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Oxidative Stress as Regulatory Factor for Fatty-Acid-Induced Uncoupling Involving Liver Mitochondrial ADP/ATP and Aspartate/Glutamate Antiporters of Old Rats

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Abstract—Palmitate-induced uncoupling, which involves ADP/ATP and aspartate/glutamate antiporters, has been studied in liver mitochondria of old rats (22-26 months) under conditions of lipid peroxidation and inhibition of oxidative stress by antioxidants—thiourea, Trolox, and ionol. It has been shown that in liver mitochondria of old rats in the absence of antioxidants and under conditions of overproduction of conjugated dienes, the protonophoric uncoupling activity of palmitate is not suppressed by either carboxyatractylate or aspartate used separately. However, the combination of carboxyatractylate and aspartate decreased uncoupling activity of palmitate by 81%. In this case, palmitate-induced uncoupling is limited by a stage insensitive to both carboxyatractylate and aspartate. In the presence of antioxidants, the palmitate-induced protonophoric uncoupling activity is suppressed by either carboxyatractylate or aspartate used separately. Under these conditions, palmitate-induced uncoupling is limited by a stage sensitive to carboxyatractylate (ADP/ATP antiporter) or aspartate (aspartate/glutamate antiporter). In the absence of antioxidants, the uncoupling activity of palmitate is not suppressed by ADP either in the absence or in the presence of aspartate. However, in the presence of thiourea, Trolox, or ionol ADP decreased the uncoupling activity of palmitate by 38%. It is concluded that in liver mitochondria of old rats the development of oxidative stress in the presence of physiological substrates of ADP/ATP and aspartate/glutamate antiporters (ADP and aspartate) results in an increase of the protonophoric uncoupling activity of palmitate.

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Key words: liver mitochondria, aging, oxidative stress, reactive oxygen species, fatty acids, uncoupling, ADP/ATP antiporter, aspartate/glutamate antiporter

Mitochondria of higher animals are not only highly effective power stations supplying a cell with ATP and heat, but they are also involved in its programmed death. In the latter alternative, mitochondrial function is associated with increased production of reactive oxygen species (ROS) [1-3]. Mitochondrial ROS formation involves various pathways. The one-electron reduction of oxygen (at complexes I and III) results in direct formation of superoxide anion radical; its subsequent conversions (in chemical enzymatic and nonenzymatic reactions) are accompanied by formation of hydrogen peroxide, hydroxyl radical, and other ROS [1, 4, 5]. Aging of animals is also accompanied by increased ROS generation in mitochondria of various tissues and organs [1, 4, 6]. This state, also known as oxidative stress, can be also associated with decreased activity of one of the natural antioxidants, α-tocopherol [7, 8]. The development of oxidative stress is accompanied by mutation of mitochondrial DNA, oxidative damage of proteins, and lipid peroxidation; according to one of the theories of aging, oxidative stress is one of the factors responsible for age-related changes in organs and tissues [1, 4, 6].

One of the ways suppressing mitochondrial ROS production is the decrease of proton electrochemical potential difference ($\Delta\mu_{H^+}$) across the inner mitochondrial membrane [3, 9, 10]. This can be achieved by the increase of proton conductance of the inner mitochondrial membrane by means of natural uncouplers of oxidative phosphorylation, free (non-esterified) fatty acids [10]. Several mechanisms of the uncoupling effect of fatty acids are known [9, 11]. In the absence of calcium ions (in the

Abbreviations: DNP) 2,4-dinitrophenol; FCCP) carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ROS) reactive oxygen species.

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presence of EGTA), the protonophore uncoupling effect of fatty acids observed in liver mitochondria mainly involves protein carries of the inner mitochondrial membrane: ADP/ATP and aspartate/glutamate antiporters [9, 11-13]. This conclusion is based on the fact, that the specific inhibitor of ADP/ATP antiporter carboxyatractylate and substrates of the aspartate/glutamate antiporter, aspartate and glutamate, inhibit the uncoupling effect of fatty acids [9, 11-13]. It is suggested that involvement of ADP/ATP and aspartate/glutamate antiporters in the uncoupling effect of fatty acids consists in transfer of fatty acid anion from the inner membrane monolayer onto the outer one, whereas subsequent trans-bilayer transfer of a nondissociated form of fatty acid occurs via the flip-flop mechanism, which does not employ proteins [9, 11]. It is suggested that modification of ADP/ATP antiporter by products of lipid peroxidation can result in the increase of proton conductance of the inner mitochondrial membrane in the presence of fatty acids [11]. Our pilot experiments have shown that in contrast to liver mitochondria from young rats, the liver mitochondria from old rats are characterized by resistance of the uncoupling effect of palmitate to inhibition by carboxyatractylate or aspartate used separately [14]. It is interestingly to suggest that excessive ROS production in mitochondria from old rats would result in the increase of the rate of fatty acid anion transfer from the inner monolayer of membrane onto the outer one by means of ADP/ATP and aspartate/glutamate antiporters and the development of insensitivity of this process to inhibition by physiological ligands of these carriers.

