Age-Dependent Changes of Mitochondrial Functions in Ca\(^{2+}\)-Induced Opening of Permeability Transition Pore\(^1\)


Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow region, 142290 Russia; e-mail: tazarash@rambler.ru

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Abstract—Mitochondria are intracellular organelles, which provide cells with energy and participate in multiple processes of cell vital functions. Within one of the numerous theories of aging, dysfunction of mitochondria is considered to lead to tissue degeneration and induce the initial stage in developing of degenerative diseases. Since mitochondria play a clue role in apoptosis/necrosis processes, it was suggested that dysfunction of mitochondria observed under aging is related with disturbance of programmed cell death regulation. In the present study, a comparative examination of parameters of the functional states of mitochondria isolated from young (2–3-months old) and old (20–22-months old) rats under conditions of opening of unselective pore (PTP, permeability transition pore) has been performed. Ca\(^{2+}\) accumulation rate in mitochondria isolated from old rats was found to be decreased by 25–30%, threshold calcium concentration was lowered to 50%, and the swelling of mitochondria loaded by calcium was stimulated 3–4-fold. Production of reactive oxygen species (ROS) has been also determined in these mitochondria. In old mitochondria superoxide anion level was increased. In addition, H\(_2\)O\(_2\) content was found to be 2 times higher in mitochondria with PTP opened. Using electron microscopy method, a decreased amount of cristae in mitochondria was revealed under aging.

Key words: mitochondria, age-dependent changes, permeability transition pore, aging

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According to one of the theories of aging, mitochondria are the pacemakers of tissue and cell aging due to the continuous production of oxygen free radical species (ROS) and selective oxidative damage that leads to mitochondrial dysfunction and accumulation of damages in a cell. Mitochondria are the main source of ROS and a clue site of apoptosis initiation. Therefore, the free radical theory of aging considers the mitochondria functioning as a particular case of realization of this mechanism [1–9]. Mitochondria have their own apparatus of DNA repair and yet mitochondrial DNA is rather sensitive to damages by exogenous and endogenous agents, which are usually free radicals. Mitochondrial DNA was found to be close to the inner mitochondrial membrane, where mitochondrial respiratory complexes are located and ROS production takes place determining DNA damages. Besides, the amount of histones in mitochondria is not enough for preventing DNA from damages. Mutations in DNA play an important role in damages of neurons as well as in development of neurodegenerative diseases [1–5]. In 1982, Umansky supposed that aging can be a consequence of programmed cell death, apoptosis [8]. It turned out that aging of many cell types is related with changes in their sensitivity to apoptosis. As mitochondria play a clue role in apoptosis/necrosis, it was suggested that mitochondrial dysfunction associated with aging is linked with disturbances of apoptosis regulation [3–15]. Dysfunction of mitochondria in liver and brain can occur also as a result of diminished activities of respiratory complex I and IV due to a decreased rates of electron transfer along respiratory chain [13, 14] as well as lowering of ATP production due to a decreased ATP synthase activity [12, 15–17]. Mitochondria isolated from the brain of aged animals show an increased accumulation of the oxidation products of phospholipids, proteins, and DNA [9]. Mutations in DNA could lead to defects in protein synthesis and disturbance of ion channel expression as well as the changes of the activity of non-mutated channels. Dysfunction of mitochondrial channels such as ATP-sensitive K\(^+\) channel, Ca\(^{2+}\) carriers, voltage-dependent anion channel (VDAC), and permeability transition pore (PTP) was reported to be a reason of mitochondrial channelopathies that are able to accelerate the aging process [18]. Mitochondria isolated from old rats are characterized by lowered respiratory control, inability to accumulate calcium and retain it in the matrix, decreased membrane potential as well as increased capacity to swelling [12, 18].
It is known that initiation of apoptosis in a cell correlates with an increase of permeability of the inner mitochondrial membrane. Formation and opening of Ca\(^{2+}\)-induced CsA-sensitive nonselective pore occurs in the contact sites between the outer and inner membrane of mitochondria in the presence of the threshold Ca\(^{2+}\) concentrations in matrix or in response to oxidative stress. As a result of the PTP opening, ions and Ca\(^{2+}\) can penetrate through the inner membrane, and pro-apoptotic proteases, such as cytochrome c, apoptosis-induced factor (AIF), endonuclease G, can be released from the intermembrane space [21]. Hence, PTP opening is characterized by Ca\(^{2+}\)-release from mitochondria, accompanied with depolarization of the inner membrane, swelling, and a release of pro-apoptotic proteins.

Importance of mitochondria in the aging is doubtless (see reviews of Crompton [22]); however, the role of PTP in these processes is not fully understood. Rotenberg et al. showed that mitochondria in lymphocytes isolated from spleen of 24-month-old mice were more sensitive to activation of PTP [19]. Similar effect was observed in isolated liver and brain mitochondria of old hybrid B6D2F1 mice [20].

