ISSN 0006-2979, Biochemistry (Moscow), 2024, Vol. 89, No. 2, pp. 299-312 © Pleiades Publishing, Ltd., 2024.

Age-Dependent Changes in the Production of Mitochondrial Reactive Oxygen Species in Human Skeletal Muscle

Mikhail Yu. Vyssokikh[1,2,3,a]*, Maksim A. Vigovskiy[4], Vladislav V. Philippov[4], Yakov R. Boroday[4], Mariya V. Marey[2], Olga A. Grigorieva[4], Tatiana F. Vepkhvadze[3], Nadezhda S. Kurochkina[3], Ludmila A. Manukhova[2], Anastasiya Yu. Efimenko[4], Daniil V. Popov[3], and Vladimir P. Skulachev[1#]

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia*

[2]*National Medical Research Center for Obstetrics, Gynecology and Perinatology Named after Academician V. I. Kulakov, 117997 Moscow, Russia*

[3]*Institute of Biomedical Problems, Russian Academy of Sciences, 123007 Moscow, Russia*

[4]*Medical Research and Education Center, Lomonosov Moscow State University, 119192 Moscow, Russia*

[a]*e-mail: mikhail.vyssokikh@gmail.com; mike@genebee.msu.ru*

Received December 12, 2023
Revised January 30, 2024
Accepted February 1, 2024

Abstract—A decrease in muscle mass and its functionality (strength, endurance, and insulin sensitivity) is one of the integral signs of aging. One of the triggers of aging is an increase in the production of mitochondrial reactive oxygen species. Our study was the first to examine age-dependent changes in the production of mitochondrial reactive oxygen species related to a decrease in the proportion of mitochondria-associated hexokinase-2 in human skeletal muscle. For this purpose, a biopsy was taken from m. vastus lateralis in 10 young healthy volunteers and 70 patients (26-85 years old) with long-term primary arthrosis of the knee/hip joint. It turned out that aging (comparing different groups of patients), in contrast to inactivity/chronic inflammation (comparing young healthy people and young patients), causes a pronounced increase in peroxide production by isolated mitochondria. This correlated with the age-dependent distribution of hexokinase-2 between mitochondrial and cytosolic fractions, a decrease in the rate of coupled respiration of isolated mitochondria and respiration when stimulated with glucose (a hexokinase substrate). It is discussed that these changes may be caused by an age-dependent decrease in the content of cardiolipin, a potential regulator of the mitochondrial microcompartment containing hexokinase. The results obtained contribute to a deeper understanding of age-related pathogenetic processes in skeletal muscles and open prospects for the search for pharmacological/physiological approaches to the correction of these pathologies.

DOI: 10.1134/S0006297924020093

Keywords: aging, skeletal muscle, mitochondria, mitochondrial reactive oxygen species, hexokinase

INTRODUCTION

Aging is a complex process inherent in all organisms that includes a number of common features, in particular, a progressive decrease in the functional and regenerative capabilities of the organism, developing against the background of impaired adaptation to external and internal stressors, and ultimately leading to death. One of the most developed theories of aging is the mitochondrial theory, first proposed by Harman in 1956, according to which the trigger of aging is considered to be mitochondrial dysfunction associated with their production of reactive oxygen species and oxidative damage to biological macromolecules [1].

Abbreviations: mROS, mitochondrial reactive oxygen species.

* To whom correspondence should be addressed.

Deceased.

One of the integral signs of aging is a decrease in the mass and strength of skeletal muscles, leading to a pronounced decrease in physical performance and quality of life. The last of the above is due to the fact that skeletal muscles not only provide movement and maintain posture, but also play an important role in regulating the metabolism of the organism. Thus, normally, skeletal muscles, constituting up to 40% of body mass, consume from 20 to 30% of the energy produced [2] and are one of the main insulin-dependent consumers of glucose [3]. Unlike other tissues, decline in skeletal muscle mitochondrial function is thought to be a major mediator of declines in muscle mass and strength in older adults [4].

The most important role in the regulation of metabolism and consumption of glucose from the blood [5] is played by the enzyme hexokinase (EC 2.7.1.1), which catalyzes the rate-limiting reaction of glycolysis and transfers the phosphoryl from ATP to glucose with the formation of glucose-6-phosphate, an intermediate of glycolysis and the pentose phosphate pathway. In muscles, as in other insulin-sensitive tissues, I and II of the five known isozymes of hexokinase are predominantly expressed; they have high similarity in amino acid sequences, however, they differ quite significantly in kinetic parameters and metabolic functions (for review, see [6]). In skeletal muscle, most hexokinase is localized on the surface of mitochondria, forming a metabolic compartment that uses ATP synthesized in mitochondria to phosphorylate hexoses, which stimulates mitochondrial respiration and functionally couples glycolysis with oxidative phosphorylation [7]. The binding of hexokinase to mitochondria is mediated by protein–protein interactions with VDAC1, the main protein of the outer membrane of mitochondria, which ensures the transport of various compounds between mitochondria and the rest of the cell [8]. The formation of this protein complex leads to the convergence of mitochondrial membranes and stabilization of the so-called contact sites, which are the structural basis of the metabolic microcompartment of mitochondria, responsible for the shuttle movement of ATP/ADP between the active centers of ATP synthase and hexokinase [9, 10].

Approximately 1-2% of molecular oxygen in the cell, with the participation of complexes I and III of the respiratory chain, is converted into superoxide, spontaneously or with the help of enzyme systems that regulate the production of hydrogen peroxide with the subsequent formation of mitochondrial reactive oxygen species (mROS), which can damage biological macromolecules [11]. It has been established that an imbalance in the production and utilization of ROS (reactive oxygen species) underlies oxidative stress and the induction of programmed cell death, which is one of the key reasons for the development of cardiovascular and neurodegenerative pathologies [12]. It is important to note that a slight decrease in mitochondrial membrane potential almost completely stops the generation of mROS [13]. In particular, in isolated mitochondria, activation of oxidative phosphorylation by the addition of ADP led to a decrease in transmembrane potential by a small amount – approximately 20% (mild depolarization), preventing the formation of H_2O_2. Investigating this effect, A. Galina with colleagues described a new antioxidant mechanism consisting in the cyclic movement of ADP, formed during phosphorylation of glucose at the expense of ATP, produced during oxidative phosphorylation and reaching the active center of hexokinase through the VDAC1 channel [14].

Previously, using models of short- and long-lived rodents, we showed that mild depolarization of mitochondria, associated with activation of bound hexokinase, leads to a decrease in the production of mROS in various tissues (including skeletal muscles), and disruption of this mechanism correlates with the age of short-lived animals [15]. The purpose of this study was to investigate the age-related distribution of hexokinase, as well as the dependence of the rate of hydrogen peroxide production by mitochondria on the activation of mitochondrial hexokinase by its substrates in human skeletal muscle.

MATERIALS AND METHODS

Study design. The study was approved by the Biomedical Ethics Committee of the State Scientific Center of the Russian Federation – Institute of Biomedical Problems of the Russian Academy of Sciences and the Local Ethics Committee of the Lomonosov Moscow State University (protocol no. 2/20 dated March 16, 2020). All volunteers signed a voluntary consent to participate in the study.

The study involved 10 healthy volunteers (age from 25 to 43 years) and 62 patients with long-term primary arthrosis of the knee/hip joint (age from 26 to 85 years), divided into groups of young (n = 8, 39 (26-45) years), middle (n = 20, 59 (58-62) years) and elderly (n = = 42, 72 (66-83) years) age (Table 1). This pathology was previously used as a model to study the effects of decreased physical activity and chronic inflammation on the skeletal muscles of the thigh, including during aging [16-19]. The criteria for non-inclusion of volunteers into the study were: a history of cancer or systemic diseases; mental, physical, and other reasons that do not allow you to adequately assess behavior and correctly comply with the conditions of the research protocol; a history of any significant, in the opinion of the research physician, condition/disease or circumstance that prevents inclusion in the study; contraindications

for surgical treatment at the time of inclusion; pregnancy and lactation. All participants had an anamnesis collected and the subjective level of physical capabilities (questionnaire SF12 [20]) and fasting blood glucose and insulin levels were assessed. A biopsy of the lateral head of the quadriceps muscle (~500 mg) was taken from patients at the start of elective hip/knee replacement surgery. In healthy volunteers, samples from the vastus lateralis muscle (~200 mg) were collected by needle biopsy with aspiration under local anesthesia (1 ml of 2% lidocaine) as previously described [21]. The muscle tissue sample was immediately placed into a tube containing a cardioplegic solution (Custodiol, USA); after 10 min, part of the sample was taken for histological and biochemical studies (see below), and the remaining part was transferred to a fresh aliquot of Custodiol solution to isolate mitochondria.

Histological analysis. Skeletal muscle samples were fixed in 10% buffered neutral formalin for 24-48 h. Histological processing was carried out according to standard methods using 8 changes of isopropyl alcohol (total duration – 5.5 h at 37°C) and 3 changes of paraffin (total duration – 5 h at 62°C). Then the preparations were embedded in paraffin blocks, sections 1 μm thick were prepared and mounted on glass slides (Menzel GmbH & Co KG, Germany). Staining was performed according to standard techniques using Mayer's hematoxylin and eosin (PanReac AppliChem, Spain). Microscopic examination was carried out on a Leica DM600B microscope with a Leica DFC 420X camera (Leica Microsystems GmbH, Germany), using representative fields of view to obtain microphotographs. Image processing and analysis were performed using LasX software (Leica Microsystems GmbH) and FiJi.

