

DECREASED RESPIRATORY FUNCTION IN LIVER MITOCHONDRIA ISOLATED FROM HEALTHY OLD RATS

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Manuscript received: January 2014

Abstract

Mitochondrion dysfunction has been constantly associated with ageing yet a significant variation across animal species and investigated tissues has been reported. We have previously demonstrated that healthy ageing is associated with impairment in heart mitochondria function. The purpose of the present study was to compare mitochondrial respiratory function in liver mitochondria of from old (20-24 months) vs. adult (4-6 months) healthy rats. Mitochondria were isolated by differential centrifugations and the respiratory function was assessed at 37°C using a Clark-type oxygen electrode in the presence of NAD⁺-linked substrates (glutamate/malate) and a FAD-linked substrate (succinate in the presence of rotenone), respectively. Basal (State 2) and ADP-stimulated (State 3) respiratory rates were measured and expressed as natoms O₂/min/mg of mitochondrial protein. The respiratory control ratio (RCR) was calculated as the ratio of State 3 and State 2 respiratory rates. The intactness of mitochondria was verified by adding exogenous cytochrome *c*. Our data showed a significant decline in both State 2 and 3 respiratory rates in mitochondria isolated from old as compared to adult rodents when using either NAD⁺ (20.68 ± 2.43 vs. 24.46 ± 2.38, $p < 0.001$; 101.11 ± 5.40 vs. 121.60 ± 17.74, $p < 0.001$, respectively) or FAD⁺ (33.55 ± 3.28 vs. 42.75 ± 1.83, $p < 0.0001$; 123.92 ± 5.02 vs. 140.69 ± 15.37, $p < 0.001$, respectively) linked substrates. In conclusion, the impairment of respiratory parameters in the senescent group supports the hypothesis that a decrease of respiratory function in liver mitochondria occurs during healthy ageing.

Rezumat

Existența disfuncției mitocondriale asociată procesului de îmbătrânire este unanim acceptată, dar există o variabilitate semnificativă în funcție de specie și de țesutul investigat. Am demonstrat într-un studiu anterior că funcția mitocondriilor cardiace este alterată la șobolanii bătrâni sănătoși. Scopul acestui studiu a fost de a compara funcția respiratorie a mitocondriilor hepatice izolate de la șobolani bătrâni (20-24 luni) și respectiv, adulți (4-6 luni). Mitocondriile hepatice au fost izolate prin tehnica centrifugărilor diferențiale și respirația mitocondrială a fost măsurată cu ajutorul unui electrod de oxigen de tip Clark la 37°C în prezența substratelor NAD⁺-dependente (glutamat/malat) și respectiv, FAD-dependente (succinat, în prezența rotenonei). Respirația bazală (Stadiul 2) și cea stimulată cu ADP (Stadiul 3) au fost înregistrate și exprimate ca natomi de O₂ consumați per minut și per mg proteine mitocondriale. Integritatea membranei externe mitocondriale a fost testată prin adăugarea citocromului *c*. Indicele respirator sau raportul controlului respirator (RCR) a fost calculat ca raportul dintre rata respiratorie în Stadiul 3 și respectiv 2. Rezultatele noastre au indicat o scădere semnificativă atât a respirației bazale (Stadiul 2), cât și a celei active (Stadiul 3) la șobolanii senescenti comparativ cu cei adulți, atât în prezența substratelor NAD⁺-dependente (20.68 ± 2.43 vs. 24.46 ± 2.38, $p < 0.001$; 101.11 ± 5.40 vs. 121.60 ± 17.74, $p < 0.001$), cât și a celor FAD⁺-dependent (33.55 ± 3.28 vs. 42.75 ± 1.83, $p < 0.0001$; 123.92 ± 5.02 vs. 140.69 ± 15.37, $p < 0.001$). În concluzie, alterarea parametrilor respiratori la nivelul mitocondriilor hepatice izolate de la șobolanii senescenti pledează pentru o scădere a funcției mitocondriale asociată îmbătrânirii fiziologice.

