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A COMPARATIVE STUDY OF SUCCINATE-SUPPORTED RESPIRATION AND ATP/ADP TRANSLOCATION IN LIVER MITOCHONDRIA FROM ADULT AND OLD RATS

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SUMMARY

This study was undertaken to compare the rates of succinate-supported hepatic mitochondrial respiration between 12 months (adult) and 29 months (old) male Fischer 344 rats. Experiments were also performed to determine the activity of adenine nucleotide translocase and the effect of its inhibition on mitochondrial respiration. Succinate-supported state 3 mitochondrial respiration was found to decline 20% between 12 and 29 months of age in rat liver, along with a similar 25% decrease in the respiratory control ratio with age. Adenine nucleotide translocase activity is shown to decrease 39% from adult to old rat liver mitochondria. This decrease does not, however, account for the decline in state 3 respiration, since translocase activity is approximately 50% greater than state 3 respiration in both adult and old rats. Therefore, adenine nucleotide translocase is not rate-limiting for state 3 mitochondrial respiration.

Neither the rate of succinate permeation into the mitochondrion nor the rate of electron transport is rate-limiting for state 3 respiration, indicated by the greatly increased oxygen consumption with addition of the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (*m*-CCCP). These processes, therefore, are not responsible for the observed decline in state 3 respiration. The implications and possible cause of the age-related decrease in the maximal rate of ATP-synthesis are discussed.

Key words: Liver mitochondria; Respiration; Adenine nucleotide translocase; Aging

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INTRODUCTION

In healthy mitochondria, respiration is tightly coupled to the production of ATP which, in turn, is translocated across the inner mitochondrial membrane from the matrix of these organelles to the cytosol by a stoichiometric exchange with cytosolic ADP. This exchange is carried out by a membrane-bound transport protein, the adenine nucleotide translocase (ANT). There are numerous reports in the literature that the rate of mitochondrial ATP production when supported by NAD^+-linked substrates declines with aging (see Ref. 1 for a review with relevant references) and this effect has been attributed by several investigators to changes in the composition and the physical properties of the mitochondrial membrane [2—4]. When NAD^+-linked substrates are used, the ratio between the rates of respiration in the presence of excess ADP (state 3) and in its absence (state 4), which is termed the respiratory control ratio (RCR), has also been found to decline with aging in rat liver mitochondria [1,5]. This is caused by a decline in the rate of state 3 respiration, with very little change in the rate of state 4 respiration. It is generally believed that state 4 respiration is thermodynamically controlled [6]; however, the mechanism of control of state 3 respiration is still being disputed and a role for the ANT as the rate-limiting step has been suggested by some [7—13] and disputed by others [14—16].

In contrast to the results obtained with NAD^+-linked substrates, the rate of succinate-supported state 3 respiration was found by most investigators to be undiminished with age in various tissues including rat liver [17—19]. Others reported decreases in the rate of state 3 respiration [5,20,21] and under certain conditions also in the P/O ratio [5]. The observation that the decrements in mitochondrial respiration with aging seem to be substrate-specific may be explained if one assumes that with some substrates the step which is rate-limiting for respiration is the entry of metabolites into the electron transport chain (for succinate this may be the rate of its permeation into the mitochondrion or the rate of its oxidation by succinate dehydrogenase). In such cases the decline in state 3 respiration, though still existing, becomes masked. Only when the entry of substrate into the pathway is not rate limiting does the age-related decline in respiration rate become evident. It is thus not clear, from published work, whether there is no age-related decline in the rate of respiration supported by FAD-linked substrates, or is this decline present but undetected because substrate permeation or oxidation is rate limiting for ATP production.

In a recent study a significant age-related reduction in the rate of succinate-supported respiration was found when high concentrations of ADP were used, while no decline was observed with aging when suboptimal concentrations of ADP were employed [22]. A significant age-related decrease in the respiration rate was also found for uncoupled mitochondria and it was concluded that the changes in respiration originate in differences, with aging, in the electron transport chain.

