [26]. Also in these conditions only state 4 respiration shows a moderate decline, while state 3 respiration remains unchanged. Thus, the increase of RCI that we found in the 24-month-old group in the presence of glutamate or succinate as substrates, in comparison to the 3 months group, may be interpreted as a functional adaptation of mitochondria to a condition of cardiac hypertrophy that occurs in aged animals [23]. The lack of age-dependent changes in mitochondrial phosphorylating activity suggests that the lower levels of ATP found in the cardiac muscle of old rats [23] may be due to a reduced availability of mitochondrial substrates. For example, decreased citrate synthase [27], isocitrate dehydrogenase [27] and malate dehydrogenase activites [28] have been described in the aged heart of various animals. Furthermore, Hansford [24] found a marked impairment of fatty acid oxidation caused by a reduction of both acyl-CoA synthase and 3-hydroxyacyl-CoA dehydrogenase activities and myocardial carnitine concentration. Other mitochondrial modifications such as reduction in adenine nucleotide translocase activity [29] and age related variations in the mitochondrial turnover [30–32] and shape [33] can be responsible in the imbalance of ATP/ADP in the cardiac muscle of senescent rats. Therefore, all these observations confirm the idea that in the heart muscle a reduction of mitochondrial biochemical processes, rather than a diminution of the mitochondrial phosphorylating activity, might be the event responsible for the impairment of cardiac energetics of the aged rats. Additionally, middle aged rats (14 months) exhibit some mitochondrial changes that are difficult to explain, but they could be important signals in the adaptation of the cardiac muscle during ageing. In fact we found an increased O_2^- mitochondrial production (Fig. 2), paralleled with an elevation of the tissue GSSG levels (Fig. 3) probably because glutathione reductase activity is very low in the heart muscle [34]. However, we can not exclude that the alteration of glutathione metabolism observed at 14 and 18 months of age could be caused by a modification in NADPH availability or glutathione peroxidase and reductase activities. These data seem to indicate that in the 14 month group the heart muscle supports a kind of oxidative stress, that according some recent observations might represent a signal to accentuate mitochondrial biogenesis [35] or protein turnover [36]. Both these events might favour the adaptation of the heart muscle toward a condition of compensative hypertrophy in response to the alterations of the cardiac vascular system caused by ageing. Moreover, in the 14-month-old group there was a reduction of the succinate-induced

respiration, probably caused by a specific deterioration of the complex II of the respiratory chain, while changes were not evident in glutamate-induced respiration as well as in the formation of peroxidative damage. Thus, the heart muscle in this period did not show signs of severe oxidative damage, while these effects, in the form of lipid fluorescent materials, were progressively evident from 18 to 24 months of age. The fact that TBARS content is not changed in our experimental conditions is in agreement with the observation that malondialdehyde is readily metabolized by mitochondria [37]. It is also possible that a low level of TBARS might be caused by a continuous conversion of TBARS into conjugated Schiff base products leading to enhanced levels of the fluorescent end products. It is significant that the major amount of fluorescent products is formed in the mitochondria beginning from 14 months of age, where the mitochondrial O_2^- production is more evident.

In conclusion, the present results suggest that the respiratory function of cardiac mitochondria isolated from rats at different ages is not substantially affected by the ageing process. However, the increase of mitochondrial oxidative stress observed in the middle age group (14 months) might represent a signal for the age-dependent accumulation of lipid peroxidative end-products in the cardiac tissue.

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