Keywords: rat liver mitochondria, ageing, respirometry

Introduction

The multifactorial process of ageing is characterized by a gradual impairment of body functions and tissue homeostasis [1-3]. The hallmarks of ageing at

cellular level are a higher number of mtDNA mutations or deletions, an increase in lipid peroxidation, accumulation of nondigested debris and higher levels of reactive oxygen species (ROS) [4].

Mitochondria are the major intracellular sources of ROS and also the main targets of their harmful effects. Cellular ROS are common by products of respiration mostly generated at the level of respiratory chain complexes I and III [5-7] that are responsible, when excessively produced, for oxidative damage [5].

Known for more than half century as the "power-houses" of the cells [5, 8], mitochondria have also emerged as the major culprit responsible for ageing that has been associated with both an increased production of free radical species and a decline in mitochondrial respiratory function [9, 10].

Impairment of mitochondrial respiratory function occurring with ageing is still a matter of debate. Several studies showed that State 3 (active respiration after adenosine diphosphate (ADP) addition) measured in the presence of different substrates such as: glutamate, malate, succinate, is decreased in liver mitochondria isolated from aged rats whereas State 2 (basal respiration measured after addition of substrates) showed no changes [11, 12]. Other studies confirmed the decrease of active respiration, but reported a decline in the basal respiration, too [13, 14]. Finally, there are reports in the literature where State 3 was found to be not influenced by ageing in different tissues, including liver [15, 16].

We have recently demonstrated a decline of bioenergetics parameters for both complex I and complex II supported-respiration, and an increased mitochondrial ROS production in rat heart mitochondria isolated from old vs. adult rats [17]. Moreover, one of the most important criticisms addressed to preclinical studies is related to the fact that they are conducted in healthy young animals, without considering the role of ageing and comorbidities [18]. Therefore, the purpose of the present study was to assess the parameters of respiratory function in rat liver mitochondria isolated from old vs. adult rats.

Materials and Methods

Animals

All procedures and experimental protocols used in this study are in agreement with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Research Ethics of "Victor Babeş" University for Medicine and Pharmacy of Timișoara, Romania. Animals were fed *ad libitum* and kept in the University animal facility under standard conditions (constant temperature of $22.5 \pm 2^\circ\text{C}$ and relative humidity of $55\% \pm 5\%$, 12 h (light) – 12 h (dark) cycle). Twenty-four hours prior

to the experiment, solid food was withdrawn, but there was no limitation in water supply. Two groups of male Sprague–Dawley (SD) rats were used: the adult group (4–6 months, $n = 6$) and the old group (20–24 months, $n = 8$).

Reagents

All reagents used in the study were of the highest quality available and were purchased from Sigma and Merck Chemicals.

Mitochondria isolation

Rat liver mitochondria (RLM) were isolated by differential centrifugation at 4°C , in compliance with a previously described method [19]. In brief, the animal was anesthetized using an intraperitoneal injection of ketamine (Vetased[®], 30 mg/kg body mass) and xylazine (Xylazin[®], 10 mg/kg body mass), in the following step the liver was quickly removed, discarded of connective tissue, fat and blood and rinsed in ice-cold 0.9% KCl solution. Afterwards it was minced to obtain fragments smaller (< 0.5 cm) in a Petri dish and manually homogenized in a glass-teflon pestle homogenizer in 25 mL Buffer 1 (210 mM mannitol, 70 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 125 mg bovine serum albumin (BSA), 5 mg/mL and 0.25 mL ethylene glycol tetra acetic acid (EGTA) 1 mM, pH = 7.4).