In the present study the rates of state 3 and state 4 respiration were studied, using

succinate as substrate, in mitochondria from livers of adult and old rats. Both at saturating and at low concentrations of ADP a clear decline in the rate of state 3 respiration was found with aging, while the rates of oxygen consumption during state 4 respiration as well as by uncoupled mitochondria were unaffected. The changes induced in the rates of state 3 respiration with increasing degrees of inhibition of respiration by the specific ANT inhibitor carboxyatractyloside were also followed, in both adult and old mitochondria. The effects of aging on state 3 respiration under these conditions, where ATP/ADP translocation becomes rate limiting, are discussed.

MATERIALS AND METHODS

Materials

Rompun (xylazine) was obtained from Mobay Corp., Shawnee, KS. Ketaset (ketamine) was obtained from Aveco., Inc., Fort Dodge, IO. Carboxyatractyloside (CAT), carbonyl cyanide *m*-chlorophenyl hydrazone (*m*-CCCP) and all other reagents were of the highest quality available and obtained from Sigma Chemical Co., St. Louis, MO.

Animals

Male Fischer 344 rats from the NIA Barrier colony were obtained from the Charles River Co., Wilmington, MA. The rats were housed (two animals/cage) in a pathogen-free environment for 9 weeks before being sacrificed. They were fed ad libitum (Purina Chow #5001) and kept on a 12-h light/12-h dark cycle. Two groups were used: adult (12 months old at time of sacrifice), 11 animals; and old (29 months old at time of sacrifice) 15 animals.

Mitochondrial preparation

The rats were anesthetized with a Rompun (13 mg/kg body wt.) Ketaset (87 mg/kg body wt.) mixture and then decapitated. Livers were removed and mitochondria were immediately isolated by differential centrifugation according to the procedure of Johnson and Lardy [23]. All respiration experiments were conducted on isolated mitochondria within 3—7 h after decapitation. Protein was determined by solubilizing the mitochondria in a 50-fold excess of a medium containing 3% Triton X-100, 150 mM Na_2SO_4 0.5 mM EDTA, 20 mM Tris buffer brought to pH 8.0 with NaOH, and using the BCA protein assay reagent (Pierce Chemical Co., Ref. 24). Bovine serum albumin (fatty acid free) in the same solubilizing buffer was used as a standard.

Mitochondrial respiration experiments

Mitochondrial respiration was determined polarographically at room temperature using a Yellow Springs Instruments oxygen monitor equipped with a Clark oxygen

electrode based on the procedure described by Estabrook [25]. The monitor was calibrated before and after addition of excess sodium dithionite to distilled water. It is noteworthy that the rate of decrease in [oxygen] measured under these conditions was much greater than in any of the mitochondrial respiration experiments performed. The electrode response time, therefore, did not affect the respiration rates measured. For each respiration experiment, a 0.25-ml aliquot of mitochondria (4.5—5.5 mg protein total) was added to 4.55 ml respiration medium consisting of 0.3 M sucrose, 1.0 mM EGTA, 5.0 mM Mops, 5.0 mM KH_2PO_4, 0.1% bovine serum albumin (fatty acid free), brought to pH 7.4 with KOH. Succinate (5.0 mM final) from a 500 mM stock solution was added to initiate state 4 respiration, and this rate was measured once linearity was reached (approx. 1 min). ADP was then added (usually to 0.30 mM final) from a 10 mM stock solution to initiate state 3 respiration. Once linearity was again reached (1 min), carboxyatractyloside (1—10 nmol) from a 1.0 mM stock solution was added and inhibition of state 3 respiration measured. Approximately 1 min passed before linearity was reached after the final addition. Uncoupled oxygen uptake rates were measured after addition of *m*-CCCP (25 μM final) from a 2.5 mM, stock solution in 5% ethanol. All respiration experiments were performed at 20°C.

The rates of state 3 and state 4 respiration were evaluated from the linear slopes of oxygen uptake vs. time plots. The respiratory control ratio (RCR) was calculated as the ratio of the rates of state 3 and state 4 respiration. The P/O ratio is expressed as the number of nmol of ADP phosphorylated per ng atom of oxygen consumed during state 3 respiration. This ratio was calculated from the amplitude of the jump in oxygen consumption which follows the addition of a known amount of ADP, as described below under Results.