The goal of this study was to investigate how oxidative stress induced in liver mitochondria by endogenous processes, associated with aging of animals, influences the protonophore uncoupling effect of fatty acids attributed to functioning of ADP/ATP and aspartate/glutamate antiporters. To achieve this goal the following parameters have been investigated in liver mitochondria of old rats (characterized by increased lipid peroxidation): the effect of ligands of the carriers, carboxyatractylate, ADP, and aspartate on palmitate-induced respiration assayed in the absence and in the presence of antioxidants (thiourea, Trolox, or ionol) as well as the uncoupler FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone).

MATERIALS AND METHODS

Male albino rats were used in the experiments. According to the age classification [15], they have been subdivided into two age groups: young rats (6-9-month-old, body mass of 200-250 g) and old rats (22-26-month-old, body mass of 420-500 g). These groups of rats are defined as "young" and "old" rats. Animals of these two age groups were kept, fed, and sacrificed under identical conditions corresponding to requirements given in [15]. Liver mitochondria were isolated by the conventional method of differential centrifugation followed by subsequent removal of endogenous fatty acids by means of treatment with fat-free BSA as described in [16]. The isolation medium contained 250 mM sucrose, 1 mM EGTA, 5 mM Mops-Tris (pH 7.4). During experiments, mitochondrial suspension (70-80 mg of mitochondrial protein per ml) was kept in an Eppendorf-type (Germany) conical tube. Protein concentration was determined by the biuret method using bovine serum albumin as standard.

Mitochondrial respiration was registered at 25°C using a Clark type oxygen electrode and a LP-9 polarograph. The concentration of mitochondrial protein in the oxygen cell was ~1.2-1.3 mg/ml. The incubation medium contained 200 mM sucrose, 20 mM KCl, 5 mM potassium succinate, 2 mM $MgCl_2$, 0.5 mM EGTA, and 10 mM Mops-Tris (pH 7.4). Oligomycin (2 μg/ml) and 2 μM rotenone were added into the oxygen cell right after the mitochondria. In the case of determination of the respiration rate during oxidative synthesis of ATP (State 3), the incubation medium also contained 5 mM KH_2PO_4 but without oligomycin. In this case, 200 μM ADP was added 2 min after addition of rotenone. The values of ADP/O were determined by the pulse method [17]. The recoupling effects of carboxyatractylate, ADP, or aspartate expressed as percent were determined as the ratio of the value of inhibition of respiration in the presence of palmitate by one of the recoupling agents used to the value of stimulation of respiration by palmitate, according to the formula: $100 \cdot \Delta J_u/(J_u - J_o)$, where J_u and J_o are respiration rates in the presence and absence of palmitate and ΔJ_u is the value of the decrease in the respiration rate by a recoupling agent. Specific protonophore activity of palmitate was determined by our own, previously developed approach [18] by the palmitate-induced increase of mitochondrial respiration rate referred to its concentration, according to the formula $(J_u - J_o)/[U]$, where $[U]$ is palmitate concentration. The specific protonophore activity is expressed in μM O_2/min per μM of palmitate.

The content of mitochondrial-conjugated dienes was determined by the spectrophotometric method after their extraction into heptane [19]. The increase in content of mitochondrial-conjugated dienes during incubation of mitochondria under controlled conditions was determined as follows. Mitochondria (1.4 mg protein per ml) were suspended in the incubation medium at 25°C under stirring. Oligomycin (2 μg/ml) and 2 μM rotenone were added together with the mitochondria. According to the goal of this study, the other compounds were also added together with these inhibitors. Aliquots (0.7 ml each) were taken twice: right after these additions and 5 min later. The increase in content of mitochondrial conjugated dienes was expressed as the difference between optical density values of the heptane extract at 233 nm at the initial moment of mitochondrial incubation (ΔA_0) and 5 min

later (ΔA) or in relative units as the factor α, determined according to the formula: $\alpha = (\Delta A - \Delta A_0)/\Delta A_0$.