Isolation of mitochondria. Mitochondrial isolation was performed as described previously [15]. Biopsy samples of muscle tissue were rinsed with an ice-cold solution of 0.9% NaCl, dried with filter paper, weighed, and fragmented into pieces of 0.5-1 mm in size with cooled and washed with the isolation medium (see below) using scissors with curved ends. The resulting fragments were homogenized using a Potter microhomogenizer (glass/Teflon) with a clearance of 200 microns for 2 min at 4°C, in a ratio of 10/1 (volume/mass) in an isolation medium of the following composition: 300 mM mannitol, 0.5 mM EGTA, 20 mM HEPES/ NaOH, pH 7.6 and 0.1% BSA. The homogenate was centrifuged at 1000*g* for 10 min at 4°C on a 5410 centrifuge (Eppendorf, Germany). The supernatant was collected and centrifuged at 9000*g* under the same conditions. The pellet was suspended in the same volume of isolation medium without BSA (by a microhomogenizer) and centrifuged at 10,500*g* for 10 min and 4°C. The resulting pellet was suspended in a minimal volume, the typical protein concentration of the resulting mitochondrial preparation was 90-100 mg/ml. The protein content in mitochondrial preparations was determined with bicinchoninic acid and 1 mg/ml BSA solution as a standard according to manufacturer's instructions (Pierce, USA). All procedures for the isolation and storage of mitochondria during the experiments were carried out at 4°C.

Respiratory rate measurement. The rate of oxygen consumption by mitochondria was measured at 30°C using a closed-type Clark electrode on an oxygraph Hansatech (UK), as previously described [15, 22]. Mitochondria (0.05-0.1 mg protein) were incubated in an oxygraph cell containing 0.5 ml of MIR05 respiratory medium [23] (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/liter bovine serum albumin (free from fatty acids), 100 μM potassium diadenosine pentaphosphate) and assessed respiration efficiency in the presence of 5.5 mM pyruvate/malate or 10 mM succinate / 2 μM rotenone, 1 μM oligomycin, 0.1 mM ADP, 5 mM glucose, 10 nM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (all Sigma-Aldrich, USA).

Measuring the rate of peroxide production. The rate of hydrogen peroxide production by mitochondria was estimated using the method of Chow et al. [24] with the modifications we described earlier [25, 26]. For registration of H_2O_2 production Amplex reagent Red (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen, USA) was used. Resorufin, a product of H_2O_2-induced oxidation of Amplex Red, was measured by monitoring its fluorescence (excitation/emission maxima ~550/595 nm) on a spectrophotometer Cary Eclipse (Agilent, USA) for 10-15 min. Mitochondria (0.15 mg/ml mitochondrial protein) were incubated at 37°C with stirring with a built-in magnetic stirrer in MIR05 medium containing 5 μM Amplex Red, horseradish peroxidase (12 units/ml, Sigma, USA) and superoxide dismutase (45 units/ml, Sigma, USA). The reaction was initiated by adding 10 mM succinate, after reaching the maximum rate of peroxide production, ADP (up to 0.1 mM final concentration) and glucose (final concentration 5 mM) were added. The rate of H_2O_2 production was calculated from the change in fluorescence intensity, as described previously [27]. Calibration curves were obtained by adding freshly diluted H_2O_2 to the analytical medium (the concentration of the hydrogen peroxide stock solution was checked at 240 nm using a molar extinction coefficient of 43.6).

Enzyme activity measurement. Determination of the enzymatic activity of hexokinase was carried out in unfractionated muscle tissue homogenate, cytosolic and mitochondrial fractions according to the method of Scheer et al. [28] with minor modifications. The assay buffer contained 50 mM Tris-HCl, 5 mM mercaptoethanol, 5 mM ATP, 10 mM $MgCl_2$, 0.5 mM glucose, 0.8 mM NAD^+ and 1 U/ml glucose-6-phosphate dehy-

drogenase from Leuconostoc mesenteroides (Roche) at pH 7.5. All assays were performed at 25°C in a total volume of 1.0 ml, activity was determined using a spectrophotometer Cary Varian 300 (Agilent, USA). Enzyme activity was determined by measuring the increase in optical density at a wavelength of 340 nm, where 1 unit of enzyme was defined as the amount that catalyzes the conversion of 1 μmol of substrate to product within 1 min. Calculations were carried out considering the dilution factor, which was different for different fractions. Three technical replicates were performed for each measurement. Reagents from Sigma-Aldrich were used.

PAGE and Western blot analysis of hexokinase content in mitochondrial preparations. Electrophoresis in polyacrylamide gel was carried out according to the Laemmli method. [29]. Mitochondria were lysed in buffer: 150 mM sodium chloride, 50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 1% sodium deoxycholate, 0.5% sodium dodecyl sulfate with a protease inhibitor cocktail (ThermoFisher Scientific, USA). After separation (30 μg of protein per lane), proteins from a 12% polyacrylamide gel were transferred to a nitrocellulose membrane using a combined electrophoresis/immunoblotting system (Bio-Rad, USA) and immunoblotting was performed as previously described [30]. Primary mouse monoclonal antibodies against human isozymes of hexokinase I and II (ab150013, ab227198, Abcam, USA) and against the mitochondrial outer membrane protein VDAC1 (ab186321, Abcam) were used, as well as secondary antibodies conjugated to horseradish peroxidase (ab97023, Abcam) in accordance with the manufacturer's recommendations. To visualize the signal, a Novex ECL Kit (Invitrogen) and a ChemiDoc scanner (Bio-Rad) were used.

Lipid extraction and cardiolipin analysis by high-performance thin-layer chromatography (HPTLC). Lipid extraction was carried out according to the Bligh and Dyer method in a nitrogen flow with oxygen-free solutions bubbled overnight with 99.9% N_2 [31]. Extracted lipids were dissolved in chloroform/methanol 2 : 1 (v/v) and stored under nitrogen at –80°C in a silica gel desiccator. Thin layer chromatography was carried out according to the method [32], using organic solvents of analytical grade and HPTLC chromatographic plates with silica gel on an aluminum substrate (Merck, Germany). Before application of the sample or standard on the day of use, 10 by 10 cm HPTLC plates were prepared as follows: immersed once in 2.3% boric acid in ethanol, dried for 2 h in a fume hood, and activated at 110°C for 20 min in a sand bath. Samples were applied using a homemade glass capillary applicator with a ball valve (actuated by a stream of nitrogen) in the form of strips 10 mm long at a distance of 15 mm from the edge of the plate with a constant application rate of about 200 nl/s while drying continuously with a stream of nitrogen at a pressure of 1.5-2.0 bar. For phospholipid standards (Avanti Polar Lipids, France) prepared a stock solution (1 mg/ml) in a mixture of chloroform/methanol (2 : 1, v/v). Elution was carried out in a glass chamber equilibrated with eluent vapor for at least 1 h before chromatography. The eluent consisted of a mixture of chloroform/ethanol/triethylamine /water (3/3.5/3.5/0.7, v/v). Chromatography was stopped when the front reached 10 mm from the upper edge of the plate, after two hours of drying in a fume hood the plate was stained by immersing for 2 min in 0.5% copper sulfate (w/v) in 1.16 M phosphoric acid, drying in a fume hood for 2 h at room temperature in a stream of nitrogen and developed in a sand bath for 15 min at 155°C. The plates were photographed in a ChemiDoc scanner (Bio-Rad). The measured phospholipid band intensities were integrated and the peak surface area values were expressed in arbitrary units using the ImageJ tool, and the concentrations of cardiolipin and monolysocardiolipin (nmol/mg mitochondrial protein) were then calculated using calibration curves for the corresponding standards.

Statistical methods. Group comparisons were made using analysis of variance and Tukey's multiple comparison test. Data are presented as median with interquartile range; $p < 0.05$ were considered significant. Correlation analysis was performed using Spearman's rank correlation coefficient. Statistical processing was carried out using the Prism 7.0 program (Graph Software Inc., USA).

RESULTS

Characteristics of volunteer groups. Clinical and anamnestic characteristics of study participants presented in Table 1; it was shown that the subjective assessment of physical capabilities in patients is significantly lower than in young healthy people, and the body mass index and insulin resistance index are increased only in middle-aged and elderly patients.

Histological analysis revealed progressive impairment of muscle fibers in patients with age, namely: muscle fiber atrophy, displacement of nuclei from the periphery of the fiber, infiltration by immune cells and an increase in the distance between fibers, presumably associated with an increase in the proportion of the connective tissue component (Fig. 1).

