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Dietary Supplementation of Old Rats with Hydrogenated Peanut Oil Restores Activities of Mitochondrial Respiratory Complexes in Skeletal Muscles

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Abstract—The effect of dietary supplementation of old rats (26-33 months) with hydrogenated peanut oil on the activity of mitochondrial enzymes in skeletal muscles has been studied. The activities of NADH-coenzyme Q1 oxidoreductase, cytochrome *c* oxidase, and citrate synthase were determined spectrophotometrically in muscle homogenates. The activities of respiratory complexes I and IV were shown to significantly decrease with the age compared to the activity of the same enzymes in young animals, while the activity of citrate synthase was virtually unchanged. The fatty acid composition of muscle homogenates of old rats differed from that of young animals by a reduced content of myristic, oleic, linoleic, and α-linolenic acids and enhanced content of dihomo-γ-linolenic, arachidonic, and docosahexaenoic acids. Per oral supplementation of the old rats with hydrogenated peanut oil completely restored the activity of complex IV and increased the activity of complex I to 80% of the value observed in muscles of young animals, reducing the content of stearic, dihomo-γ-linolenic, arachidonic, eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids relative to that in the groups of old and young rats. The content of oleic and linoleic acids increased relatively to that in the group of the old rats, as well as young animals. The possible mechanisms of the restoration of the activity of the respiratory enzymes under the administration of hydrogenated peanut oil are discussed.

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Key words: mitochondrial respiratory chain, oxidative stress, aging, fatty acid composition of lipids, skeletal muscles, vegetable oil

The problems of prolonging life and improving its quality in the elderly have always been of great concern. Recovering of the activities of the enzymes of the mitochondrial respiratory chain is of current interest, since normal functioning of mitochondria is disordered in a number of neurological diseases [1] as well as during aging [2]. The hypothesis stated more than 50 years ago that oxidative stress can be the main reason for damage to tissues and cells currently prevails in explaining age-induced changes [3]. Reactive oxygen species (ROS) formed mainly in mitochondria are the main injuring factor of these changes [4]. Sharp increase in the production of ROS in the case of the high trans-membrane potential in mitochondria [5] damages proteins, lipids, and mitochondrial DNA, decreasing the activity of the respiratory enzymes. Constant use of antioxidants for the defense of tissues is impossible, since normally ROS play an important role in both cellular and intramitochondrial signaling [6, 7]. Mechanisms of the formation of ROS in mitochondria and the mechanisms of the defense against ROS are the subject of intensive investigations. Searching for substances that are able to defend mitochondria from injury by ROS [5, 8] is not only of practical significance, but also can help in understanding the mechanisms of their excessive formation.

The state of the electron-transport chain of mitochondria after supplementation of rodents with vegetable oils that are composed mainly from fatty acids has been studied for the last two decades [9-15]. Recently, it was shown that free fatty acids could either decrease or increase the rate of the ROS formation in mitochondria [10, 11]. The fatty acid composition of phospholipids of

Abbreviations: HNE, 4-hydroxy-2-nonenal, unsaturated aldehyde; ROS, reactive oxygen species.

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the mitochondrial membranes influences the activity of the membrane-bound enzymes and can be modified by supplementation of animals with purified fatty acids or vegetable oils [12-15]. The goal of the present work was to investigate the effect of hydrogenated peanut oil added into the ration of old animals on the activity of the respiratory chain enzymes of the mitochondria of their skeletal muscles.

MATERIALS AND METHODS

Animals. In the experiments, we used Sprague–Dawley rats that were kept in a vivarium in individual cages under natural light duration. The animals were fed with a standard nutrition mixture (complete combined feed of PK-120 formula) with free access to food and water. The age of the animals was in the range of 26-33 months. During 6 weeks the rats daily obtained 0.5-0.7 ml of hydrogenated peanut oil (Fluka, Germany) added to a small amount of oat flakes boiled with water. The fatty acid composition of the oil is presented in Table 1. The control old animals obtained an analogous portion of oat flakes without the oil. Young control rats were 5-6-months old. Immediately before the experiment the rats were decapitated after preliminary anesthesia under CO_2 according to the international protocols.