The following reagents were used in this study: Mops, Tris, palmitic acid, FCCP, oligomycin, potassium succinate, potassium aspartate, carboxyatractylate, thiourea, and fatty acid free BSA (Sigma, USA); Trolox or ionol (Aldrich, USA); rotenone and EGTA (Serva, Germany); ADP, 2,4-dinitrophenol (DNP), and sucrose (Fluka, Germany); KCl and $MgCl_2$ (Merck, Germany). Other chemicals of chemically pure and especially pure grades were produced in Russia. Palmitic acid was used as 20 mM solution in ethanol.

RESULTS AND DISCUSSION

Accumulation of lipid peroxidation products (including conjugated dienes) is considered as one of indicators of increased cellular generation of ROS, i.e. the development of oxidative stress [19-21]. Figure 1 shows that the mitochondrial level of conjugated dienes is higher in old than in young rats. Controlled-state incubation of liver mitochondria from old rats for 5 min results in the increase of conjugated dienes by 0.023 ± 0.003 ΔA/mg protein ($n = 6$). Under identical conditions, incubation of liver mitochondria from young rats caused a smaller increase of the conjugated dienes: 0.015 ± 0.001 ΔA/mg protein ($n = 9$). Formation of conjugated dienes as primary products of lipid peroxidation is known to be associated with dislocation of a double bond induced by free radicals, including superoxide anion-radical [19-21]. Consequently, these results may be considered as evidence that aging of animals is accompanied by the development of oxidative stress in mitochondria and associated with increase of lipid peroxidation. Intensification of oxidative stress in liver mitochondria of old rats may be determined by reduced protective effect of natural antioxidants. The latter is consistent with known data on decreased activity of α-tocopherol in liver mitochondria during aging of rats [7, 8]. However, we did not find differences in the State 3 respiration rate and the ADP/O coefficient between liver mitochondria from old and young rats (data not shown). This is consistent with known data that increased ROS production in liver mitochondria is not necessarily accompanied by the decrease in parameters of respiratory coupling and oxidative phosphorylation during aging of animals [22, 23].

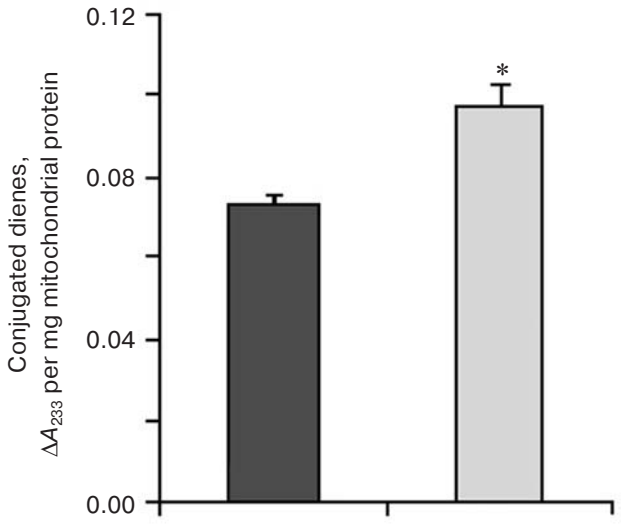

Fig. 1. Conjugated diene content in liver mitochondria from young and old rats (black and gray columns, respectively). Data represent mean ± SEM ($n = 6$-8), * $p < 0.01$ (Student's test).

Table 1. Effect of antioxidants on accumulation of conjugated dienes (α) in liver mitochondria from old rats under controlled conditions

Experimental conditions	n	α, relative units
Control	8	0.235 ± 0.022
Thiourea, 0.2 mM	5	0.060 ± 0.022*
Trolox, 20 μM	5	0.074 ± 0.024*
Ionol, 10 μM	4	0.081 ± 0.023*
FCCP, 20 nM	4	0.077 ± 0.021*

Note: Experimental conditions and composition of the incubation medium are given in "Materials and Methods". The compounds used in this study were added right after the mitochondria. Data represent mean ± SEM.

* Differences between experimental (in the presence of antioxidant) and control (without antioxidant) groups are statistically significant, $p < 0.01$ (Student's test).