RESULTS

Typical respiration experiments using mitochondria from old rat livers are presented in Fig. 1, which depicts oxygen consumption as a function of time under different experimental conditions. The addition of mitochondria to the incubation medium devoid of ADP and substrate consistently induced a small, rapid, decrease in the reading of the oxygen electrode; however, the system stabilized before the addition of succinate at approximately 2 min after mitochondrial addition. State 4 respiration was followed for 2—3 min and an aliquot of ADP was then added to the sample. This induced an abrupt and marked increase in the rate of oxygen consumption, as seen in Fig. 1A, which is typical of state 3 respiration and continued until all the ADP (the limiting substrate here) has been phosphorylated, at which time the rate of oxygen uptake declined back to that of state 4 respiration. The rates of state 3 and state 4 respiration were determined from the corresponding slopes and the RCR was obtained from their ratio. The respiration rates and RCR values obtained for mitochondria from adult and old rats, from experiments as described in Fig. 1,

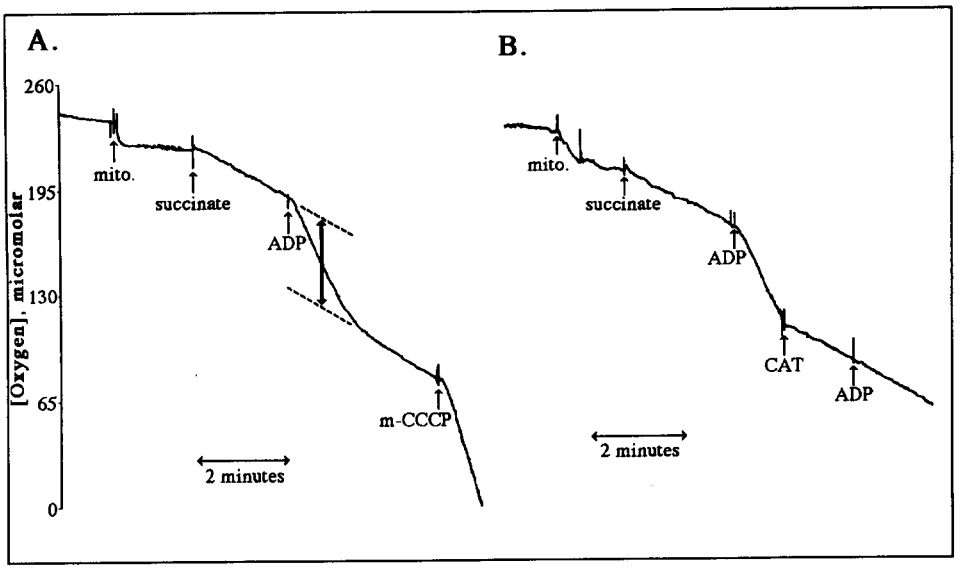

Fig. 1. A typical respiration experiment monitoring changes in oxygen concentration vs. time for a sample containing 29-month-old rat liver mitochondria. The addition of succinate, ADP, *m*-CCCP and CAT are indicated by the marked arrows. (A) Mitochondria (4.63 mg protein) were added first (indicated mito.) to 4.55 ml of respiration medium. Succinate was added subsequently (5.0 mM final), followed by ADP (100 μM final), and finally *m*-CCCP (25 μM final). (B) Mitochondria (mito.), succinate and each of the two ADP additions to the respiration medium were identical to those in A. CAT was added to a final concentration of 5.5 nmol/mg protein. More experimental details are given under Materials and Methods.

are summarized in Table I. The absolute oxygen consumption rates for state 3 and state 4 obtained in our experiments are similar to corresponding ones in several reports under similar conditions [5,26,27].