Preparation of samples. After decapitation, ~50 mg of the thigh quadriceps were immediately homogenized on ice with a glass homogenizer in 10 mM phosphate buffer, 0.1 mM EDTA, pH 7.4, containing protease inhibitor cocktail (10 μl per ml homogenate). The homogenate was filtered through a kapron tissue. The resulting suspension was additionally sonicated (4 times for 20-30 sec, using half of the maximal power of an UZDN-1 ultrasonic disintegrator). To compare the activity of the respiratory complexes in the homogenate with that in isolated mitochondria, the mitochondria were isolated from the thigh quadriceps using medium containing 120 mM KCl, 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, and 2 mg BSA per ml of the buffer. A piece of the muscle tissue (2-3 g) was carefully minced with scissors, supplemented with 10 ml of the buffer with 3-5 mg of Nagarse, incubated for 5 min at 3°C, and then supplemented with 10-20 ml of cold buffer. The minced tissue was filtered through a kapron tissue or centrifuged. Then the mitochondria were isolated using a standard procedure: the tissue was homogenized in 15 ml of the buffer using a glass homogenizer, the mitochondria were centrifuged and suspended in buffer containing 250 mM sucrose, 2 mM HEPES, pH 7.4, 0.2 mM EGTA, and then again centrifuged. The mitochondria were purified in a Percoll density gradient according to the protocol of the reagent. Protein concentration in the homogenate and in the suspension of the mitochondria was determined by the Lowry method. The enzyme activity was calculated per mg protein.

Table 1. Fatty acid composition of hydrogenated peanut oil

Fatty acids	Content, %
Palmitic ($C_{16:0}$)	8.5
Stearic ($C_{18:0}$)	16.8
Oleic ($C_{18:1}$)	67.2
Linoleic ($C_{18:2}$)	0.27
α-Linolenic ($C_{18:3}$)	0.2
Arachidic ($C_{20:0}$)	1.55
Behenic ($C_{22:0}$)	2.54
Lignoceric ($C_{24:0}$)	1.42
Saturated fatty acids	31.02
Monounsaturated fatty acids	68.3
Polyunsaturated fatty acids	0.47

Enzyme activities were determined using the procedures described earlier with minor modifications [16-19]. Methods were chosen after their experimental testing. Slight modifications seem to be noncritical, but the change of the buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 7.4 [18], to 10 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA during tissue homogenization maintained the activity of complex I in the homogenate, and the use of a high concentration of reduced cytochrome *c* (above 50 μM) allowed more accurate determination of the activity of complex IV [19]. The reactions were initiated by the addition of an aliquot of the homogenate into a 1-ml cuvette at 30°C, and the kinetics of the reaction were monitored for 5-8 min. The activity of NADH-coenzyme Q1 oxidoreductase was determined after 10 min of incubation of the homogenate at room temperature in the presence of 30-50 μM NADH [20]. The activity of complex I was determined by the rate of electron transfer from NADH to CoQ1, which was monitored at 340 nm in the absence or in the presence of rotenone. The kinetics of the citrate synthase reaction were monitored at 412 nm by the accumulation of CoA, which was detected by its reaction with the Ellman reagent. The activity of complex IV was determined at 550 nm by the rate of oxidation of cytochrome *c*. Complexes I and IV were assayed for sensitivity to the specific inhibitors rotenone and cyanide, which was found to be ~70-90 and 97-99%, respectively. All reagents used for the spectrophotometric determination of the activities of mitochondrial enzymes were from Sigma (Germany). A Specord M-40 spectrophotometer (Carl