We have used well known antioxidants, free radical scavengers, differing in chemical structure and by solubility in water and lipids: strictly water soluble thiol-containing compounds (thiourea) [24], a water/lipid soluble analog of α-tocopherol (Trolox) [25], and also highly effective lipid soluble phenolic antioxidant (ionol, also known as butylated hydroxytoluene) [26]. Pilot experiments have shown that the employed concentrations these antioxidants did not influence respiration and oxidative phosphorylation in liver mitochondria from old rats (data not shown). Table 1 shows that all these antioxidants decrease accumulation of conjugated dienes in liver mitochondria from old rats during their incubation under controlled conditions. Similar effects of these antioxidants have been observed in liver mitochondria from young rats, where accumulation of conjugated dienes is less intensive. Inhibition of conjugated diene accumulation was also observed during incubation of mitochondria in the presence of the protonophore uncoupler (FCCP, Table 1).

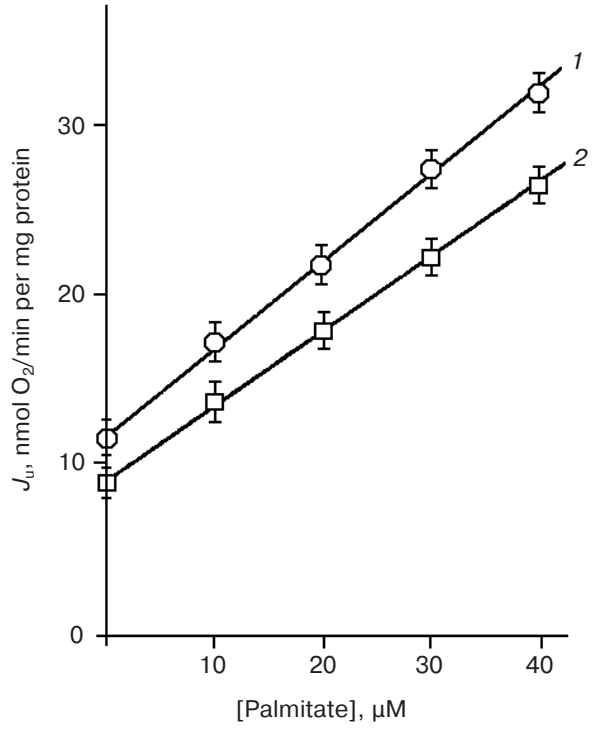

Fig. 2. Dependence of respiration rate of liver mitochondria from young (*1*) and old (*2*) rats on palmitate concentration. Data represent mean ± SEM (n = 4-6).

In the presence of magnesium ions, oligomycin, and EGTA, protonophoric activity of fatty acids can be evaluated by stimulation of mitochondrial respiration [13, 18]. Under these conditions, the dependence of the respiration rate of mitochondria from young and old rats on palmitate concentration exhibited almost linear behavior up to 40 μM palmitate concentration (Fig. 2). It should be noted that the respiration rate of mitochondria from old rats was lower than the respiration rate of mitochondria from young rats (Fig. 2). In all subsequent experiments, the concentration of palmitate was 30 μM.

Respiration of mitochondria from young rats was effectively inhibited by carboxyatractylate and aspartate irrespectively to the order of their addition (Fig. 3, curves *a* and *b*). However, in the presence of palmitate, respiration of mitochondria from old rats was basically insensitive to inhibition by carboxyatractylate, whereas subsequent addition of aspartate caused significant inhibition of respiration (Fig. 3, curve *c*). Addition of these reagents in the reverse order caused the reversed effect (Fig. 3, curve *d*). Table 2 shows results of the study on the effects of the antioxidants thiourea, ionol, and Trolox on respiration of liver mitochondria from old rats under controlled conditions in the presence of palmitate and following subsequent addition of carboxyatractylate and aspartate. This table shows that none of the antioxidants influenced mitochondrial respiration under controlled conditions, in the presence of palmitate and in the presence of DNP (under conditions of maximal stimulation of respiration). In the presence of antioxidants, carboxyatractylate and aspartate acquire the ability to inhibit mitochondrial respiration following their addition after palmitate (Table 2).

Inhibition of the fatty acid uncoupling effect by carboxyatractylate and aspartate can be quantitatively expressed as the recoupling effect [12, 13]. In the absence of carboxyatractylate (Fig. 3, curve *c*) or aspartate (Fig. 3, curve *d*), the influence of uncoupling their recoupling effects are zero; during combined action (Fig. 3, curves *c* and *d*) the recoupling effect of carboxyatractylate and aspartate is 81%. In the presence of thiourea, ionol, or Trolox, the recoupling effect of carboxyatractylate is 44.2 ± 1.1% (n = 5), 42.1 ± 0.4% (n = 6), and 45.6 ± 1.4% (n = 4), respectively, and the effect of aspartate is 35.0 ± 1.8% (n = 5), 37.5 ± 1.2% (n = 6), and 36.9 ± 1.5% (n = 4), respectively.