Distribution of hexokinase between cellular fractions. No differences in hexokinase activity were found between healthy volunteers and young patients, either in the homogenate or in the isolated fractions (Fig. 2). Elderly patients showed a decrease in total hexokinase activity compared to other groups ($p < 0.01$ for HV, $p < 0.05$ for YP and EP, Fig. 2a) and a significant

Table 1. Characteristics of individuals in various groups

Individuals	*n*	Age, years	BMI, kg/m^2	HOMA-IR, c.u.	Physical capabilities SF-12, c.u.
Norm	—	—	*18.5-25.0*	*<2.7*	*40-60*
Healthy volunteers (HV)	10	25-43 (34.5)	18.9-29.4 (22.5)	0.14-3.3 (1.3)	52.0-59.8 (56.3)
Young patients (YP)	8	26-44 (38)	20.8-30.9 (25.7)	1.43-4.65 (2.7)	16.1-31.7 (26.8)
Middle-aged patients (MP)	20	52-64 (59)	21.3-49.3 (34.5)	0.81-12.5 (4.6)	19.1-29.8 (25.2)
Elderly patients (EP)	42	65-85 (72)	20.8-45 (30.4)	1.12-10.96 (3.4)	15.5-37.7 23.0

Note. Medians, minimum and maximum values are presented. BMI, body mass index; HOMA-IR, insulin resistance index.

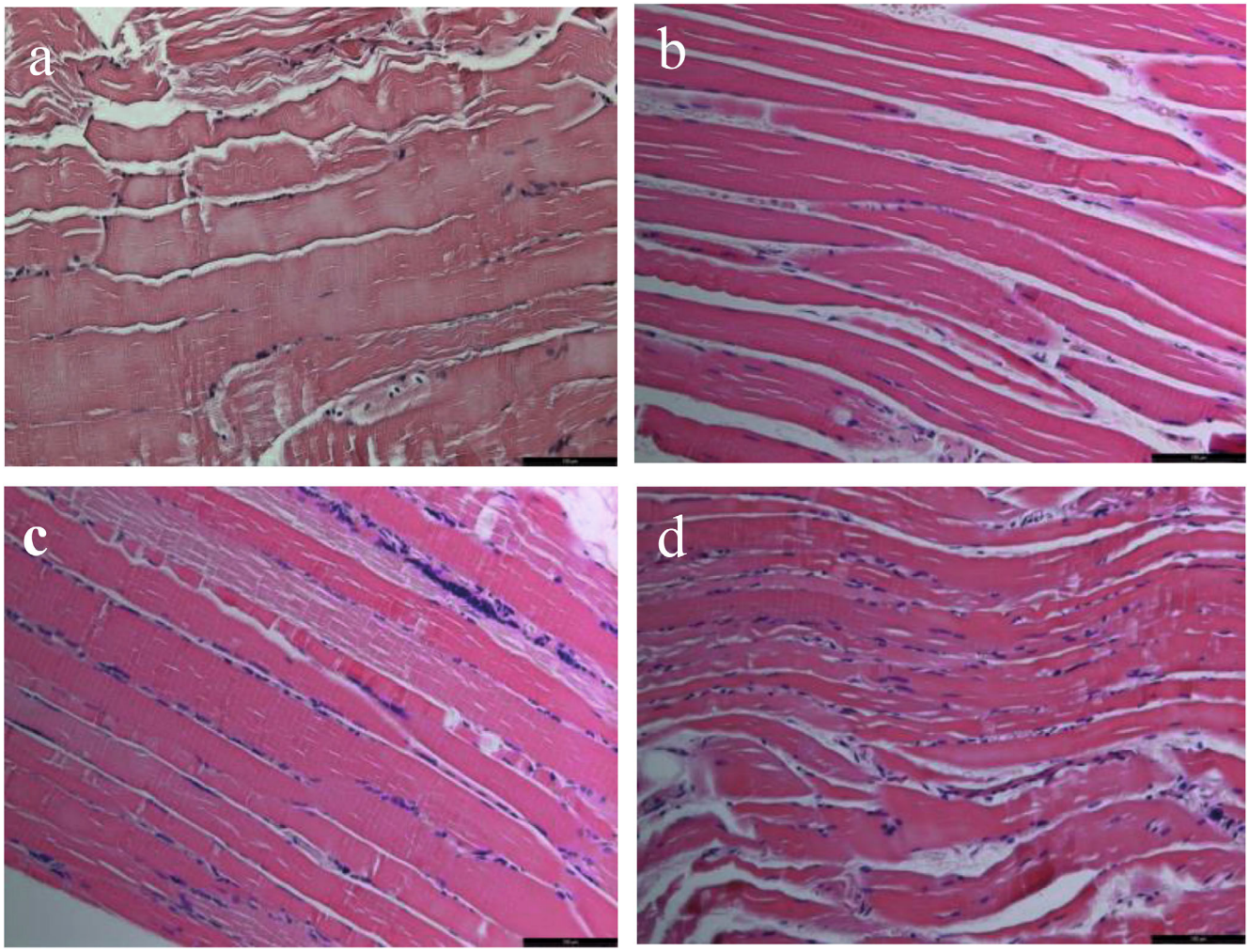

Fig. 1. Representative microphotographs of longitudinal sections of skeletal muscle stained with hematoxylin-eosin. Lens 20×. a) Sample of a 35-year-old healthy individuals (HV group, n = 10); b) sample of a 37-year-old patient (YP group, n = 8); c) sample of a 56-year-old patient (MP group, n = 20); d) sample of a 72-year-old patient (EP group, n = 42).

increase in activity for the cytosolic fraction compared to the group of young patients ($p < 0.05$, Fig. 2b). However, in middle-aged and elderly patients, hexokinase activity in the mitochondrial fraction was lower compared to both healthy volunteers ($p < 0.01$ and 0.001, respectively) and young patients ($p < 0.05$ and 0.01, respectively); moreover, differences were found between middle-aged and elderly patients ($p < 0.05$) (Fig. 2c).

It is known that in human skeletal muscles the ratio of I and II hexokinase's isozymes varies and, according to different authors, ranges from 1/10 to 1/3 [5, 33, 34]. At the same time, more than 90% isozyme I associated

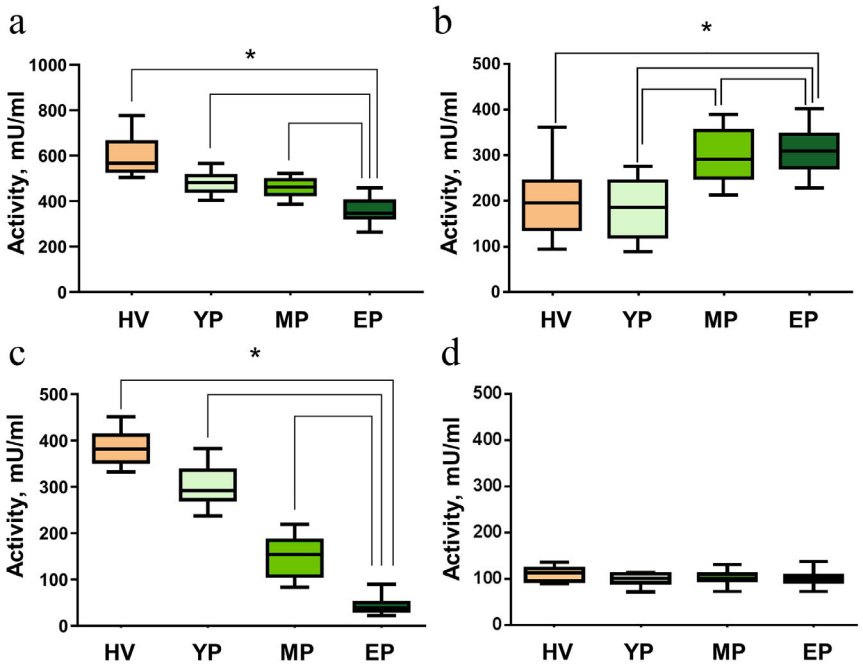

Fig. 2. Hexokinase activity in skeletal muscle of young healthy volunteers (HV, n = 10), young (YP, n = 8), middle-aged (MP, n = 20) and elderly (EP, n = 42) patients. a) Total hexokinase activity in the homogenate; b) hexokinase activity cytosolic fraction; c) activity of hexokinase-2 associated with mitochondria; d) activity of hexokinase-1 associated with mitochondria. Medians and interquartile ranges are presented. * $p < 0.05$.

with mitochondria [35] and the level of expression of this gene, as well as the protein representation in skeletal muscle, do not depend on metabolic load, physical activity, or the concentration of glucose or insulin in the blood [36]. On the contrary, for isozyme II, this dependence is clearly expressed – mRNA expression and protein concentration for hexokinase II in skeletal muscle can change significantly with changes in the concentration of glucose or insulin in the blood, as well as with changes in the level of physical activity [2, 5, 8, 37, 38].

Taking into account the different levels of binding of these isozymes to the mitochondrial membrane and the different regulation of their expression, activity measurements were carried out at 42°C, when the overwhelming majority of hexokinase II is inactivated, and hexokinase activity I changes slightly [5]. It was shown that there were no differences in hexokinase I activity in the mitochondrial fraction, regardless of age and clinical status (Fig. 2d).

Data from the analysis of isozyme distribution in groups of different ages were confirmed using mitochondrial protein electrophoresis followed by immunoblotting. Representative blots are shown in Fig. 3.