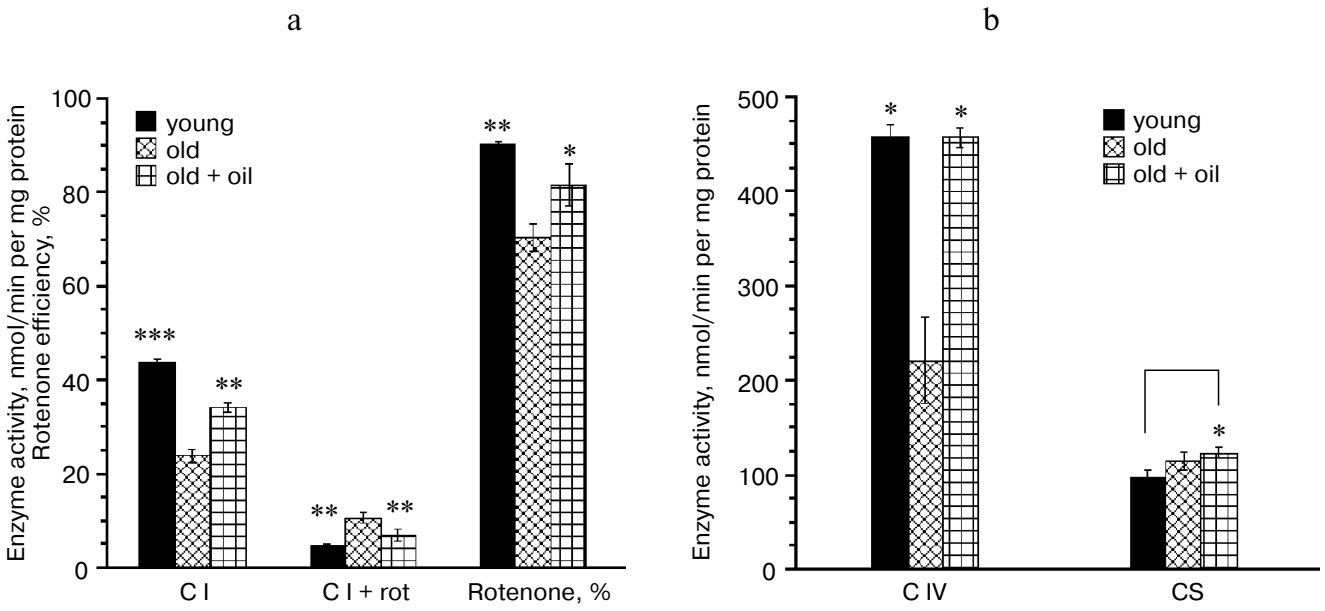
Zeiss, Germany) was significantly upgraded to obtain data in electronic form, which increased its sensitivity and stability approximately 10-fold. The data from the spectrophotometer were saved as *.txt files and imported into KaleidaGraph version 4 for subsequent computations.

Determination of fatty acid composition of muscle tissue. The buffer for homogenization was supplemented with 0.5% ionol (2,6-di-tert-4-methylphenol). The homogenate from 50 mg of muscle tissue was dried in a Savant SpeedVac vacuum concentrator (Savant Instruments, USA). Methyl esters of higher fatty acids were obtained using the method described earlier [21]. Fatty acid composition was determined using a GC 3900 analytical gas chromatograph (Varian, USA) equipped with a flame ionization detector (temperature, 260°C). Methyl esters of fatty acids were separated in a SUPELCOWAX-10 quartz capillary column (15 m × 0.25 mm × 0.3 μm) with immobilized stationary phase (Supelco, Switzerland). Samples (2 μl) were injected without partition of the carrier gas (helium) flow. The flow partition regime was started after 12-30 sec depending on concentration of the investigated substances. The temperature varied from 90 (0.5 min) to 240°C (5 min) at the rate of 6°C/min. The signal was detected using the Multikhrom-1.5x computer program (ZAO Ampersend, Russia). To determine the amount of fatty acids, the method of internal standards was used with preliminary calculation of the corresponding calibration coefficients from the chromatogram of the mixture of the investigated fatty acids with margaric acid.