It has been mentioned above that conjugated diene production is suppressed under conditions of increased

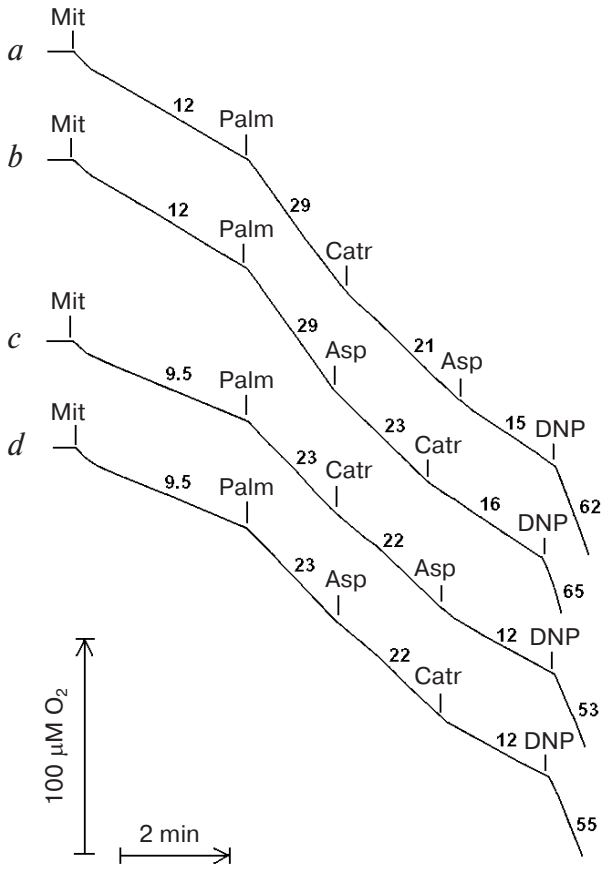

Fig. 3. Respiration of liver mitochondria from young (curves *a* and *b*) and old (curves *c* and *d*) rats in the presence of palmitate and during subsequent addition of carboxyatractylate, aspartate, and DNP. Additions: 1.2 mg mitochondrial protein (Mit), 30 μM palmitate (Palm), 1 μM carboxyatractylate (Catr), 3 mM aspartate (Asp), and 50 μM DNP (DNP).

Table 2. Effect of antioxidants on respiration of liver mitochondria from old rats under conditions of the uncoupling effect of palmitate and following subsequent addition of carboxyatractylate and aspartate (added in different order: carboxyatractylate before aspartate (A) and carboxyatractylate after aspartate (B))

Additions	Respiration rate, nmol O_2/min per mg protein			
	control ($n = 8$)	thiourea ($n = 5$)	ionol ($n = 6$)	Trolox ($n = 4$)
A				
Without additions	9.5 ± 0.3	9.6 ± 0.4	9.8 ± 0.3	9.6 ± 0.4
Palm	23.2 ± 0.6	23.8 ± 1.1	24.0 ± 0.9	23.5 ± 0.8
Palm + Catr	21.8 ± 0.4	17.6 ± 0.8	18.0 ± 0.5	17.5 ± 0.6
Palm + Catr + Asp	12.2 ± 0.3	12.5 ± 0.6	12.7 ± 0.3	12.1 ± 0.4
Palm + Catr + Asp + DNP	52.6 ± 1.3	58.0 ± 6.5	51.0 ± 2.4	50.6 ± 3.2
B				
Without additions	9.7 ± 0.3	9.3 ± 0.5	9.9 ± 0.3	9.4 ± 0.4
Palm	23.0 ± 0.7	22.8 ± 1.2	23.1 ± 0.6	22.8 ± 0.9
Palm + Asp	22.0 ± 0.5	18.1 ± 1.1	18.1 ± 0.5	18.0 ± 0.5
Palm + Asp + Catr	12.2 ± 0.4	12.4 ± 0.7	12.5 ± 0.3	11.7 ± 0.5
Palm + Asp + Catr + DNP	52.2 ± 1.7	53.4 ± 4.9	53.8 ± 2.9	51.5 ± 3.1

Note: All the compounds studied (0.2 mM thiourea, 10 μM ionol, and 20 μM Trolox) were added right after the mitochondria. Other additions: 35 μM palmitate (Palm), 1 μM carboxyatractylate (Catr), 3 mM aspartate (Asp), and 50 μM DNP. Data represent mean ± SEM.

proton conductivity of the inner mitochondrial membrane induced by FCCP (Table 1). Table 3 shows that FCCP induced carboxyatractylate inhibitory activity on mitochondrial respiration in the presence of palmitate. In this case, the recoupling effect of carboxyatractylate is 46.3 ± 2.3% ($n = 4$). This is close to the values obtained in the presence of thiourea, Trolox, or ionol. These and the other (abovementioned) data suggest that inhibition of free radical and peroxide reactions by antioxidants observed in rat liver mitochondria from old rats under controlled conditions is accompanied by appearance of suppression of palmitate-induced uncoupling by carboxyatractylate or aspartate.