The P/O ratio was obtained from the amount of oxygen consumed during state 3 respiration, in response to the addition of a given amount of ADP. In calculating

TABLE I

COMPARISON OF SUCCINATE-SUPPORTED RESPIRATION IN LIVER MITOCHONDRIA FROM ADULT AND OLD RATS

Respiration	*12 Months*	*n*	*29 Months*	*n*
P/O Ratio	1.74 ± 0.22	(*n* = 6)	1.83 ± 0.26	(*n* = 8)
State 4	22.6 ± 2.1	(*n* = 11)	23.5 ± 2.2	(*n* = 15)
State 3	137 ± 14	(*n* = 9)	109 ± 15	(*n* = 11)
RCR	6.08 ± 0.84	(*n* = 19)	4.53 ± 0.28	(*n* = 20)

Respiration was measured and the RCR calculated as described in Materials and Methods. The units for respiration are ng atoms O/min × mg protein. The higher *n*-values for RCR are due to the fact that several experiments were not calibrated for absolute [oxygen]. Those experiments were only used to determine RCR values, in which only relative rates of oxygen consumption are used.

oxygen consumption, the assumption was made that the basal (state 4) respiration continues with its unperturbed rate also in presence of ADP (i.e., is included in the observed rate of state 3). The amount of oxygen consumed to support ADP phosphorylation was thus evaluated from the amplitude of the vertical shift between the two phases of state 4 before and after ADP addition. This amplitude is indicated by the bold arrow in Fig. 1A. The P/O ratios obtained in all our experiments were in the range of 1.5—2.2 (summarized in Table I) as expected for succinate, for both adult and old mitochondria.

Figure 1A also shows that upon addition of the uncoupler *m*-CCCP the rate of respiration greatly increases, to a level even higher than that of state 3. Indeed, for mitochondria from both adult and old rats the rate of uncoupled respiration varied among different experiments between 1.3 and 2.0 times that of state 3. This observation demonstrates that during state 3 respiration, the rate of oxygen consumption was never limited by electron transfer. The ratio of 0.13—0.16 between the rate of state 4 respiration and the uncoupled rate, found for mitochondria from both age groups, indicates tight coupling of these organelles [5]. Also, there was no change in the uncoupled rate from adult to old mitochondria (approximately 170 ng atoms O/min × mg protein). This differs from a recent report [22] of decreases with age in the uncoupled rate.

Table I summarizes the results of experiments, as depicted in Fig. 1, performed with mitochondria from both adult and old rats. Our data show a significant age-related change in the rate of state 3 respiration, this rate being about 20% lower in old mitochondria than in the adult organelles. Darnold et al. recently reported an identical age-related decline for liver mitochondria [22], while Murfitt and Sanadi reported a similar decrease in heart mitochondria [2]. In our experiments this decline in the state 3 respiration value was, however, apparent with ADP concentrations as low as 100 μM (final), while Darnold et al. used 650 μM ADP (final) stating that at lower concentrations of nucleotide no age-related differences are observed. Table I reveals no significant change in the rate of state 4 respiration with aging. As a result, the respiratory control ratio is 25% lower in old mitochondria compared with the adult organelles. These results are in good agreement with previous reports on age-related decreases in RCR in both liver and heart mitochondria (2,5].

Carboxyatractyloside is an irreversible inhibitor of the ANT which binds to this protein with a high affinity ($K_D = 2 \times 10^{-8}$ M) [28]. When CAT was added in excess over ANT (Fig. 1B) the rate of state 3 respiration instantly (on our time scale) declined to the rate of state 4, reflecting a complete inhibition of ADP/ATP translocation. It should be mentioned that CAT inhibition was not always instantaneous and lag times up to 3 min were observed when subsaturating amounts of inhibitor were used. The reasons for this effect are unclear. Also in Fig. 1B, addition of more ADP failed to induce any change in the rate of respiration, while addition of the uncoupler *m*-CCCP still was capable of enhancing respiration as in the absence of CAT (data not shown). This is to be expected since CAT does not inhibit electron transport reactions.