Statistical analysis. The enzyme activity and fatty acid composition were determined in groups containing 3-6 animals. In each sample, the activity was determined in 4-6 repetitions. The statistical significance of the differences was determined using the unpaired Student's *t*-test or ANOVA test.

RESULTS

Investigation of the activity of the enzymes of the mitochondrial respiratory chain in the homogenate of the muscle tissue of old rats showed a statistically significant decrease in the activity of complexes I and IV ($p \le 0.0003$ and $p \le 0.008$, respectively) compared to young animals (figure, panels (a) and (b)). Statistically significant increase in the activity of NADH-coenzyme Q1 oxidoreductase in the presence of rotenone in the preparation from the control old rats relative to the analogous activity in the preparations from the other two groups of animals (figure, panel (a)) is presumably due to the activity of other enzymes and is not related to complex I. The activity of citrate synthase in the muscle tissue was similar in three investigated groups of animals (figure, panel (a)) [16]. To demonstrate that the isolated mitochondria can-



Activity of mitochondrial enzymes of the thigh quadriceps in three groups of rats (young, old control, and old obtaining peanut oil). a) C I, the activity of complex I in the absence of rotenone; C I + rot, the activity of NADH-coenzyme Q1 oxidoreductase in the presence of rotenone (the same as the nonspecific component of complex I activity); rotenone (%), percentage of inhibition of complex I in three groups of rats. b) C IV, the activity of complex IV; CS, the citrate synthase activity (significantly increased in the old rats obtaining peanut oil compared to young animals). The activities of complexes I and IV in muscles of old rats (medium column) are compared with the activities of the enzymes in the muscles of young rats and old rats obtaining peanut oil. The data are presented as the mean value of three experiments ± standard error. Statistical significances of the differences are designated as: * $p \le 0.01$-0.05; ** $p \le 0.001$-0.005; *** $p \le 0.0001$-0.0005

Table 2. Activity of respiratory complexes in homogenates and in mitochondria isolated from muscle tissue of young and old rats

Animals, preparation	Complex I	Complex IV
Young, homogenate	47.6 ± 0.3 (2)*	453 ± 20 (2)
Young, mitochondria	50.0 ± 0.8 (1)	488 ± 50 (1)
Old, homogenate	25.2 ± 1.4 (2)	241 ± 72 (2)
Old, mitochondria	45.4 ± 0.2 (2)	528 ± 224 (2)

* Number of experiments is indicated in parentheses.

not serve as a criterion of aging of animals, the activity of the mitochondrial complexes was measured simultaneously in tissue homogenates and in the mitochondria isolated from muscles (Table 2). As seen from Table 2, the enzymatic activities in the mitochondria isolated from the tissues of the young and old rats are similar or coincide, which does not reflect the real situation. This was the reason for using muscle tissue homogenates rather than isolated mitochondria. The activity of citrate synthase was not investigated, since it varied little with the age of the animals.

Fatty acid composition of the muscle tissue homogenate of the old rats differed from that of the young animals by a decreased content of myristic, oleic, linoleic, and α-linolenic acids. At the same time, the ratio of dihomo-γ-linolenic, arachidonic, and docosahexaenoic acids was enhanced (Table 3).

Supplementation of old rats with hydrogenated peanut oil resulted in statistically significant restoration of the activity of complex I ($p \leq 0.0015$), this constituting 80% of the complex I activity in the muscles of young animals. The activity of complex IV in the old rats obtaining hydrogenated peanut oil was equal to the activity of this

Table 3. Fatty acid composition of homogenates of thigh quadriceps of young rats, old control rats, and old rats obtaining hydrogenated peanut oil (percentage; data are presented as means of six experiments)