Table 3. The effect of FCCP on respiration of mitochondria from old rats under conditions of uncoupling effect of palmitate followed by subsequent addition of carboxyatractylate and aspartate

Additions	Respiration rate, nmol O_2/min per mg protein	
	control ($n = 4$)	FCCP, 10 nM ($n = 4$)
Without additions	9.2 ± 0.3	14.4 ± 0.6
Palm	22.9 ± 0.6	28.6 ± 1.0
Palm + Catr	21.7 ± 1.2	22.1 ± 0.7
Palm + Catr + Asp	11.5 ± 0.4	16.8 ± 1.0
Palm + Catr + Asp + DNP	50.3 ± 1.8	55.0 ± 3.6

Note: FCCP was added right after the mitochondria; other additions as indicated in Table 2. Data represent mean ± SEM.

The data suggest that the development of oxidative stress in liver mitochondria from old rats results in changes of the general mode of the uncoupling effect of palmitate. However, it should be noted that in the absence of antioxidants carboxyatractylate together with aspartate inhibits the uncoupling effect of palmitate as well as in the presence of these antioxidants (Table 2). Consequently, under conditions of oxidative stress the uncoupling effect of palmitate also involves ADP/ATP and aspartate/glutamate antiporters. As mentioned above, the involvement of ADP/ATP and aspartate/glutamate antiporters consist in transfer of fatty acid anion from the inner membrane monolayer to the outer one, whereas the subsequent transfer of the fatty acid nondissociated form of the fatty acid through the bilayer follows the flip-flop mechanism, which does not involve proteins [9, 11]. Consequently, the uncoupling process consists of at least two sequential stages, which employ the recoupling effect of carboxyatractylate or aspartate in only one of them. It would be tempting to suggest that the development of oxidative stress in mitochondria is accompanied by change in the rate-limiting step at the uncoupling stage. It should also be noted that in all the cases, the mitochondrial electron

transport chain does not limit uncoupling because the maximal mitochondrial repatriation rate measured in the presence of optimal concentration of DNP was significantly higher than the concentration usually employed for studies of uncoupling (Table 2).

It is known that the rate-limiting stages of mitochondrial energy transformation can be detected by means of corresponding enzyme inhibitors (using analysis of the inhibitor titration curves) [27, 28]. Using this approach, we earlier found that palmitate uncoupling in liver mitochondria of adult but not old rats is limited by the stage of fatty acid anion transfer from the inner membrane monolayer to the outer one, and this involves ADP/ATP and aspartate/glutamate antiporters [12, 13]. In contrast to this process, the titration curves for carboxyatractylate (Fig. 4a) or aspartate (Fig. 4b) obtained in the case of palmitate uncoupling of mitochondria of old rats are characterized by sigmoid shape: during the first additions the recoupling substances inhibit respiration to a lesser degree than during subsequent additions. These results suggest that in mitochondria from old rats the uncoupling induced by palmitate is limited by the stage insensitive to carboxyatractylate or aspartate or the transfer of nondissociated form of fatty acid through the bilayer via the flip-flop mechanism. During thiourea addition to mitochondria, the suppression of the palmitate uncoupling occurred within the first additions of carboxyatractylate (Fig. 4a) or aspartate (Fig. 4b). Consequently, suppression of the production of reactive oxygen species in mitochondria of old rats results in transition of the rate-limiting step of uncoupling, and in this case the rate-limiting step consists in transfer of fatty acid anion from the inner monolayer to the outer one by means of the carriers.

The results suggest that oxidative stress in mitochondria of old rats modifies the ADP/ATP and aspartate/glutamate antiporters. These modifications result in increased transport activity with respect to transfer of fatty acid anion from the inner membrane to the outer one. This results in the change of the rate-limiting process of uncoupling from fatty acid anion transport (sensitive to carboxyatractylate or aspartate) to the transfer of the protonated fatty acid molecule from the outer membrane monolayer to the inner one (insensitive to carboxyatractylate or aspartate). In this case, removal of one of these carriers from the uncoupling process would not be accompanied by decreased uncoupling activity of palmitate due to the excessive activity of the other one.

