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Control of oxidative phosphorylation by Complex I in rat liver mitochondria: implications for aging

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Abstract

We compared NAD-dependent state 4 and state 3 respiration, NADH oxidation and Complex I specific activity in liver mitochondria from 4- and 30-month-old rats. All the activities examined were significantly decreased with aging. In both groups of animals, the flux control coefficients measured by rotenone titration indicated that Complex I is largely rate controlling upon NADH aerobic oxidation while, in state 3 respiration, it shares the control with other steps in the pathway. Moreover, we observed a trend wherein flux control coefficients of Complex I became higher with age. This indication was strengthened by examining the rotenone inhibition thresholds showing that Complex I becomes more rate controlling, over all the examined activities, during aging. Our results point out that age-related alterations of the mitochondrial functions are also present in tissues considered less prone to accumulate mitochondrial DNA mutations.

Keywords: Rat liver mitochondria; Aging; Respiratory chain; Flux control; Complex I

1. Introduction

The progressive decline of cell functions with age has been attributed to either a genetic program inborn in all organisms or to the accumulation of stochastic errors in somatic cells leading to loss of cell functions [1].

Among the stochastic theories the mitochondrial theory of aging [2,3] proposes that mitochondria are involved both as producers and as targets of reactive oxygen species (ROS) whose aggressive behavior can result in oxidative damage of all biological molecules.

The consequence of oxidative stress at the level of mitochondria will be the failure of enzymatic, transport or receptor systems. A vicious circle [4,5] has been proposed to occur: any damage to the respiratory chain may enhance ROS production [6] and induce a progressive and continuous perpetuation of damage through somatic mutations of mitochondrial DNA (mtDNA) and defective mtDNA-encoded proteins. Although this idea is controversial and rejected by some authors [7], an increase in both ROS pro-

Abbreviations: ROS, reactive oxygen species; mtDNA, mitochondrial DNA; CoQ, coenzyme Q, ubiquinone; DB, decylubiquinone; RCR, respiratory control ratio; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; EDTA, ethylenediaminetetraacetic acid; I_{50}, rotenone concentration eliciting half-inhibition

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duction [8,9] and mtDNA deletions [10] was described during aging; the existence of mtDNA damage in senescence is supported by the observation of an aging-related accumulation in human and animal mtDNA of oxidative and alkylation derivatives of nucleotides [11,12], of small insertions and of large deletions [13]. Recently, it has been reported that specific heteroplasmic mutations in the mitochondrial control region for replication and transcription (D-loop) accumulate, sometimes in high numbers, in fibroblasts from normal old individuals, but not in young ones [14].

It is proposed that such alterations, which affect exclusively subunits belonging to the four mitochondrial complexes involved in energy conservation (i.e. Complex I or NADH-ubiquinone oxidoreductase, Complex III or ubiquinol cytochrome *c* oxidoreductase, Complex IV or cytochrome *c* oxidase and the H^+-translocating ATP synthase complex), would result in defective electron transfer and oxidative phosphorylation [15].

If the energetic impairment derives from the stochastic damage to the mitochondrial genes, then it is important to select the mitochondrial activity that is likely to be most severely affected. Since seven out of 13 polypeptides encoded by mtDNA belong to Complex I (NADH-coenzyme Q reductase, EC 1.6.99.3) it is expected that this enzyme should be mostly affected by aging [16]. The so-called ND subunits of the enzyme, encoded by mtDNA, are involved in binding of the electron acceptor, i.e. coenzyme Q (CoQ, ubiquinone) and of several inhibitors, such as rotenone, and seem also to contribute to the proton-translocating machinery of Complex I [17,18].

A decreased activity of Complex I was indeed found in different tissues from aged animals and in bioptic specimens from old individuals [19–21]; nevertheless it was found that direct assay of Complex I using artificial quinone acceptors may undervalue the real enzyme activity [22]. We have previously reported that significant decreases in NADH-CoQ reductase activity, undetected by the direct assay, were revealed in liver and heart mitochondria from 24-month-old rats [23] by exploiting the 'pool equation' [24] whereby the rate of CoQ reduction is related to the total rate of NADH oxidation by oxygen and to the rate of ubiquinol oxidation. Another approach for recognition of possible early functional changes in the subunits encoded by mtDNA was provided by the decreased rotenone sensitivity of the enzyme observed by us in rat brain [25] and in human platelet [26] mitochondria.