The data obtained suggested that, firstly, the observed differences in the distribution of hexokinase activity between mitochondria and the cytosol are largely determined by age rather than by a decrease in motor activity/chronic inflammation, and secondly, the identified differences are associated with changes in hexokinase content II, not I.

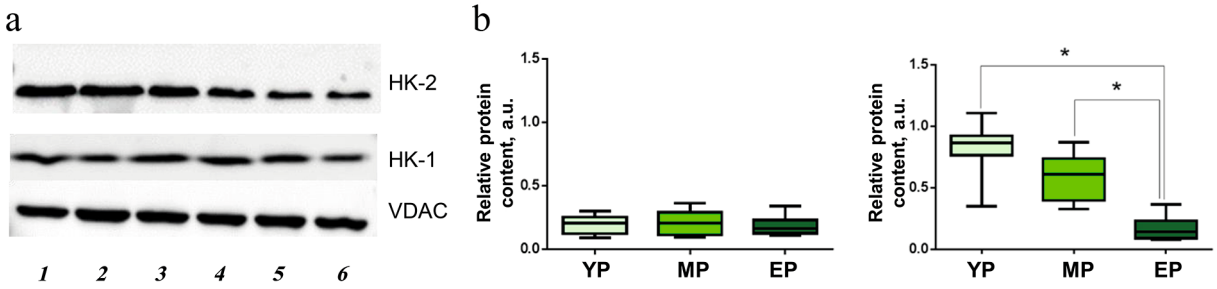

Fig. 3. Contents I and II isozymes hexokinase (GK-1 and 2, respectively) in skeletal muscle mitochondria of patients of different ages. a) Representative electrophoregrams: *1* and *2*) young patients (YP) 37 and 39 years old, *3* and *4*) middle-aged patients (MP) 59 and 60 years old, *5* and *6*) elderly patients (EP) 77 and 81 years old. Membrane protein VDAC1 is used as a control for mitochondrial protein loading; b) age-related changes in the content of GK-1 (left) and GK-2 (right), for all groups of patients n = 8, * $p < 0.001$.

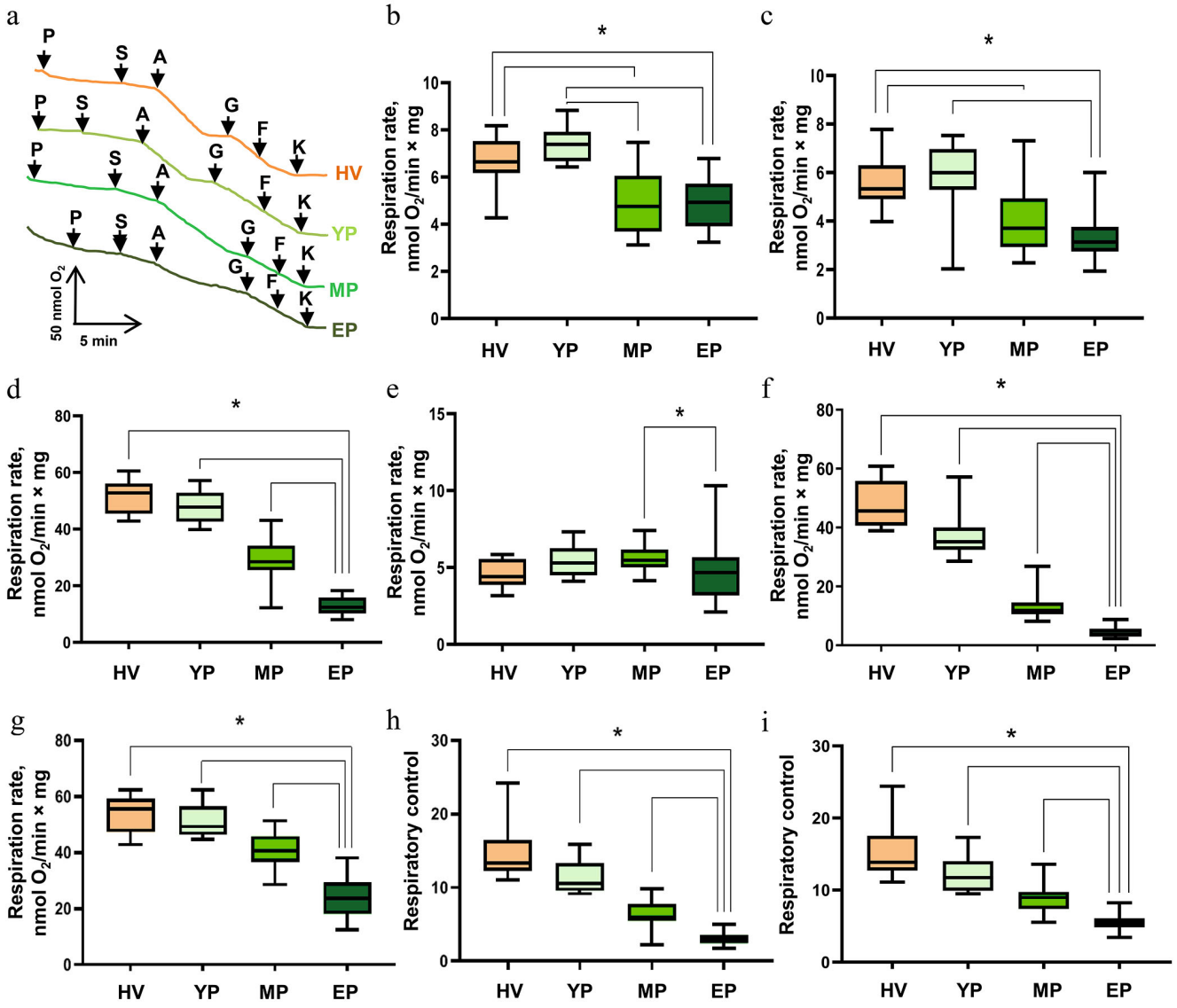

Fig. 4. Respiration of skeletal muscle mitochondria in young healthy volunteers (HV, n = 10), young (YP, n = 8), middle-aged (MP, n = 20), and elderly (EP, n = 42) patients. a) Representative polarograms: P, pyruvate + malate; S, succinate + rotenone; A, ADP; G, glucose; F, FCCP; K, KCN; b) respiration rate on pyruvate with malate; c) respiration rate on succinate in the presence of rotenone; d) rate of phosphorylating respiration on succinate with rotenone in the presence of ADP (state 3); e) respiration rate on succinate with rotenone in state 4; f) rate of phosphorylating respiration on succinate with rotenone in the presence of ADP and glucose; g) rate of uncoupled breathing in the presence of FCCP; h and i) respiratory control by ADP and FCCP, respectively. Medians and interquartile ranges are presented; * $p < 0.05$.

Respiration of isolated mitochondria. The respiration rate of isolated mitochondria was determined using a polarograph, assessing the change in oxygen concentration over time. Representative polarograms are presented in Fig. 4a. The obtained values of the respiration rate of skeletal muscle mitochondria in different states depending on the age of the study participants are presented in Fig. 4, b-g. Endogenous respiration (state 1) was initiated by adding the respiratory substrate pyruvate with the addition of malate, as described above, and after adding the complex I inhibitor – rotenone, respiration was again activated by succinate, which somewhat stimulated respiration; it was now limited by the absence of ADP (energy acceptor state 2). Addition of 0.1 mM ADP increased respiration rate to a maximum level (state 3); however, within minutes, O_2 consumption decreased to levels prior to the addition of ADP as ADP was depleted upon phosphorylation to ATP (state 4). The maximum rate of O_2 consumption was achieved after the addition of the uncoupler of respiration and phosphorylation (FCCP). Blocking of respiration was achieved by adding 1 mM potassium cyanide (KCN), the rate of respiration in its presence was subtracted from all previously obtained values [22].

The data shown in Fig. 4, a, f, and g, demonstrate a change in the respiration rate when 5 mM glucose

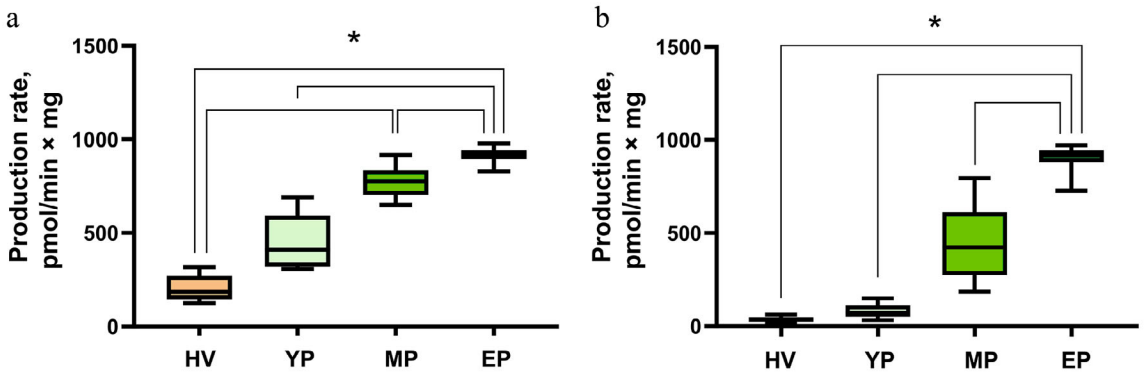

Fig. 5. Hydrogen peroxide production by skeletal muscle mitochondria of young healthy volunteers (HV, n = 10), young (YP, n = 8), middle-aged (MP, n = 20), and elderly (EP, n = 42) patients. a) Products in the presence of succinate; b) production in the presence of succinate upon activation of mitochondrial hexokinase by substrates of the enzymatic reaction. Medians and interquartile ranges are presented; * $p < 0.05$.

is added to mitochondria in state 4 in the presence of succinate after the depletion of ADP. At the same time, in fact, state 3 continues and respiration occurs at almost maximum speed, as if the added ADP had not been exhausted, which indicates its constant level in the microcompartment due to the hexokinase reaction at a saturating glucose concentration and access to mitochondrial ATP, which fully confirms the previously obtained S. Bessman and D. Wilson data [7, 39]. Thus, it can be argued that hexokinase bound on the surface of mitochondria retained its functional conjugacy within the microcompartment during mitochondrial fractionation, i.e., after isolation mitochondria were a highly coupled (Fig. 4, g-i).