A titration of mitochondrial ANT with increasing concentrations of CAT is presented in Fig. 2, where the inset depicts the inhibition of state 3 respiration both for adult and old rat liver mitochondria. The main Fig. 2 was derived from the combined data of several experiments as shown in the inset, and presents state 3 minus state 4 respiration rates as a function of the concentration of the inhibitor. The inhibition plots are biphasic with both adult and old mitochondria being uninhibited at concentrations of CAT below about 70 nmol/g mitochondrial protein, while at CAT concentrations above this value the translocation rates decrease linearly to a final level identical to the rate of state 4 respiration. Similar biphasic inhibition patterns for mitochondria from heart or liver tissues (in studies which did not address aging effects) have been reported before, using atractyloside [27] and, more markedly, with CAT [14,29,30] as inhibitors. The linearity of the declining portion of the inhibition curves with CAT concentration reflects the high affinity of this inhibitor towards the ANT, such that at subsaturating concentrations of CAT, its binding to the ANT is practically complete under the conditions used in our study. Since CAT

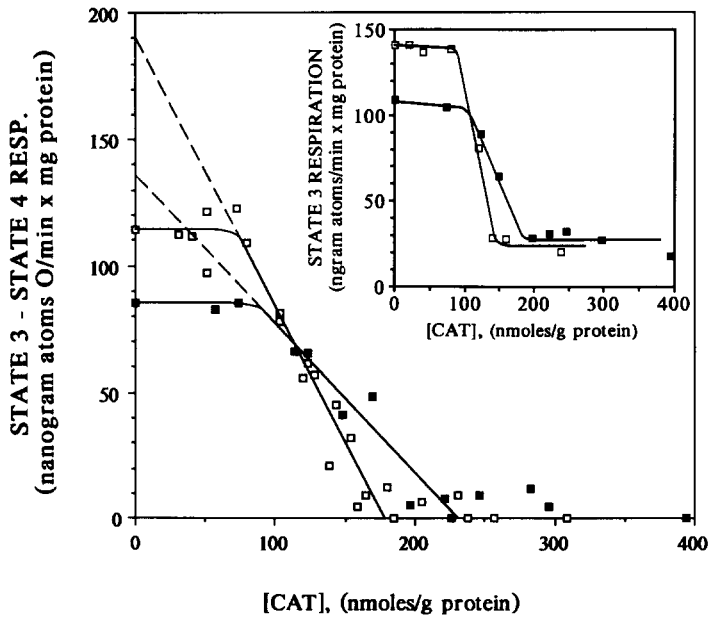

Fig. 2. Inhibition of mitochondrial respiration by carboxyatractyloside. (□), mitochondria from 12-month rat liver; (■), mitochondria from 29-month rat liver. The main figure presents the rates of consumption of oxygen utilized for ATP synthesis (evaluated from the difference between the rates of state 3 and state 4 respiration) as observed after addition of varying amounts of CAT to a respiration medium containing approximately 5 mg mitochondria (by protein), 5.0 mM succinate, and 0.30 mM ADP. Each point was calculated from the rates obtained in an experiment similar to that shown in Fig. 1. The results were totalled from experiments using 3 adult and 2 old rats. The inset depicts a typical experiment where mitochondrial state 3 respiration was determined after addition of varying amounts of CAT and without subtracting the rates of state 4 respiration from the data. The conditions were identical to those used in the main figure, except that these results were obtained from experiments using 1 adult and 1 old rat.

addition has no effect on the rate of state 4 respiration (see Fig. 1), one can evaluate the concentration of the mitochondrial ANT sites by determining the CAT concentration needed to fully inhibit the excess rate of state 3 over state 4 (i.e., bring respiration rate in the presence of ADP to that of state 4). This was done by extrapolating the linearly declining section of the inhibition curves shown in Fig. 2, and gave values of 180 and 230 nmol ANT binding sites per g mitochondrial protein for adult and old mitochondria, respectively. These values for both adult and old rat mitochondria are in good agreement with previously reported values [15,28].

The turnover number of the ANT during uninhibited state 3 respiration under the conditions of our experiments was calculated from the rates of respiration in absence of inhibitor, using the value of 2.0 for the P/O ratio. The turnover number was found to be 21 s^{-1} for adult mitochondria, which is similar to the value (22 s^{-1}) reported by Forman and Wilson [14] and 13 s^{-1} for the old organelles. A large decline with aging is thus evident. It is, however, pertinent to note that these turnover numbers are limited by the rate of state 3 respiration and do not represent the maximal turnover numbers for the translocase. Indeed when these maximal turnover numbers were determined by extrapolating the slopes of the CAT inhibition curves to zero inhibitor as shown in Fig. 2, their values were found to be 33 s^{-1} for adult mitochondria and 20 s^{-1} for the old organelles.