A decrease in an individual enzyme activity in a metabolic pathway is meaningful only if it is able to affect the rate of the whole pathway, and this will depend on the degree of flux control exerted by the individual step itself [27], in other words on how much the step is rate controlling upon the whole pathway. From the data in the literature, it appears that Complex I activity is among the major rate-limiting steps in mitochondrial oxidative phosphorylation [28,29].

In mitochondrial diseases affecting Complex I, such as Leber's hereditary optic neuropathy, the flux control coefficient at site I of the respiratory chain in permeabilized cells was found to dramatically increase [29]; this point has not been addressed in studies on aging. Our previous studies, indicating a decrease of aerobic NADH oxidation in rat liver, heart [23] and brain cortex mitochondria [25], have provided only an indirect answer to this question.

In the present work, the metabolic control theory has been applied to the study of mitochondrial metabolism for determining the level of control exerted by Complex I on the rate of respiration in rat liver mitochondria and for identifying and quantifying enzymatic defects occurring with aging. A preliminary account of this work was published in the Proceedings of the 11th European Bioenergetics Conference [30].

2. Experimental procedures

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Milan, Italy) and all solvents were pure reagents from J.T. Baker (Deventer, The Netherlands).

2.2. Animals

Two groups of 10 male albino rats of the Wistar strain aged 4 months (young) and 30 months (old), respectively, were used for this study. The animals, bred under conditions of microbiological barriers,

were purchased from Harlan Nossan Italy and used within 2 weeks from arrival. During this period, they were kept under conditions of constant temperature and humidity and with a 12 h light–12 h dark cycle. Animals were sacrificed by cervical shock and decapitation, in accordance with the Italian law on the use of experimental rats, under veterinary control and approval by local bioethical committees.

Livers were immediately removed and washed in ice-cold buffer solution (mannitol 0.22 M, sucrose 0.07 M, Tris 2 mM, ethylenediaminetetraacetic acid (EDTA) 1 mM, HEPES 20 mM, pH 7.2).

2.3. Mitochondrial preparation

Liver mitochondria were isolated according to the procedure of Kun et al. [31] with albumin (0.4%) but without digitonin. Albumin was eliminated in the last step before protein assay by washing mitochondria in buffer. Protein concentration was determined by a biuret method [32] with the addition of 10% sodium deoxycholate and using bovine serum albumin as standard.

2.4. Oxygraphic measurements

Freshly prepared mitochondria were assayed for oxygen consumption at 30°C by means of a thermostatically controlled oxygraph apparatus equipped with a Clark's electrode and a rapid mixing device. In a typical experiment, mitochondria (2 mg of protein) were incubated for 5 min at 30°C in the following respiration medium (100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM KH_2PO_4, 10 mM Tris–HCl and 50 μM EDTA, pH 7.4; final volume: 1700 μl) before respiratory substrates (10 mM glutamate and 5 mM malate or 10 mM succinate) were added. State 4 respiration was recorded for 2 min, then state 3 respiration was induced by addition of 0.6 mM ADP according to Chance and Williams [33]. The respiratory rates were expressed in μgatom O_2/min/mg protein.

2.5. Enzymatic determinations

NADH oxidase and Complex I specific activities were determined in succession, according to an experimental procedure designed in our laboratory. Mitochondria, which had been stored at −80°C, were thawed and pulse sonicated five times at 10 s periods (150 W) with 50 s intervals in an ice water bath under nitrogen gas. The assay of both enzymatic activities was performed using 80 μg of mitochondrial protein/ml in buffer solution (50 mM KCl, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4) at 30°C in a Sigma-Biochem ZWS 2 dual wavelength spectrophotometer equipped with a rapid mixing apparatus of our own design. The oxidation of NADH (75 μM) was followed at the wavelength couple of 340 minus 380 nm ($\varepsilon = 5.5\ mM^{-1}\ cm^{-1}$) first in the absence and then in the presence of antimycin A (2 μM) plus K-cyanide (1 mM) as downstream inhibitors and decylubiquinone (DB, 60 μM) as electron acceptor.

The NADH-ferricyanide reductase activity was assayed spectrophotometrically at 30°C according to Minakami et al. [34] in the same buffer used for the assay of the NADH oxidase, except for including 10 mM KCN in the buffer solution. The rate of reduction of 2 mM K-ferricyanide was measured at the wavelength couple 420 minus 500 nm ($\varepsilon = 1\ mM^{-1}\ cm^{-1}$) in the presence of 150 μM NADH and 14 μg protein/ml of mitochondrial suspension totally inhibited by rotenone (2000–3000 pmoles/mg protein). The specific activity of ferricyanide reduction was used as a parameter proportional to the content of Complex I in the membrane [35].