It was shown that substrate respiration in state 2 significantly decreases, both with activation of complex I and complex II (Fig. 4, b and c) for groups of middle-aged and elderly patients in comparison not only with healthy volunteers, but also with young patients. At the same time, stimulated respiration (ADP or FCCP) did not differ between healthy volunteers and young patients, but showed a pronounced (multiple) age-related decrease: for the “MP” and “EP” groups compared with healthy volunteers and for “EP” compared with young patients (Fig. 4, d and g*)*. The effect of continued state 3 (see above) when measuring the respiratory rate in the presence of glucose disappeared with age, and for the “EP” group the differences were significant in relation to all other groups, including middle-aged patients (Fig. 4f).

Peroxide production by mitochondria. It has been shown that the generation of peroxide by mitochondria, energized by succinate, tends to increase even in young patients compared to healthy volunteers of the same age (Fig. 5a), however, a significant increase in this indicator was observed with increasing age of patients, which correlated with the degree of dissociation of hexokinase with the mitochondrial membrane (Fig. 2, Table 2) and a decrease in the rate of phosphorylating respiration (Fig. 3, Fig. S1c and Table 2). Confirming the data previously obtained by us and other researchers [15, 40-46], we found a significant decrease in the generation of hydrogen peroxide by mitochondria upon stimulation of respiration in the presence of the substrate of the hexokinase reaction ($p < 0.05$ for “HV” and “YP” in Fig. 5, a and b, Fig. S1e) and revealed the absence of significant changes for the “MP” and “EP” groups. In addition, it turned out that for young people, regardless of clinical status, the rate of peroxide production significantly decreases in the presence of glucose, i.e., upon activation of the ATP/ADP shuttle in the microcompartment hexokinase and the outer membrane of mitochondria, while in middle and old age, peroxide production with the addition of a hexokinase reaction substrate remains high (as in the presence of succinate) (Fig. 5, a and b, Fig. S2).

Cardiolipin content in mitochondria. We have previously shown the importance of maintaining the intact structure and total content of the mitochondrial lipid cardiolipin, and also demonstrated that cardiolipin is present in the mitochondrial proteolipid complexes, including hexokinase associated with VDAC1 [10, 47]. In this study, significant differences were identified in the content of cardiolipin in the mitochondria of skeletal muscles of elderly patients compared with a group of healthy volunteers ($p < 0.01$) and a group of young patients ($p < 0.05$) (Fig. 6b*)*, while for monolysocardiolipin, significant differences were observed between the following groups: middle-aged patients in relation to healthy volunteers ($p < 0.01$) and young patients ($p < 0.05$); elderly patients and healthy volunteers ($p < 0.001$); elderly patients and young patients ($p < 0.01$). Thus, a decrease in the content of mitochondrial cardiolipin and accumulation of monolysocardiolipin does not occur in young patients in relatively healthy people, but is observed with increasing age of patients (Fig. 6), and this correlates with the production of mROS (Table 2).

Table 2. Correlation (Spearman correlation coefficient) of indicators characterizing mitochondrial functions in human skeletal muscles in various groups

Groups	VH (n = 10)	YP (n = 8)	MP (n = 20)	EP (n = 42)
HK bound/age	no	r = –0.809 p = 0.015	r = –0.953 p = 0.0012	r = –0.866 p = 0.0002
Respiration (succ. + ADP)/age	r = –0.814 p = 0.004	no	r = –0.88 p = 3.2e-7	r = –0.514 p = 3.6e-4
Respiration (succ.+ ADP + gluc.)/age	r = –0.843 p = 0.002	r = –0.8 p = 0.017	r = –0.917 p = 0.001	r = –0.746 p = 0.002
Respiration (succ. + ADP + gluc.)/HK bound.	r = 0.793 p = 0.006	r = 0.921 p = 0.001	r = 0.836 p = 4.5e-6	r = 0.824 p = 6.37e-12
H_2O_2 production (succ.)/age	r = 0.87 p = 0.01	r = 0.77 p = 0.025	r = 0.872 p = 5.5e-7	r = 0.555 p = 9.57e-5
H_2O_2 production (succ.)/HK bound	r = –0.761 p = 0.037	no	r = –0.887 p = 1.85e-7	r = –0.556 p = 8.92e-5
H_2O_2 production (succ.)/respiration (succ. + ADP + gluc.)	r = –0.703 p = 0.023	no	r = –0.762 p = 9.34e-5	r = –0.477 p = 0.001
H_2O_2 production (succ. + ADP + gluc.)/respiration (succ. + ADP + gluc.)	r = –0.765 p = 0.01	no	r = –0.791 p = 3.25e-5	r = –0.766 p = 1.35e-9
H_2O_2 production (succ. + ADP + gluc.)/HK bound	no	no	r = –0.932 p = 2.32e-9	r = –0.813 p = 2.07e-11
H_2O_2 production (succ. + ADP + gluc.)/age	r = 0.94 p = 5.21e-5	r = 0.882 p = 0.004	r = 0.925 p = 5.67e-9	r = 0.674 p = 5.22e-7
H_2O_2 production (succ. + ADP + gluc.)/respiration (succ. + ADP)	r = –0.754 p = 0.012	no	r = –0.854 p = 1.7e-6	no
HK total/age	no	no	no	r = –0.361 p = 0.016
Cardiolipin content/respiration (succ. + ADP + gluc.)	no	no	no	r = 0.622 p =6.7e-06
Cardiolipin content /HK bounded	no	no	no	r = 0.841 p =9.53e-13
Cardiolipin content/H_2O_2 production (succ.)	no	no	no	r = –0.521 p = 0.00029
Cardiolipin content/H_2O_2 production (succ. + ADP + gluc.)	r = –0.802 p = 0.005	no	no	r = –0.612 p =1.03e-05
MLCL content/ respiration (succ. + ADP + gluc.)	no	r = –0.71 p = 0.048	no	r = –0.594 p = 2.13e-05
MLCL content/HK bound	no	r = –0.805 p = 0.016	no	r = –0.718 p = 3.99e-08
MLCL content/H_2O_2 production (succ.)	no	no	no	r = 0.432 p = 0.003

Table 2 (*cont.*)

Groups	VH (n = 10)	YP (n = 8)	MP (n = 20)	EP (n = 42)
MLCL content/H_2O_2 production (succ. + ADP + gluc.)	no	no	no	r = 0.544 p = 0.00014
Respiratory control (FCCP)/age	no	no	no	r = –0.874 p = 0.0002
Respiration (succ. + ADP)/BMI	no	r = 0.719 p = 0.044	no	no
HOMA-IR/HK bound	no	no	no	no
HOMA-IR/respiration (succ. + ADP)	no	no	no	no
HOMA-IR/age	r = 0.94 p = 5e-5	r = 0.882 p = 0.004	r = 0.925 p = 0.0001	r = 0.674 p = 2e-5
HOMA-IR/BMI	no	no	r = 0.453 p = 0.045	r = 0.472 p = 0.001
HOMA-IR/respiration (succ. + ADP + gluc.)	no	no	r = 0.448 p = 0.047	no

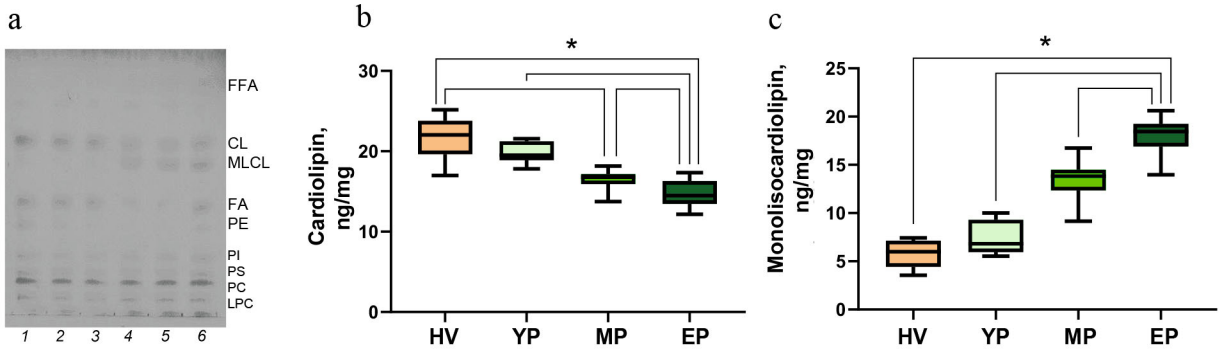

Fig. 6. Cardiolipin content in skeletal muscle mitochondria of young healthy volunteers (HV), young (YP), middle-aged (MP), and elderly (EP) patients, for all groups n = 8. a) Representative thin-layer chromatogram: *1-3*) young patients, *4-6*) elderly patients. FFA, free fatty acids; CL and MLCL, cardiolipin and monolysocardiolipin; FA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC and LPC, phosphatidylcholine and lysophosphatidylcholine. b and c) Averaged values of the content of cardiolipin and monolysocardiolipin, normalized to the content of phosphatidylcholine.