Maximal (state 3) respiratory activities that could potentially be supported by the existing amounts of ANT in adult and old mitochondria were also calculated by extrapolating the linear inhibition curves depicted in Fig. 2 to zero inhibitor concentration. These values were found to be 213 ng atoms oxygen/mg protein per min for adult mitochondria (155% of the observed uninhibited rate) and 160 ng oxygen/mg protein per min for old mitochondria (147% of the observed uninhibited rate). These values clearly demonstrate that in both mitochondrial preparations, the ANT is not rate limiting for respiration but rather is present in excess over the amount needed to support state 3 respiration. This 'reserve translocating ability', as expressed in the fraction of ANT molecules which can bind CAT before any decrease in state 3 respiration is observed, is 30—39%. It has been suggested by some investigators that a partial extraction of cytochrome *c* occurs during mitochondrial preparation and leads to a reduction in the normal rate of respiration [2,31]. Indeed, Murfitt and Sanadi showed that addition of excess cytochrome *c* to preparations of mitochondria from young and old rats induced a 35—50% stimulation of state 3 respiration, with the old mitochondria being somewhat preferentially stimulated [2]. If a similar stimulation, by cytochrome *c*, could be induced in the rates of state 3 observed for mature and old mitochondria in the present study, these rates would approach the maximal rates of the ANT thereby making translocation the rate-limiting step in respiration. Our data do, however, provide good evidence that in the experiments reported here the maximal (uninhibited) rate of state 3 respiration is not limited by electron transfer (which depends on cytochrome *c*). This evidence comes from the experiments done in the presence of *m*-CCCP (i.e., in uncoupled mitochondria),

where the rate of oxygen consumption observed was on the average 160% that of the rate of state 3 respiration (for mature as well as old mitochondria). We thus conclude that under the conditions employed in our experiments, the availability of cytochrome *c* (and more generally the electron transfer rate) is not rate-limiting for respiration.

DISCUSSION

The experimental results of the present study unequivocally show the rate of succinate-supported state 3 respiration to significantly decline in rat liver mitochondria between 12 and 29 months of age. In contrast, the rates of state 4 respiration were found not to vary between these two age groups. These rates, presented in Table I, have been normalized for the amount of protein. While changes in mitochondrial protein composition with age could affect these respiration rates, it appears to not be the case here. This is shown by the similar 25% decline in the respiratory control ratio between adult and old mitochondria, which is independent of the amount of mitochondrial protein. This conclusion is also supported by the fact that there is either no change or even a slight increase in active ANT/mg protein with age, as determined from the *x*-intercepts of the inhibition curves in Fig. 2B. The decline in RCR we found is similar to the value reported by Horton and Spencer [5], while Kim et al. [26] recently found only a small change in RCR from 12 to 29 months, when succinate was used as substrate. While the reason for this discrepancy is not clear, it should be noted that the RCR values found by the last authors for 4-months- and 12-months-old mitochondria are significantly smaller than ours. This could reflect differences in experimental conditions (most notably the temperature which was 35°C in Ref. 26 and 20°C in our experiments), which may preferentially affect the young membrane. Evidence that the reduced RCR observed by us in old mitochondria is not due to a decline, with age, in the rate of introduction of succinate into the mitochondrial matrix is provided by our finding that the rate of oxygen consumption in mitochondria uncoupled by *m*-CCCP is not significantly affected by age. Moreover, in both age groups studied the respiration of uncoupled mitochondria was significantly faster than the maximal (uninhibited) rate of state 3 respiration in coupled mitochondria. The rate of succinate permeation into old mitochondria is, thus, more than adequate and is not rate limiting for respiration.