Fatty acid	Young animals	Old animals	Old animals + + peanut oil
Myristic acid ($C_{14:0}$)	1.55 ± 0.08	1.21 ± 0.05##	1.12 ± 0.11#
Palmitic acid ($C_{16:0}$)	23.97 ± 0.35	23.33 ± 0.45	19.83 ± 1.50#*
Palmitoleic acid ($C_{16:1}$)	3.01 ± 0.32	2.12 ± 0.38	3.23 ± 0.65
Stearic acid ($C_{18:0}$)	8.05 ± 0.60	9.18 ± 0.54	5.28 ± 0.34##***
Oleic acid ($C_{18:1}$)	19.08 ± 0.93	14.03 ± 1.54#	27.30 ± 1.25###***
Vaccenic acid ($C_{18:1}$ *cis*-isomer)	2.26 ± 0.06	3.25 ± 0.11###	3.87 ± 0.20#*
Linoleic acid ($C_{18:2}$)	27.09 ± 1.10	22.54 ± 0.26##	30.23 ± 0.64#***
α-Linolenic acid ($C_{18:3}$)	0.84 ± 0.12	0.20 ± 0.01###	0.34 ± 0.06###*
Dihomo-γ-linolenic acid ($C_{20:3}$)	0.42 ± 0.05	0.60 ± 0.03##	0.29 ± 0.02#***
Arachidonic acid ($C_{20:4}$)	7.69 ± 0.69	13.97 ± 0.77###	5.07 ± 0.37##***
Eicosapentaenoic acid ($C_{20:5}$)	0.097 ± 0.009	0.088 ± 0.014	0.0433 ± 0.006###*
Docosapentaenoic acid ($C_{22:5n-3}$)	1.16 ± 0.17	1.45 ± 0.14	0.66 ± 0.03#***
Docosahexaenoic acid ($C_{22:6}$)	4.60 ± 0.63	7.89 ± 0.60##	2.54 ± 0.17#***
Saturated fatty acids	33.57	33.72	26.23
Monounsaturated fatty acids	24.35	19.39	34.40
Polyunsaturated fatty acids	41.90	46.77	39.17

\# Differences are statistically significant relative to young animals.
* Differences are statistically significant relative to old animals.
#* $P \leq 0.01–0.05$.
##** $P \leq 0.001–0.005$.
###*** $P \leq 0.0001–0.0005$.

enzyme in young animals (figure, panel (b)). Simultaneously, the content of stearic, dihomo-γ-linolenic, arachidonic, eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids significantly decreased compared to the groups of young and old animals. The content of oleic acid significantly increased with respect to both old and young rats. The content of linoleic acid was restored to a level that slightly exceeded that of the young animals (Table 3).

The sensitivity of complex I to the specific inhibitor rotenone for the young rats was 90%, reducing to 70% in for the old control rats ($p \leq 0.002$; figure, panel (a)). Supplementation of the old animals with hydrogenated peanut oil restored the sensitivity of complex I to rotenone to 82% ($p \leq 0.075$). The activity of citrate synthase was virtually independent of the age of the animals, but supplementation of the old rats with the oil resulted in a small but statistically significant increase in the enzyme activity (figure, panel (b)).

The fatty acid composition of the hydrogenated peanut oil determined in our work is shown in Table 1. It contains a small amount of polyunsaturated fatty acids. Resistant to oxidation saturated stearic and palmitic acids and monounsaturated oleic acid constitute 99% of the fatty acids of this oil.

DISCUSSION

It is known that the mitochondrial respiratory complexes from both skeletal muscles and other tissues are damaged during aging with complete or partial loss of their activities [2, 4, 12, 22]. As mentioned above, the main damaging factors are ROS. According to the literature, during aging of rodents the most pronounced damages are observed in mitochondrial complexes I and IV. In a number of works damages of complexes II and III were demonstrated [10, 11, 23-25]; however, in our experiments we observed no statistically significant decrease in the activities of complexes II and III in the muscles of old rodents [26].