DISCUSSION

The study examined the influence of physical inactivity/chronic inflammation in young people, as well as age in patients with arthrosis of the knee/hip joint on the regulation of mROS production in human skeletal muscle. For the first time, an association of an age-dependent increase in the production of hydrogen peroxide by mitochondria with a decrease in the proportion of hexokinase-2 associated with mitochondria was shown for human muscle tissue. This is confirmed by data on an age-dependent change in the distribution of hexokinase-2 between the mitochondrial and cytosolic fractions and a decrease in the rate of coupled respiration of isolated mitochondria and respiration upon stimulation with glucose – the substrate of hexokinase. These observations are in good agreement and complement our previous data on the production of mROS by tissues of long- and short-lived rodents [15].

It can be assumed that the age-dependent decrease in the efficiency of coupled respiration of skeletal muscle mitochondria that we described (Fig. 4d) is associated with an increase in energy deficiency in aging muscle, which was previously shown in studies on rodents and primary human myoblasts (for a review, see [48, 49]). A pronounced age-related decrease in the sensitivity of isolated mitochondria to ADP (Fig. 4d) confirmed the effect of a decrease in the ADP-induced decrease in peroxide production in permeabilized muscle fibers of elderly people compared to young

people shown by G. Holloway and colleagues [50]. At the same time, the lack of effect of the addition of hexokinase on respiration and peroxide generation in the cited work can be explained by the fact that, firstly, to maintain the microcompartment in an intact state during mitochondrial fractionation, the presence of Mg^{2+} ions in the medium is necessary; secondly, when isolating mitochondria, reassociation hexokinase with the outer membrane does not undergo mirrored dissociation and requires special conditions; thirdly, the added enzyme is approximately ten times less effective, in comparison with the adsorbed one, in stimulating the respiration of ADP, formed during the phosphorylation of glucose [39, 51].

Pathological conditions of skeletal muscles caused by oxidative stress are especially pronounced during aging, leading to apoptosis, atrophy, and muscle dysfunction [52-54]. Increasing evidence in the literature points to a link between oxidative stress caused by excess mROS production by hyperpolarized mitochondria, and hyperglycemia [13, 15, 44, 55]. Our data confirm this observation and, in part, explain the mechanisms responsible for these changes [52]. Hexokinase II, unlike other isozymes of this enzyme, has a second (noncatalytic) glucose binding site. Occupation of this site caused by hyperglycemia blocks chaperone binding to it HSP7C (Heat shock cognate 71, encoded by the *HSPA8* gene), inducing proteolytic degradation of hexokinase [44]. This leads to an increase in the intracellular content of hexokinase II and the accumulation of glucose-6-phosphate, which has a solubilizing effect and causes desorption of hexokinase from the surface of mitochondria. Apparently, these events underlie the age-dependent destruction of the outer membrane microcompartment, including hexokinase II, observed by us and other authors [14, 15], which leads not only to an increase in membrane potential, increased production of mROS and oxidative stress, but also may induce apoptosis by binding the proapoptotic protein Bax to VDAC1 [56].

We found an age-correlated decrease in cardiolipin content in skeletal muscle mitochondria (Fig. 5, Table 2). It is known that ADP/ATP translocase 1 (ANT), which is part of the microcompartment and localized in the inner membrane, is part of the proteolipid complex of mitochondrial contact sites [57], which includes 3 molecules of tightly bound cardiolipin; in this case, the oxidation of cardiolipin leads to disruption of the structure of this complex [58, 59]. It was previously shown that disruption of ANT conformations leads to the destruction of its complex with VDAC1 and mitochondrial contact sites [60]. It can be assumed that the age-dependent decrease in cardiolipin content in human skeletal muscle mitochondria that we discovered, which correlates with the amount of bound hexokinase, the production of mROS and phosphorylating respiration, is part of the general mechanism of impaired soft depolarization of skeletal muscle mitochondria during aging. This assumption opens up prospects for further research into the mechanisms underlying age-dependent disruption of the structure of mitochondrial contact sites, which are associated with remodeling cardiolipin during oxidative stress. The study of these mechanisms seems promising for the search for pharmacological/physiological approaches to the correction of age-related skeletal muscle pathologies caused by disturbances in glucose oxidation and mROS production.

Supplementary information. The online version contains supplementary material available at https://doi.org/10.1134/S0006297924020093.

Contributions. M.Yu.V., A.Yu.E., D.V.P., and V.P.S. concept and management of the work; M.Yu.V., M.A.V., V.V.Ph., Ya.R.B., M.V.M., O.A.G., T.F.V., N.S.K., and L.A.M. conducting experiments; M.Yu.V., M.A.V., A.Yu.E., D.V.P., and V.P.S. discussion of the research results; M.Yu.V., A.Yu.E., and D.V.P. text writing; M.Yu.V., A.Yu.E., and D.V.P. editing the text of the article.

Funding. The work was supported by the Russian Science Foundation (grant no. 21-15-00405, study with healthy volunteers, experiments with mitochondrial respiration) and by the State Assignment of Lomonosov Moscow State University (study with patients, clinical and histological studies).

Ethics declarations. All studies were conducted in accordance with the principles of biomedical ethics as outlined in the 1964 Declaration of Helsinki and its later amendments. Each participant in the study provided a voluntary written informed consent after receiving an explanation of the potential risks and benefits, as well as the nature of the upcoming study. The authors of this work declare that they have no conflicts of interest.

REFERENCES

1. Harman, D. (1972) The biologic clock: the mitochondria?, *J. Am. Geriatr. Soc.*, **20**, 145-147, doi: 10.1111/j.1532-5415.1972.tb00787.x.
2. Anderson, C. M., Hu, J., Barnes, R. M., Heidt, A. B., Cornelissen, I., and Black, B. L. (2015) Myocyte enhancer factor 2C function in skeletal muscle is required for normal growth and glucose metabolism in mice, *Skelet. Muscle*, **5**, 7, doi: 10.1186/s13395-015-0031-0.
3. Hargreaves, M. (2004) Muscle glycogen and metabolic regulation, *Proc. Nutr. Soc.*, **63**, 217-220, doi: 10.1079/PNS2004344.
4. Fealy, C. E., Grevendonk, L., Hoeks, J., and Hesselink, M. K. C. (2021) Skeletal muscle mitochondrial network dynamics in metabolic disorders and aging, *Trends*

Mol. Med., **27**, 1033-1044, doi: 10.1016/j.molmed.2021.07.013.