Ubiquinol cytochrome *c* reductase was assayed in the same buffer of NADH oxidase, with 1 mM KCN, and using 50 μM ferricytochrome *c* and 15 μM decyl-ubiquinol as substrates. The activity was evaluated by monitoring the absorbance change of cytochrome *c* upon reduction, at 550 minus 540 nm in a Sigma-Biochem ZWS-2 dual wavelength spectrophotometer equipped with a rapid mixing apparatus, and using an extinction coefficient of 19.1 $mM^{-1}\ cm^{-1}$. The chemical reaction between ubiquinol and cytochrome *c* was subtracted.

The enzymatic activities were expressed in μmol/min/mg protein.

2.6. Titration curves and flux control coefficients

The inhibition curves of the mitochondrial respiratory rate were obtained at state 4 and state 3 using increasing amounts of rotenone (0–100 pmoles/mg protein), a specific inhibitor for Complex I, added

in the incubation chamber and preincubated for 5 min prior to assay.

The inhibition curves of NADH oxidase and Complex I specific activity were determined experimentally in rotenone titrated mitochondria, preincubated for 5 min with increasing amounts (0–100 pmoles/mg protein) of a concentrated solution of the inhibitor. The rotenone concentration eliciting half-inhibition (I_{50}) was taken into consideration as an indicator of Complex I sensitivity to rotenone. Since rotenone is an almost stoichiometric inhibitor, the I_{50} was corrected for Complex I content by expressing the value relative to the rate of NADH-ferricyanide activity.

For determination of flux control, the activity rates were expressed as percentage of the control which had no inhibitor present and plotted against the rotenone concentration. Flux control coefficients were calculated according to the metabolic control theory described by Kacser and Burns [36,37] which defines the control that every single step in a pathway has over the global flux of that pathway. The control coefficients were calculated using the following equation:

$$C_i = (dJ/dI)_{I=0}/(dv_i/dI)_{I=0} \quad (1)$$

where $(dJ/dI)_{I=0}$ is either the rate of change of state 4 and state 3 respiration or that of NADH oxidase activity (global flux) while $(dv_i/dI)_{I=0}$ is the rate of change of Complex I activity (individual step). All listed rates were measured at low concentrations of rotenone by calculating the initial slope of the inhibition profile as described in Section 2.8.

Control experiments were performed of stepwise inhibition of succinate oxidase with mucidin (strobilurin A), an inhibitor of Complex III [38], in comparison with stepwise inhibition of ubiquinol cytochrome *c* reductase activity, in order to determine flux control exerted by Complex III on coupled respiration. The choice of mucidin is dictated by its specific inhibition of Complex III [38], whereas myxothiazol also inhibits Complex I [39]; the commonly used antimycin A in our hands gave a sigmoidal inhibition of ubiquinol cytochrome *c* reductase (cf. also [40]).

2.7. Threshold curves and determination of threshold values

The threshold curves were obtained from the titration curves by plotting the percent rate of the examined enzyme activities as a function of the Complex I inhibition percentage for the same rotenone concentration. The threshold value was defined as the abscissa of the intersection point between the two regression lines performed on the first and last points of the threshold plot [41]. This parameter represents the percentage of Complex I inhibition above which a sensible reduction of the global activity (i.e. aerobic respiration or NADH oxidase activity) can be observed.

2.8. Mathematical analysis and statistics

The titration curves were obtained with a nonlinear (sigmoidal) regression fitting procedure performed on the experimental data using the application program SigmaPlot [42]. The initial slope of each curve was calculated as the limit of the derivative of the sigmoidal function for rotenone concentration tending to zero. The flux control coefficients were obtained by the ratio of the initial slopes of the two fitted curves (global flux and single step, respectively) plotted from all the raw titration values of every set of experimental data. Owing to this kind of mathematical analysis, the flux control coefficients shown in this paper could not be in the form of mean values with standard errors. However, as a gauge of the accuracy of the data, it can be emphasized that the correlation coefficient (r^2) of each fitted curve was higher than 0.93.

The statistical analysis reported for some of the biochemical parameters was performed using Student's *t*-test.

3. Results

3.1. Biochemical parameters

The enzyme activities assayed in rat liver mitochondria from young and old animals are reported in Table 1.