Liver mitochondria were isolated by differential centrifugation (Rotina 38R, Hettich) technique according to the following protocol: the first centrifugation (750xg for 5 min at 4°C) was performed in order to discard the nuclei and unbroken cells. The supernatant, obtained by filtration, which contained the mitochondrial fraction, was further centrifuged at 7000xg for 10 min at 4°C . The resulting mitochondrial pellet was cleansed in Buffer 2 (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH = 7.4). The final centrifugation was performed at 7000xg for 10 min at 4°C and the final mitochondrial pellet was gently resuspended in 0.25 mL Buffer 2 and kept on ice throughout the experiment. Protein concentration was determined by means of the Biuret method [20] after mitochondria solubilisation with 1% deoxycolate, using BSA as standard.

Oxygen consumption measurements

Mitochondrial oxygen consumption was measured at 37°C using a Clark-type oxygen electrode (Strathkelvin 782 Oxygen System). All measurements were realized using mitochondria suspension (1.0 mg mitochondrial protein/mL) in the incubation medium (100 mM KCl, 2 mM KH_2PO_4 , 10 mM HEPES and 1 mM MgCl_2 , pH=7.4). Oxygen consumption rates were expressed as natoms of O_2 / minute / mg of mitochondrial protein.

Basal respiration (State 2) was started after the addition of either complex I dependent-substrates (glutamate 5 mM and malate 5 mM) or the complex II dependent-substrates (succinate 15 mM). Before the addition of succinate (complex II dependent-substrate), rotenone (1 mM) was added to inhibit complex I respiratory activity and to guarantee that the energy flux through the electron transport chain is controlled through FADH₂-linked substrates, i.e. succinate [21]. Active respiration (State 3) was measured after addition of ADP (3 mM). The addition of oligomycin (2 µg/mL) led to the suppression of oxidative phosphorylation by inhibiting ATP synthase activity. In order to verify the uncoupling of oxidative phosphorylation it was used FCCP (carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone, 0.1 mM) stepwise titration. The integrity of the outer mitochondrial membrane was evaluated after addition of exogenous cytochrome c (32 µM). An increase of the respiratory rates after the exogenous addition of cytochrome c was correlated to the effluence of endogenous cytochrome c due to the damage of outer mitochondrial membrane during the isolation process.

Respiratory control ratio (RCR) was calculated as the ratio of State 3 and 2 respiratory rates and was correlated with the relationship between mitochondria respiration and oxidative phosphorylation [13].

Statistical analysis

The differences between groups were determined using Student's unpaired *t*-test. Results are presented as means ± SEM. Values for *p* < 0.05 were considered statistically significant.

Results and Discussion

Impaired Complex I-Supported Respiration in Old vs. Adult Rats

The effect of ageing on mitochondrial respiratory parameters in the presence of complex I-dependent substrates was depicted in Figure 1. A significant decline in State 2 respiratory rate (Figure 1A) and of ADP phosphorylation rate (Figure 1B) using glutamate and malate as substrates, were found in the old vs. the adult group. No significant changes were found for RCR between the two analysed groups (data not shown).