5. Vogt, C., Yki-Jarvinen, H., Iozzo, P., Pipek, R., Pendergrass, M., Koval, J., Ardehali, H., Printz, R., Granner, D., Defronzo, R., and Mandarino, L. (1998) Effects of insulin on subcellular localization of hexokinase II in human skeletal muscle *in vivo*, *J. Clin. Endocrinol. Metab.*, **83**, 230-234, doi: 10.1210/jcem.83.1.4476.
6. Wilson, J. E. (2003) Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function, *J. Exp. Biol.*, **206**, 2049-2057, doi: 10.1242/jeb.00241.
7. Gots, R. E., Gorin, F. A., and Bessman, S. P. (1972) Kinetic enhancement of bound hexokinase activity by mitochondrial respiration, *Biochem. Biophys. Res. Commun.*, **49**, 1249-1255, doi: 10.1016/0006-291x(72)90602-x.
8. Sui, D., and Wilson, J. E. (1997) Structural determinants for the intracellular localization of the isozymes of mammalian hexokinase: intracellular localization of fusion constructs incorporating structural elements from the hexokinase isozymes and the green fluorescent protein, *Arch. Biochem. Biophys.*, **345**, 111-125, doi: 10.1006/abbi.1997.0241.
9. Brdiczka, D., Knoll, G., Riesinger, I., Weiler, U., Klug, G., Benz, R., and Krause, J. (1986) Microcompartmentation at the mitochondrial surface: its function in metabolic regulation, *Adv. Exp. Med. Biol.*, **194**, 55-69, doi: 10.1007/978-1-4684-5107-8_5.
10. Vyssokikh, M., and Brdiczka, D. (2004) VDAC and peripheral channelling complexes in health and disease, *Mol. Cell Biochem.*, **256-257**, 117-126, doi: 10.1023/b:mcbi.0000009863.69249.d9.
11. Cadenas, E., Boveris, A., Ragan, C. I., and Stoppani, A. O. (1977) Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome *c* reductase from beef-heart mitochondria, *Arch. Biochem. Biophys.*, **180**, 248-257, doi: 10.1016/0003-9861(77)90035-2.
12. Morse, P. T., Wan, J., Bell, J., Lee, I., Goebel, D. J., Malek, M. H., Sanderson, T. H., and Huttemann, M. (2022) Sometimes less is more: inhibitory infrared light during early reperfusion calms hyperactive mitochondria and suppresses reperfusion injury, *Biochem. Soc. Trans.*, **50**, 1377-1388, doi: 10.1042/BST20220446.
13. Korshunov, S. S., Skulachev, V. P., and Starkov, A. A. (1997) High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria, *FEBS Lett.*, **416**, 15-18, doi: 10.1016/s0014-5793(97)01159-9.
14. Da-Silva, W. S., Gomez-Puyou, A., de Gomez-Puyou, M. T., Moreno-Sanchez, R., De Felice, F. G., de Meis, L., Oliveira, M. F., and Galina, A. (2004) Mitochondrial bound hexokinase activity as a preventive antioxidant defense: steady-state ADP formation as a regulatory mechanism of membrane potential and reactive oxygen species generation in mitochondria, *J. Biol. Chem.*, **279**, 39846-39855, doi: 10.1074/jbc.M403835200.
15. Vyssokikh, M. Y., Holtze, S., Averina, O. A., Lyamzaev, K. G., Panteleeva, A. A., Marey, M. V., Zinovkin, R. A., Severin, F. F., Skulachev, M. V., Fasel, N., Hildebrandt, T. B., and Skulachev, V. P. (2020) Mild depolarization of the inner mitochondrial membrane is a crucial component of an anti-aging program, *Proc. Natl. Acad. Sci. USA*, **117**, 6491-6501, doi: 10.1073/pnas.1916414117.
16. Callahan, D. M., Miller, M. S., Sweeny, A. P., Tourville, T. W., Slauterbeck, J. R., Savage, P. D., Maugan, D. W., Ades, P. A., Beynnon, B. D., and Toth, M. J. (2014) Muscle disuse alters skeletal muscle contractile function at the molecular and cellular levels in older adult humans in a sex-specific manner, *J. Physiol.*, **592**, 4555-4573, doi: 10.1113/jphysiol.2014.279034.
17. Callahan, D. M., Tourville, T. W., Miller, M. S., Hackett, S. B., Sharma, H., Cruickshank, N. C., Slauterbeck, J. R., Savage, P. D., Ades, P. A., Maughan, D. W., Beynnon, B. D., and Toth, M. J. (2015) Chronic disuse and skeletal muscle structure in older adults: sex-specific differences and relationships to contractile function, *Am. J. Physiol. Cell Physiol.*, **308**, C932-C943, doi: 10.1152/ajpcell.00014.2015.
18. Miller, M. S., Callahan, D. M., Tourville, T. W., Slauterbeck, J. R., Kaplan, A., Fiske, B. R., Savage, P. D., Ades, P. A., Beynnon, B. D., and Toth, M. J. (2017) Moderate-intensity resistance exercise alters skeletal muscle molecular and cellular structure and function in inactive older adults with knee osteoarthritis, *J. Appl. Physiol.*, **122**, 775-787, doi: 10.1152/japplphysiol.00830.2016.
19. Suetta, C., Aagaard, P., Magnusson, S. P., Andersen, L. L., Sipila, S., Rosted, A., Jakobsen, A. K., Duus, B., and Kjaer, M. (2007) Muscle size, neuromuscular activation, and rapid force characteristics in elderly men and women: effects of unilateral long-term disuse due to hip-osteoarthritis, *J. Appl. Physiol.*, **102**, 942-948, doi: 10.1152/japplphysiol.00067.2006.
20. Ware, J., Jr., Kosinski, M., and Keller, S. D. (1996) A 12-Item Short-Form Health Survey: construction of scales and preliminary tests of reliability and validity, *Med. Care*, **34**, 220-233, doi: 10.1097/00005650-199603000-00003.
21. Popov, D. V., Makhnovskii, P. A., Shagimardanova, E. I., Gazizova, G. R., Lysenko, E. A., Gusev, O. A., and Vinogradova, O. L. (2019) Contractile activity-specific transcriptome response to acute endurance exercise and training in human skeletal muscle, *Am. J. Physiol. Endocrinol. Metab.*, **316**, E605-E614, doi: 10.1152/ajpendo.00449.2018.
22. Skulachev, V. P., Bogachev, A. V., and Kasparinsky, F. O. (2013) *Principles of Bioenergetics*, Springer-Verlag Berlin Heidelberg, doi: 10.1007/978-3-642-33430-6.
23. Gnaiger, E., Kuznetsov, A. V., Schneeberger, S., Seiler, R., Brandacher, G., Steurer, W., and Margreiter, R.

(2000) Mitochondria in the Cold, in *Life in the Cold* (Heldmaier, G., Klingenspor, M., Eds.), Springer, Berlin, Heidelberg, pp. 431-442, doi: 10.1007/978-3-662-04162-8_45.

24. Zhou, M., Diwu, Z., Panchuk-Voloshina, N., and Haugland, R. P. (1997) A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases, *Anal. Biochem.*, **253**, 162-168, doi: 10.1006/abio.1997.2391.
25. Shabalina, I. G., Vyssokikh, M. Y., Gibanova, N., Csikasz, R. I., Edgar, D., Hallden-Waldemarson, A., Rozhdestvenskaya, Z., Bakeeva, L. E., Vays, V. B., Pustovidko, A. V., Skulachev, M. V., Cannon, B., Skulachev, V. P., and Nedergaard, J. (2017) Improved health-span and lifespan in mtDNA mutator mice treated with the mitochondrially targeted antioxidant SkQ1, *Aging (Albany NY)*, **9**, 315-339, doi: 10.18632/aging.101174.
26. Antonenko, Y. N., Avetisyan, A. V., Bakeeva, L. E., Chernyak, B. V., Chertkov, V. A., Domnina, L. V., Ivanova, O. Y., Izyumov, D. S., Khailova, L. S., Klishin, S. S., Korshunova, G. A., Lyamzaev, K. G., Muntyan, M. S., Nepryakhina, O. K., Pashkovskaya, A. A., Pletjushkina, O. Y., Pustovidko, A. V., Roginsky, V. A., Rokitskaya, T. I., Ruuge, E. K., Saprunova, V. B., Severina, I. I., Simonyan, R. A., Skulachev, I. V., Skulachev, M. V., Sumbatyan, N. V., Sviryaeva, I. V., Tashlitsky, V. N., Vassiliev, J. M., Vyssokikh, M. Yu., Yaguzhinsky, L. S., Zamyatnin, A. A., Jr., and Skulachev, V. P. (2008) Mitochondria-targeted plastoquinone derivatives as tools to interrupt execution of the aging program. 1. Cationic plastoquinone derivatives: synthesis and *in vitro* studies, *Biochemistry (Moscow)*, **73**, 1273-1287, doi: 10.1134/s0006297908120018.
27. Shabalina, I. G., Vrbacky, M., Pecinova, A., Kalinovich, A. V., Drahota, Z., Houstek, J., Mracek, T., Cannon, B., and Nedergaard, J. (2014) ROS production in brown adipose tissue mitochondria: the question of UCP1-dependence, *Biochim. Biophys. Acta*, **1837**, 2017-2030, doi: 10.1016/j.bbabio.2014.04.005.
28. Scheer, W. D., Lehmann, H. P., and Beeler, M. F. (1978) An improved assay for hexokinase activity in human tissue homogenates, *Anal. Biochem.*, **91**, 451-463, doi: 10.1016/0003-2697(78)90531-6.
29. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, **227**, 680-685, doi: 10.1038/227680a0.
30. Buscher, T., Luh, E., and Pette, D. (1964) Simple and Compound Optical Tests with Pyridine Nucleotides, in *Hoppe-Seyler/Thierfelder, Handbook of Physiological and Pathological Chemical Analysis* [in German], **VI/A**, 292-339, doi: 10.1007/978-3-662-11689-0_6.
31. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.*, **37**, 911-917, doi: 10.1139/o59-099.
32. Pinault, M., Guimaraes, C., Dumas, J.-F., Servais, S., Chevalier, S., Besson, P., and Goupille, C. (2020) A 1D high performance thin layer chromatography method validated to quantify phospholipids including cardiolipin and monolysocardiolipin from biological samples, *Eur. J. Lipid Sci. Technol.*, **122**, 1900240, doi: 10.1002/ejlt.201900240.
33. Kruszynska, Y. T., Mulford, M. I., Baloga, J., Yu, J. G., and Olefsky, J. M. (1998) Regulation of skeletal muscle hexokinase II by insulin in nondiabetic and NIDDM subjects, *Diabetes*, **47**, 1107-1113, doi: 10.2337/diabetes.47.7.1107.
34. Vestergaard, H., Bjorbaek, C., Hansen, T., Larsen, F. S., Granner, D. K., and Pedersen, O. (1995) Impaired activity and gene expression of hexokinase II in muscle from non-insulin-dependent diabetes mellitus patients, *J. Clin. Invest.*, **96**, 2639-2645, doi: 10.1172/JCI118329.
35. Ritov, V. B., and Kelley, D. E. (2001) Hexokinase isozyme distribution in human skeletal muscle, *Diabetes*, **50**, 1253-1262, doi: 10.2337/diabetes.50.6.1253.
36. Mandarino, L. J., Printz, R. L., Cusi, K. A., Kinchington, P., O'Doherty, R. M., Osawa, H., Sewell, C., Consoli, A., Granner, D. K., and DeFronzo, R. A. (1995) Regulation of hexokinase II and glycogen synthase mRNA, protein, and activity in human muscle, *Am. J. Physiol.*, **269**, E701-E708, doi: 10.1152/ajpendo.1995.269.4.E701.
37. Wilson, J. E. (1995) Hexokinases, *Rev. Physiol. Biochem. Pharmacol.*, **126**, 65-198, doi: 10.1007/bfb0049776.
38. Cunha, T. F., Vieira, J. S., Santos, J. B., Coelho, M. A., Brum, P. C., and Gabriel-Costa, D. (2022) Lactate modulates cardiac gene expression in mice during acute physical exercise, *Braz. J. Med. Biol. Res.*, **55**, e11820, doi: 10.1590/1414-431X2022e11820.
39. BeltrandelRio, H., and Wilson, J. E. (1991) Hexokinase of rat brain mitochondria: relative importance of adenylate kinase and oxidative phosphorylation as sources of substrate ATP, and interaction with intramitochondrial compartments of ATP and ADP, *Arch. Biochem. Biophys.*, **286**, 183-194, doi: 10.1016/0003-9861(91)90026-f.
40. Saraiva, L. M., Seixas da Silva, G. S., Galina, A., da-Silva, W. S., Klein, W. L., Ferreira, S. T., and De Felice, F. G. (2010) Amyloid-beta triggers the release of neuronal hexokinase 1 from mitochondria, *PLoS One*, **5**, e15230, doi: 10.1371/journal.pone.0015230.
41. De-Souza-Ferreira, E., Rios-Neto, I. M., Martins, E. L., and Galina, A. (2019) Mitochondria-coupled glucose phosphorylation develops after birth to modulate H_2O_2 release and calcium handling in rat brain, *J. Neurochem.*, **149**, 624-640, doi: 10.1111/jnc.14705.
42. Silva-Rodrigues, T., de-Souza-Ferreira, E., Machado, C. M., Cabral-Braga, B., Rodrigues-Ferreira, C., and Galina, A. (2020) Hyperglycemia in a type 1 Diabetes Mellitus model causes a shift in mitochondria cou-