In subsequent experiments (Fig. 5), carboxyatractylate was substituted for the physiological substrate of ADP/ATP antiporter, ADP. ADP concentration was chosen on the basis of data on its content in hepatocytes [29, 30]. There are contradictory data on the effect of ADP on fatty acid-induced protonophoric uncoupling in liver mitochondria. Some authors reported that ADP was less effective in inhibition of the uncoupling effect of palmitate than carboxyatractylate [31]; others reported that ADP was almost ineffective but it attenuated the recoupling action of carboxyatractylate [32]. We have recently

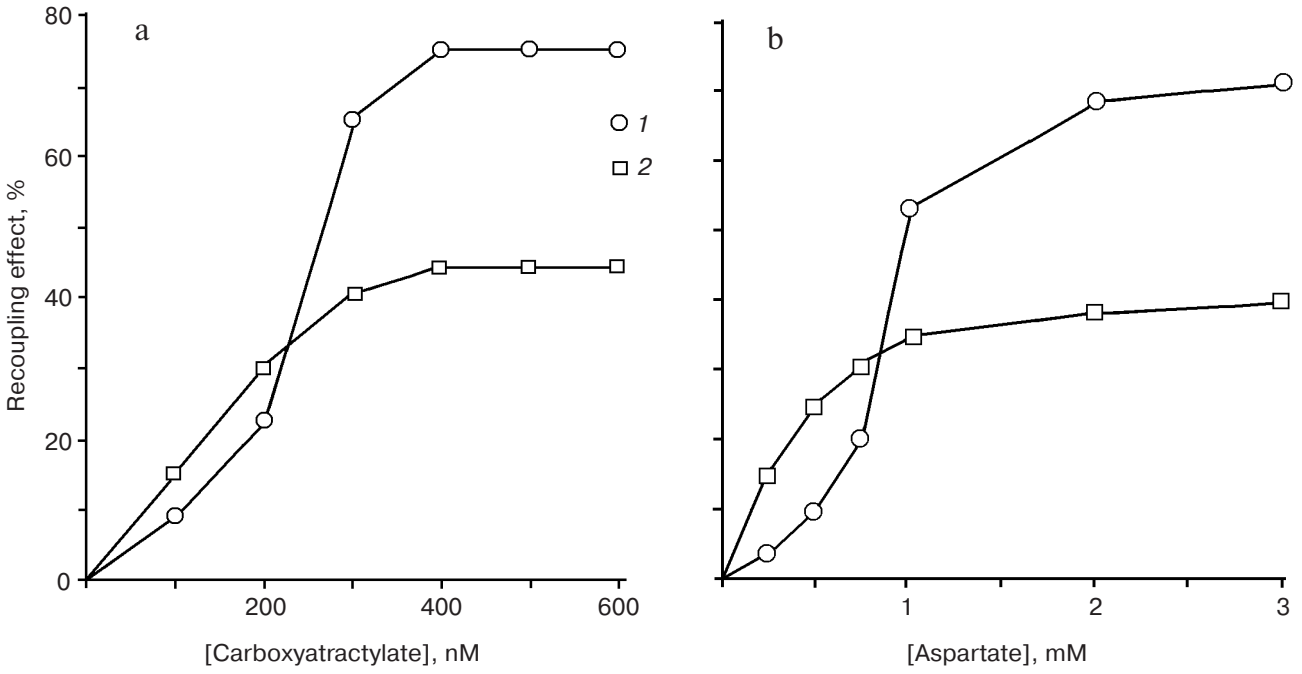

Fig. 4. Concentration dependence of the recoupling effects of carboxyatractylate in the presence of aspartate (a) and aspartate in the presence of carboxyatractylate (b) during palmitate-induced uncoupling of liver mitochondria from old rats in the absence (*1*) and in the presence of 0.2 mM thiourea (*2*). Other additions are as shown in Fig. 3.