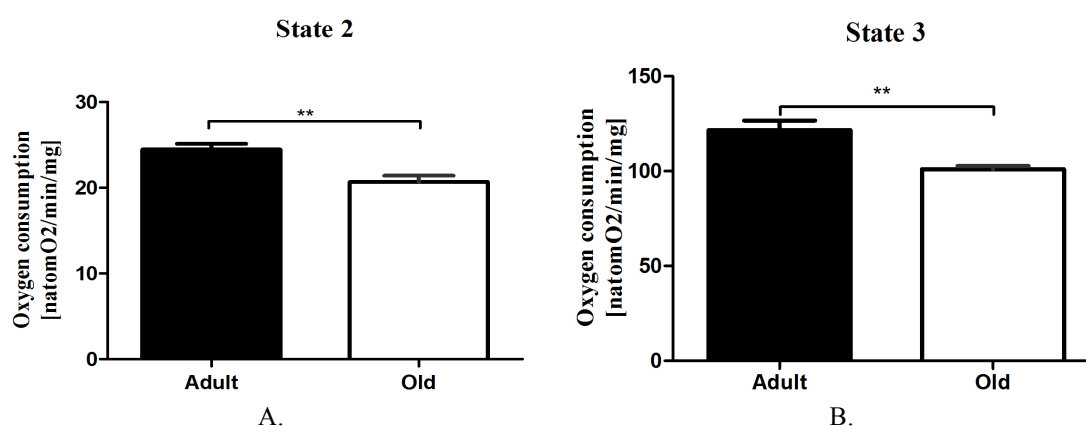


Figure 1.

Respiratory parameters for complex I-dependent respiration. State 2 (A) and State 3 (B) respiratory rates (means ± SEM) are significantly decreased in the old group (n = 8) vs. the adult group (n = 6). ** *p* < 0.001.

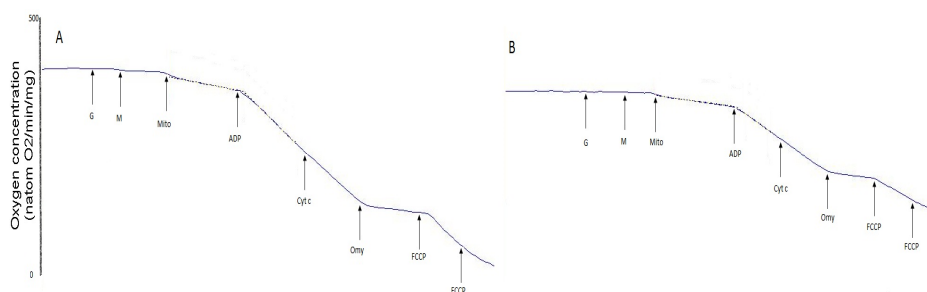


Figure 2.

Representative recordings of oxygen consumption in RLM isolated from adult (A) and old (B) rats in the presence of complex I dependent-substrates glutamate (G) and malate (M). The additions are indicated with arrows. Addition of mitochondria (Mito) induced the basal respiration whereas active respiration was stimulated via the addition of ADP. The subsequent additions were: cytochrome c (Cyt c), oligomycin (Omy) and finally, FCCP in titration. All the measurements were registered at 37°C.

Figure 2 shows representative recordings of original measurements of respirometry in adult (Figure 2A) vs. old (Figure 2B) RLM. The addition of mitochondria (1 mg/mL) in the incubation medium supplemented with complex I dependent-substrates (glutamate and malate) induced an initial decrease of oxygen concentration (basal respiration). ADP addition in the electrode chamber led to a sharp and marked decrease of oxygen concentration that indicated the stimulation of oxidative phosphorylation and continued until all the ADP was phosphorylated. This state can be limited by the addition of oligomycin. Finally, titration with the uncoupler FCCP elicited the highest rate of oxygen consumption. *Impaired Complex II-Supported Respiration in Old vs. Adult Rats*

Complex II-dependent respiration was assessed in the presence of succinate, a substrate metabolized

by succinate dehydrogenase which is a part of complex II (succinate-Q reductase complex) of the electron transport chain [9]. Thus complex II receives FADH_2 directly from succinate dehydrogenase being only dependent on the mitochondrial tricarboxylic acid (TCA) cycle [19]. As for complex I-supported respiration, oxygen consumption values recorded in the presence of succinate were significantly lower in the aged group as compared to the adult one (Figure 3). A more important decrease in State 2 respiratory rate was recorded in the case of complex II-supported respiration as compared to the complex I-supported one (Figure 3A). Active respiration showed also a statistically significant decline in senescent vs. adult group in the presence of succinate (Figure 3B).