It is known that the admissible level of damage to the mitochondrial respiratory complexes that does not affect the behavior of animals is rather high, about 80-85% [27, 28]. In our experiments we observed a decrease in the activity of mitochondrial complexes I and IV in rats more than 26 months old [26]. In most works on this subject, the damage to mitochondrial complexes of the respiratory chain was investigated on rats of ages from 3 to 12-24 months old. Supplementation with different nutrients was started at an early age and continued during their entire life [12, 22]. Also, the activities of the mitochondrial respiratory complexes were determined in isolated mitochondria. Our preliminary investigations [26] demonstrated that this approach may lead to incorrect conclusions because the procedure of isolation of mitochondria is developed for separation of the organelles of high quality that constitute only a minor part of the total pool of mitochondria. Tissues of young rodents contain mitochondria of different density, and centrifugation in a density gradient of sucrose or Percoll yields three different mitochondrial fractions. Isolation of mitochondria from tissues of old animals results in a lower yield and quality of the organelles because of the presence of large amounts of the connective tissue in these animals. Investigation of the activity of the respiratory chain complexes and citrate synthase in the mitochondria purified in a density gradient revealed no difference between old and young rats [26], so the activity of the enzymes was further studied in muscle tissue homogenates, since in this case the activity of the respiratory enzymes reflected the state of the total mitochondrial pool.

Special attention has recently given to the investigation of the structure of lipoprotein complexes [29-33]. It was shown that the lipid phase of the mitochondrion serves not only for anchoring of protein complexes and electrochemical isolation of the intramitochondrial space. It was demonstrated that the fatty acid chains of lipids and the protein backbone form complex and stable supramolecular structures that perform ATP synthesis. All of the main work on the transport of electrons and protons and transfer of the oxygen into the active site of cytochrome *c* oxidase presumably proceeds via strictly separated pathways [32-34]. Replacement of fatty acid chains by others or their damage by peroxidation disturbs the supramolecular structures, resulting in a decrease or complete loss of the activity of the enzymes, for example, because of the disruption of the interactions between the subunits of complex IV. The supplementation of old animals with vegetable oils containing free fatty acids allows modification of the fatty acid composition of the lipids of the respiratory complexes. It is known that the fatty acid composition of phospholipids changes with age [35, 36]. During aging of rodents, in the microsomes of the liver the content of unsaturated fatty acids decreases and the content of saturated fatty acids increases. However, in our experiments we observed no statistically significant increase in the content of saturated fatty acids in the muscles of old animals. Statistically significant reduction in the content of oleic, linoleic, and α-linolenic acids was observed together with growth in the content of dihomo-γ-linolenic, arachidonic, and docosahexaenoic acids. The total content of monounsaturated fatty acids decreased, while the total content of polyunsaturated fatty acids increased (Table 3). The direction of the age-related changes in the fatty acid composition in the muscles of the old rats agrees with the changes of these fatty acids in the membrane phospholipids of so-called heavy mitochondria isolated from the brain cortex of rats. It is suggested that this population of old mitochondria is characterized by disturbances in their typical functions [37].