pled-glucose phosphorylation and redox metabolism in rat brain, *Free Radic. Biol. Med.*, **160**, 796-806, doi: 10.1016/j.freeradbiomed.2020.09.017.

43. Tan, V. P., Smith, J. M., Tu, M., Yu, J. D., Ding, E. Y., and Miyamoto, S. (2019) Dissociation of mitochondrial HK-II elicits mitophagy and confers cardioprotection against ischemia, *Cell Death Dis*, **10**, 730, doi: 10.1038/s41419-019-1965-7.
44. Rabbani, N., Xue, M., and Thornalley, P. J. (2022) Hexokinase-2-linked glycolytic overload and unscheduled glycolysis-driver of insulin resistance and development of vascular complications of diabetes, *Int. J. Mol. Sci.*, **23**, 2165, doi: 10.3390/ijms23042165.
45. Bryson, J. M., Coy, P. E., Gottlob, K., Hay, N., and Robey, R. B. (2002) Increased hexokinase activity, of either ectopic or endogenous origin, protects renal epithelial cells against acute oxidant-induced cell death, *J. Biol. Chem.*, **277**, 11392-11400, doi: 10.1074/jbc.M110927200.
46. Ahmad, A., Ahmad, S., Schneider, B. K., Allen, C. B., Chang, L. Y., and White, C. W. (2002) Elevated expression of hexokinase II protects human lung epithelial-like A549 cells against oxidative injury, *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **283**, L573-L584, doi: 10.1152/ajplung.00410.2001.
47. Shilovsky, G. A., Putyatina, T. S., Ashapkin, V. V., Yamskova, O. V., Lyubetsky, V. A., Sorokina, E. V., Shram, S. I., Markov, A. V., and Vyssokikh, M. Y. (2019) Biological diversity and remodeling of cardiolipin in oxidative stress and age-related pathologies, *Biochemistry (Moscow)*, **84**, 1469-1483, doi: 10.1134/S000629791912006X.
48. Bratic, I., and Trifunovic, A. (2010) Mitochondrial energy metabolism and ageing, *Biochim. Biophys. Acta*, **1797**, 961-967, doi: 10.1016/j.bbabio.2010.01.004.
49. Pedersen, Z. O., Pedersen, B. S., Larsen, S., and Dysgaard, T. (2023) A scoping review investigating the "gene-dosage theory" of mitochondrial DNA in the healthy skeletal muscle, *Int. J. Mol. Sci.*, **24**, 8154, doi: 10.3390/ijms24098154.
50. Holloway, G. P., Holwerda, A. M., Miotto, P. M., Dirks, M. L., Verdijk, L. B., and van Loon, L. J. C. (2018) Age-associated impairments in mitochondrial ADP sensitivity contribute to redox stress in senescent human skeletal muscle, *Cell Rep.*, **22**, 2837-2848, doi: 10.1016/j.celrep.2018.02.069.
51. BeltrandelRio, H., and Wilson, J. E. (1992) Coordinated regulation of cerebral glycolytic and oxidative metabolism, mediated by mitochondrially bound hexokinase dependent on intramitochondrially generated ATP, *Arch. Biochem. Biophys.*, **296**, 667-677, doi: 10.1016/0003-9861(92)90625-7.
52. Vincent, A. M., Olzmann, J. A., Brownlee, M., Sivitz, W. I., and Russell, J. W. (2004) Uncoupling proteins prevent glucose-induced neuronal oxidative stress and programmed cell death, *Diabetes*, **53**, 726-734, doi: 10.2337/diabetes.53.3.726.
53. Bellanti, F., Lo Buglio, A., and Vendemiale, G. (2022) Muscle delivery of mitochondria-targeted drugs for the treatment of sarcopenia: rationale and perspectives, *Pharmaceutics*, **14**, 2588, doi: 10.3390/pharmaceutics14122588.
54. Russell, J. W., Golovoy, D., Vincent, A. M., Mahendru, P., Olzmann, J. A., Mentzer, A., and Feldman, E. L. (2002) High glucose-induced oxidative stress and mitochondrial dysfunction in neurons, *FASEB J.*, **16**, 1738-1748, doi: 10.1096/fj.01-1027com.
55. Green, K., Brand, M. D., and Murphy, M. P. (2004) Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes, *Diabetes*, **53**, S110-S118, doi: 10.2337/diabetes.53.2007.s110.
56. Vyssokikh, M. Y., Zorova, L., Zorov, D., Heimlich, G., Jurgensmeier, J. J., and Brdiczka, D. (2002) Bax releases cytochrome *c* preferentially from a complex between porin and adenine nucleotide translocator. Hexokinase activity suppresses this effect, *Mol. Biol. Rep.*, **29**, 93-96, doi: 10.1023/a:1020383108620.
57. Beutner, G., Ruck, A., Riede, B., and Brdiczka, D. (1997) Complexes between hexokinase, mitochondrial porin and adenylate translocator in brain: regulation of hexokinase, oxidative phosphorylation and permeability transition pore, *Biochem. Soc. Trans.*, **25**, 151-157, doi: 10.1042/bst0250151.
58. Kunji, E. R., Aleksandrova, A., King, M. S., Majd, H., Ashton, V. L., Cerson, E., Springett, R., Kibalchenko, M., Tavoulari, S., Crichton, P. G., and Ruprecht, J. J. (2016) The transport mechanism of the mitochondrial ADP/ATP carrier, *Biochim. Biophys. Acta*, **1863**, 2379-2393, doi: 10.1016/j.bbamcr.2016.03.015.
59. Duncan, A. L., Ruprecht, J. J., Kunji, E. R. S., and Robinson, A. J. (2018) Cardiolipin dynamics and binding to conserved residues in the mitochondrial ADP/ATP carrier, *Biochim. Biophys. Acta Biomembr.*, **1860**, 1035-1045, doi: 10.1016/j.bbamem.2018.01.017.
60. Allouche, M., Pertuiset, C., Robert, J. L., Martel, C., Veneziano, R., Henry, C., Sharaf el dein, O., Saint, N., Brenner, C., and Chopineau, J. (2012) ANT-VDAC1 interaction is direct and depends on ANT isoform conformation *in vitro*, *Biochem. Biophys. Res. Commun.*, **429**, 12-17, doi: 10.1016/j.bbrc.2012.10.108.