In the present work, the parameters of mitochondrial functional state of rat liver and brain mitochondria from young (2–3 months) and old (20–22 months) rats under the PTP opening were compared. Besides, for the first time the PTP parameters (threshold calcium concentrations initiating PTP opening, mitochondrial swelling, and ROS production) have been detected simultaneously.

**EXPERIMENTAL**

Mitochondria were isolated from liver and brain of the same animal using young (mature) 2–3 months old and aged (22–23-months old) rats.

**Isolation of brain mitochondria.** Mitochondria were isolated by method of Sims [23], modified in our laboratory. Rat brains were rapidly (within 30 s) removed and placed in ice-cold solution, containing 0.32 M sucrose, 0.5 mM K\(^+-\)EDTA, 0.5 mM EGTA, 0.02% BSA (fraction V), and 10 mM Tris-HCl (pH 7.4). All manipulations were carried out at 4°C. The tissue was homogenized in a glass homogenizer; the ratio of brain tissue to isolation medium was 1 : 10 (w/v). The homogenate was centrifuged at 2000 g for 3 min. The pellet of mitochondria was purified by centrifugation of the 2000 g supernatant at 12500 g for 10 min. At the next step, mitochondria were purified on Percoll gradient (15%–23%–40%) according to the procedures published in [23]. Rat brain mitochondria were suspended in ice-cold solution containing 0.32 M sucrose and 10 mM Tris-HCl (pH 7.4) and were additionally washed by centrifugation at 11500 g for 10 min. The protein concentrations in the stock mitochondrial suspensions were 25–30 mg/ml [24].

**Rat liver mitochondria** were isolated by standard procedure with differential centrifugation. Homogenization medium contained 250 mM sucrose, 70 mM mannitol, 10 mM Tris-HCl buffer (pH 7.4), 0.01% BSA, and 1 mM EGTA. BSA and EGTA were omitted from the second washing solution. Protein concentration in final mitochondrial suspensions (in solution without BSA and EGTA) was 60–65 mg per ml.

**Evaluation of mitochondrial functions.** The mitochondrial membrane potential was measured as described earlier [23] by determining the distribution of tetrathenylphosphonium (TPP\(^{+}\)) in the incubation medium with a TPP\(^{+}\)-selective electrode. Ca\(^{2+}\) transport was determined with a Ca\(^{2+}\)-sensitive electrode (Nico, Russia) [25].

Mitochondria (1.0 mg protein/ml) were incubated in the medium containing 125 mM KCl 10 mM Tris-HCl, 0.4 mM K\(_2\)HPO\(_4\), and 5 mM rotenone, pH 7.4, at 25°C. Succinate (5 mM potassium succinate) was used as mitochondrial respiratory substrate. PTP opening in rat brain mitochondria was induced by a threshold Ca\(^{2+}\) load (each addition of Ca\(^{2+}\) contained 50 n mole Ca\(^{2+}\) per mg protein). Rat liver mitochondria (1.0 mg protein/ml) were incubated in the medium containing 2 mM K\(_2\)HPO\(_4\) (instead of 0.4 K\(_2\)HPO\(_4\)) and PTP opening was induced by addition 50 nanomoles Ca\(^{2+}\) per mg. All experiments were performed in an open chamber.

Swelling of rat liver mitochondria was measured as a change in scattering of mitochondrial suspension absorbance at 540 nm (A540) using Specord M-40 spectrophotometer at 25°C. Standard incubation conditions for swelling assay were 125 mM KCl, 10 mM Tris, 2 mM KH\(_2\)PO\(_4\), 5 mM succinate, 0.5 µM oligomycin, 5 µM rotenone. The concentration of protein in the chamber was 0.5 mg protein/ml.

**Measurements of ROS production in mitochondria.** The production of superoxide anion was assessed using a selective chemiluminescent superoxide probe, 3,7-dihydro-2-methyl-6-(4-methoxyphenyl)imidazo[1,2-a]pyrazine-3-one (MCLA) [26]. The production of hydrogen peroxide was measured using fluoroscent probes dihydrofluorescein and 2,7-dichlorofluorescein diacetate. Mitochondria (0.3 mg protein/ml) were suspended in standard medium (100 µl) with succinate and rotenone (final concentrations 5 mM and 1 µg/ml, respectively). Medium also contained 5 µM MCLA (superoxide measurements) or 2 U/ml of horseradish peroxidase plus 20 µM 2,7-dichlorofluorescein diacetate or dihydrofluorescein (hydrogen peroxide measurements). Chemiluminescence and fluorescence was traced for 40 min with the Victor 3TM multiplate reader in 96 well plates at 30°C. Calcium (50–100 nmol/mg protein) and other substances were added on the third minute of the incubation. FCCP (500 nM) and hydrogen peroxide (0.01 µM–1 mM) were used for creation of calibration curves in each experiment.