The main polyunsaturated fatty acids subjected to strong oxidation in tissues of mammals are arachidonic and linoleic acids. It is known that for each oxidized molecule of monounsaturated fatty acid there are 40, 60, and 80 oxidized molecules of di-, tri-, and tetra-unsaturated fatty acids [38]. Most products of peroxidation are derivatives of linoleic acid. Among these products are malonyl dialdehyde and the unsaturated aldehyde 4-hydroxy-2-nonenal (HNE) [39, 40]. Cytochrome *c* oxidase is a potential target for reactive aldehydes and other products of peroxidation of lipids. The electron transport activity of the cytochrome *c* oxidase from bovine heart decreases when it reacts with HNE [39]. A decrease in the ratio of linoleic acid in the muscles of the old rats correlates with the reduction of the activity of complexes I and IV of the mitochondrial respiratory chain. Linoleic acid constitutes from 80 to 100% of fatty-acid chains of cardiolipin connecting cytochrome *c* oxidase with the inner mitochondrial membrane. The presence of cardiolipin is necessary for the normal functioning of this enzyme. Change in the composition of fatty-acid chains of cardiolipin and/or their peroxidation correlates with mitochondrial dysfunction [29]. The decrease in the concentration of linoleic acid and increase in the content of arachidonic and docosahexaenoic acids in the muscle tissue homogenates observed in our experiments (Table 3) agrees with the earlier described data on the reduction of the content of linoleic acid in cardiolipin in the heart mitochondria of old rats [41, 42]. This was accompanied by a growth in the content of arachidonic and especially docosahexaenoic acid. The biological role of the reduction of the content of irreplaceable linoleic and α-linolenic acids and the growth in the content of dihomo-γ-linolenic, arachidonic, and docosahexaenoic acids is unknown. It was demonstrated that a high level of docosanoic acids in the diet of the Atlantic salmon reduced the activity of mitochondrial cytochrome *c* oxidase and the content of cardiolipin in mitochondria and enhanced the activity of superoxide dismutase and caspase-3 [13]. Presumably, the increase in the ratio of docosahexaenoic acid is necessary for maintaining the decreasing with age muscle tissue function, since a high level of docosahexaenoic acid in muscles is related to high frequency of muscle contraction [43]. It is also suggested that high level of docosahexaenoic acid in phospholipids maintains the optimal activity of the ion pump of the sarcoplasmic reticulum and electron transport in mitochondria [15].

In our experiments the supplementation of old rats with hydrogenated peanut oil restored the activity of the respiratory enzymes. The restoration of the activity of complex I was statistically significant and constituted 80%, while the activity of complex IV completely coincided in the groups of the young and old animals (figure, panel (b)). At the same time, the fatty-acid composition of the homogenate of muscle tissue of old rats significantly changed (Table 3). The level of resistant to oxidation oleic acid, whose high content is responsible for the valuable effect of olive oil, was not only restored to the level corresponding to that of the young animals, but it significantly increased. The level of linoleic acid exceeded somewhat its content in the young rats, and the content of dihomo-γ-linolenic, arachidonic, eicosapentaenoic, and docosanoic acids decreased relative to both old and young animals. Thus, supplementation of old rats with hydrogenated peanut oil decreased the total content of polyunsaturated fatty acids, which presumably reduced the probability of the formation of peroxidation products. The complete restoration of the activity of complex IV in the animals obtaining hydrogenated peanut oil is likely to be due to the fact that this oil contains mostly resistant to oxidation fatty acids (Table 1). It has been found that 90% of the fatty acid chains of triglycerides efficiently maintaining the subunit composition of cytochrome *c* oxidase are resistant to oxidation palmitic (17%), stearic (12%), and oleic (61%) acids [31]. The data of X-ray analysis and model experiments suggested that the replacement of palmitate residues by stearate residues in the fatty acid chains of triglycerides blocks oxygen transport through complex IV. Besides, these fatty acids are constituents of phosphatidyl choline, phosphatidyl serine, and phosphatidyl glycerides found in the cytochrome *c* oxidase from bovine heart [31]. Presumably, the replacement of polyunsaturated fatty acids by oleic acid with some decrease in the content of stearic and palmitic acids in the muscles of old rats facilitates the restoration of the cytochrome *c* oxidase activity.

In a previous work we demonstrated that the supplementation of old mice with the antioxidant dihydroquercetin restored the activities of the enzymes of the mitochondrial respiratory chain [16]. The restoration of the activities of complexes I and IV during supplementation of old rats with hydrogenated peanut oil suggests the existence of different mechanisms of restoration of the enzymes. The restoration of the enzyme activities under the action of dihydroquercetin is likely to be due to neutralization of ROS, while fatty acids of the hydrogenated peanut oil presumably modify the mitochondrial membranes and/or the enzyme complexes.

Our data are of special interest since the therapeutic effect of hydrogenated peanut oil developed for a relatively short period of time (6 weeks). The results of this work can be useful for the development of methods for the corrections of the activity of respiratory enzymes during different neurological diseases and during aging.

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