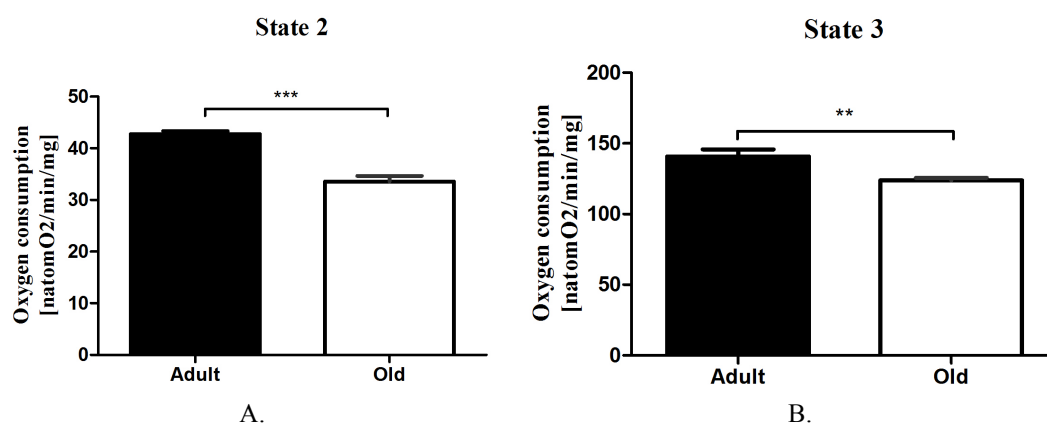


Figure 3.

Respiratory parameters for complex II-dependent respiration. State 2 (A) and State 3 (B) respiratory rates (means $\pm$ SEM) are significantly decreased in the old group (n = 8) vs. the adult group (n = 6). ** $p < 0.001$, *** $p < 0.0001$

There is a clear relationship between mitochondria and ageing considering the reported decrease in basal metabolic rates that occur with the ageing process [22, 23]. The present study aimed to assess whether changes in rat liver mitochondria respiratory function occur with healthy ageing in the SD strain of rats. As oxidizing substrates we used glutamate-malate and succinate due to their capacity to support maximum rates of respiration and, adequacy to reveal age-dependent changes in rates of electron transport or phosphorylation [11]. Our data indicated that a significant decline in mitochondrial respiratory function occurs in healthy aged SD rats as compared to the adult ones regardless the substrate used, glutamate/malate or succinate. The observed decrease in active respiration is in agreement with the findings described by Horton et al. [11] and Modi et al. [14]. Modi and co-workers reported a diminished oxidative phosphorylation not only in liver mitochondria, but also in brain mitochondria in the presence of glutamate and succinate and suggested

that impairment in oxidation of glutamate and succinate may be a primary event in ageing [14]. Tummino et al. proposed, as a literature based-explanation for the decrease of OXPHOS state, a possible non-specific leakage of ADP from the mitochondrial matrix due to age-related changes in membrane composition [12].

We also reported a significant decrease in the basal respiration, more important for succinate-supported respiration as compared to the glutamate/malate driven one in the old healthy animals. This observation confirms the early results of the group of Charles Hoppel [24] obtained in aged skeletal muscle mitochondria.

Changes in RCR values are dependent on modifications of either State 3 or State 2. No RCR changes were found between the groups when glutamate and malate were used as substrates. However, since succinate-supported basal respiration dramatically decreased in the aged group, while the active respiration state also decreased, but not so sharply, RCR values were

higher in the aged vs. adult animals (data not shown).

However, it is not clear so far whether mitochondrial dysfunction has a causal or a protective role in ageing; indeed, the role of oxidative stress in ageing has been recently questioned [25].

Conclusions

In conclusion, the impairment of respiratory parameters in the senescent group supports the hypothesis that a decrease of respiratory function in liver mitochondria occurs during healthy ageing.

Acknowledgements

Research supported by the Hungarian-Romanian cross-border project code HURO/1101/086/2.2.1. This paper was published under the frame of European Social Found, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/136893.

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