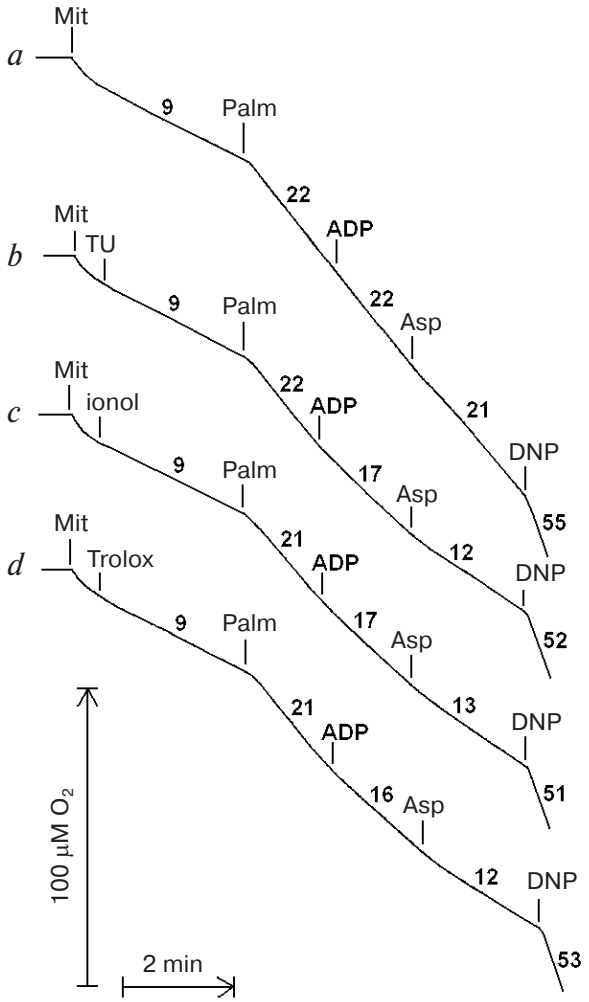

Fig. 5. Effects of antioxidants thiourea (TU) (*b*), ionol (*c*), and Trolox (*d*) on respiration of liver mitochondria (*a*) from old rats in the presence of palmitate (Palm) and following subsequent addition of ADP, aspartate (Asp), or DNP. Additions, 200 μM ADP; other additions are as shown in Fig. 3.

found that during succinate oxidation by liver mitochondria from adult but not old rats, ADP inhibited the uncoupling effect of palmitate only in the presence of thiourea [33]. One would expect that in liver mitochondria from old rats ADP would exhibit the recoupling effect only under conditions of decreased ROS production.

Figure 5 shows that in the absence of antioxidants ADP and aspartate added after palmitate do not influence respiration of the liver mitochondria from old rats. The same situation was observed during addition of these reagents in different order: palmitate, then aspartate, and finally ADP (data not shown). However, in the presence of thiourea, ionol, or Trolox, ADP and aspartate markedly inhibited mitochondrial respiration stimulated by palmitate (Fig. 5). In these experiments in the presence of thiourea, ionol, or Trolox, the recoupling effect of ADP was 38.0 ± 2.9% (n = 4), 36.1 ± 1.9% (n = 4), and 38.2 ± 2.1% (n = 4), respectively, and the recoupling effect of aspartate was 37.2 ± 2.3% (n = 4), 35.6 ± 1.6% (n = 4), and 36.3 ± 1.8% (n = 4), respectively. Specific uncoupling activity of palmitate in the presence of ADP and aspartate (but in the absence of antioxidants) was 0.441 ± 0.022 μM O_2/min per μM of palmitate, whereas in the presence of thiourea, ionol, and Trolox it decreased to 0.115 ± 0.016, 0.149 ± 0.019, and 0.119 ± 0.017 μM O_2/min per μM of palmitate, respectively. These data suggest that under conditions of overproduction of ROS in liver mitochondria from old rats, ADP (in contrast to carboxyatractylate) does not inhibit the uncoupling effect of palmitate even in the presence of aspartate. It is known that the interaction of carboxyatractylate with ADP/ATP antiporter fixes it in *c*-conformation, whereas in the presence of ADP this antiporter preferentially exists in *m*-conformation [34]. Thus, it is reasonable to suggest that in *c*-conformation the ADP/ATP antiporter is not involved into fatty acid anion transport from the inner membrane monolayer to the outer one, whereas in *m*-conformation such transport is possible but only under conditions of oxidative modification of this transporter.

Thus, the development of oxidative stress in liver mitochondria from old rats results in the significant protonophoric uncoupling activity of palmitate in the presence of physiological substrates of ADP/ATP and aspartate/glutamate antiporters—ADP and aspartate. This may be associated with the increased rate of transfer of fatty acid anion from the inner membrane monolayer to the outer one, which involves ADP/ATP and aspartate/glutamate antiporters, and removal of ADP effect to inhibit this transport. Thus, oxidative stress can be considered as one of the regulatory factors of the protonophoric uncoupling effect of palmitate involving ADP/ATP and aspartate/glutamate antiporters.

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