An alternate explanation for the observed age-related declines in state 3 respiration and in the RCR may be proposed based on the reports [12,26] that the pool of intramitochondrial adenine nucleotides is significantly decreased with aging (thus slowing the rate of state 3 but not that of state 4 or of the uncoupled reaction). This decrease in the amount of ADP available for phosphorylation could result from a non-specific leakage of ADP from the mitochondrial matrix due to age-related changes in membrane composition [26]. An increase with age in the susceptibility of rat liver mitochondria to swelling in a hypotonic medium, or in the presence of swell-

ing agents, was indeed reported [32,33] and is indicative of deterioration in the stability of the mitochondrial membrane. However, ADP leakage as a result of damage to the membrane was ruled out by Kim et al. [26] and is also not present in our preparations as the rate of state 4 respiration, which increases significantly in damaged mitochondria (and is thus a sensitive parameter for the integrity of these organelles) was found to be unaltered by age. Moreover, we found the P/O ratio to be close to its maximal value of 2.0 (for the FAD-linked substrate used) both for adult and old mitochondria.

Several studies suggest that ADP/ATP translocation, which was reported to be reduced with aging [4,12], is rate-limiting under maximal respiration conditions [6,11,27] and hence is responsible for the observed decline in the state 3 rate. We also find a large decline in ANT activity, specifically a 39% decrease in the protein's turnover number from adult to old mitochondria. This decrease in adenine nucleotide translocation is consistent with decrements in other mitochondrial transport systems, including carnitine-acylcarnitine translocation and calcium transport [1]. However, our studies also clearly show that adenine nucleotide translocation is not rate-limiting for respiration in either of the two age groups, as in both adult and old rats, the maximal ANT rate is approximately 50% greater than the maximal rate of state 3 respiration. Thus, while there may be a slight decline in the fraction of 'reserve translocating ability', from 39% to 30% with aging, both values clearly indicate excess ANT activity. Therefore, adenine nucleotide translocation is not the rate limiting step for mitochondrial respiration and consequently not the cause of the age-related decline in in state 3 respiration.

A partial loss of cytochromes during mitochondrial preparation has been demonstrated to occur under some conditions due to extraction of soluble proteins. In particular, a preferential loss of cytochrome *c* in mitochondria from old rats was reported and implicated in the reduction of respiratory activity [31]. The difference between state 3 respiration of adult and old mitochondria observed by us cannot, however, be attributed to different levels of cytochromes since the respiration rates increase markedly upon uncoupling of the mitochondria (for each age group) and, moreover, these uncoupled rates are similar. It is therefore obvious that the age-related difference resides in a component of respiration which is only expressed in coupled organelles. Therefore, we conclude from these experiments that succinate-supported maximal respiration decreases significantly with aging and that the decline is not due either to the rate of succinate permeation, to the electron transport chain or to the adenine nucleotide translocase. This decline, though, must be due to a rate-limiting step for state 3 mitochondrial respiration that significantly decreases in activity with age. One possible step is catalyzed by the F_1-ATPase. LaNoue et al. [35] concluded that the F_1-ATPase from rat liver mitochondria is not under equilibrium conditions and that a change in its activity would significantly affect mitochondrial ATP synthesis. Also, Nohl et al. [21] found a significant decrease in rat heart F_1-ATPase activity with age. Therefore, this enzyme may play an important role in the age-related decline in mitochondrial state 3 respiration.

An interesting finding of the present study is that the thermodynamic efficiency of mitochondrial respiration, as reflected in the P/O ratio, did not differ between adult and old mitochondria and an age-related decline was observed only in the rate of ATP production. Thus, under resting conditions, ATP synthesis would be expected to meet the cell's requirement with no age-related impairment in the functional ability of the cell. However, under conditions of extreme 'energy stress' i.e., when the requirement for ATP production is very high, the decline in the capacity of the old mitochondria to produce ATP may become manifested. In this context it is interesting to note the widely recognized observation that one of the most profound manifestations of biological aging is an impairment in the ability of an organism to adapt to drastic (stressful) changes in its environment. This impairment of old organisms can be manifested either as a slower response to an external stimulus, as a weaker response, or be a combination of the two. While the specific mechanistic reasons for this general phenomenon are complex and vary among cell types, tissues and whole organisms, the common result is a decline in the performance of the system under stressful conditions, when maximal output is required. The results of the present study suggest that rat liver mitochondria may provide a convenient system for future studies of this intriguing aging phenomenon.

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