The respiratory activity, measured polarographically in state 4- and in state 3-coupled mitochondria (ADP-stimulated), showed a decrease of rates with respect to age, making a difference that was statistically significant in the case of ADP-stimulated respi-

Table 1
Biochemical parameters in rat liver mitochondria from young and old animals[a]

	Young	Old
State 4 respiration rate (μg atom O/min/mg protein)	0.022 ± 0.010 (9)	0.019 ± 0.004 (7)
State 3 respiration rate (μg atom O/min/mg protein)	0.113 ± 0.057 (9)	0.059 ± 0.023 (7) $P = 0.02$
RCR	5.25 ± 2.06 (9)	3.06 ± 0.83 (7) $P = 0.01$
NADH oxidase activity (μmol/min/mg protein)	0.107 ± 0.022 (10)	0.070 ± 0.019 (10) $P = 0.0006$
Complex I activity (μmol/min/mg protein)	0.149 ± 0.037 (10)	0.112 ± 0.032 (10) $P = 0.03$
NADH-ferricyanide reductase (μmol/min/mg protein)	2.69 ± 0.40 (10)	2.51 ± 0.62 (10)
I_{50} of rotenone on Complex I activity (pmol rotenone/mg protein)/NADH-ferricyanide reductase[b]	2.6 ± 0.8 (10)	3.5 ± 0.4 (10) $P = 0.009$

Data represent mean values ± S.D. The number of animals is shown in parentheses.
[a] > Respiratory rates were assayed measuring the oxygen consumption of state 4 mitochondria and inducing state 3 by the addition of 0.6 mM ADP. NADH oxidase and Complex I specific activities were determined in succession in sonicated mitochondria following spectrophotometrically the oxidation of NADH at the wavelength couple of 340 minus 380 nm. NADH-ferricyanide reductase activity was measured in mitochondrial suspensions totally inhibited by rotenone at the wavelength couple 420 minus 500 nm (see Section 2 for further explanations).
[b]Since rotenone is an almost stoichiometric inhibitor of Complex I, the I_{50} was corrected for Complex I content by expressing the value relative to the NADH-ferricyanide activity.

ration. The respiratory control ratio (RCR) was lower in mitochondria from the old animals, suggesting the presence of slightly uncoupled mitochondria; such an uncoupling, however, may be only apparent, since the RCR decrease appears to be largely due to the decrease in state 3 respiration rather than to an increase in the state 4 rate, indicating that the actual proton leak remains unchanged.

Comparison of the data in the young and old animals also revealed a significant decrease in the aerobic oxidation of NADH, measured in sonicated mitochondria, in the aged group. This finding is paralleled by a clear decrease in Complex I specific activity measured in the same mitochondrial particles. We had previously demonstrated that some problems exist in the correct evaluation of that enzyme activity rate when experimentally assayed using low molecular weight quinones which are homologues or analogues of the natural acceptor [43]. Applying the pool equation as in [22] we have found the activity of the calculated NADH-CoQ reductase to be almost superimposable to the experimentally determined Complex I activity in the full range of rotenone concentrations. The difference with previous results [22,23] may be due to some improvements in the assay system, in particular using a much higher concentration of mitochondrial protein. In addition to the decrease in the NADH oxidation activity rate, we found a decrease in its rotenone sensitivity evidenced as the rotenone concentration eliciting 50% inhibition (I_{50}) being greater in the old group than in the young (see next paragraph for further details).

The specific activity of ferricyanide reduction, which can be used as a parameter proportional to the content of Complex I in the membrane [35], was found not to be affected by aging.

In contrast with NAD-linked activities, ubiquinol cytochrome *c* reductase was not significantly changed between young and old animals (0.82 ± 0.34 vs. 0.71 ± 0.02 μmol min^{-1} mg^{-1} protein, respectively).

3.2. Titration curves and flux control coefficients

The metabolic control exerted by a single enzyme in a metabolic pathway can be expressed by a coefficient obtained from two inhibitor titration curves (global flux and single step). The profile, particularly the initial slope, of the global flux curve is indicative of the control exerted by the examined enzyme on the considered pathway: the steeper the curve, the greater the similarity to the hyperbolic titration curve of the single enzyme step and the higher (closer to 1) the flux control coefficient [44].

In this paper we studied the variation in metabolic control of Complex I over oxidative phosphorylation and mitochondrial respiration, during aging. This requires the preliminary determination of experimental inhibition curves of the respiratory activity, the

NADH oxidase and the NADH-ubiquinone oxidoreductase activity.

Fig. 1 shows the stepwise inhibition by rotenone of NADH-ubiquinone reductase in young and old animals. The two curves, built graphically from the data given by the fitting procedure applied to the experimental points, are almost superimposable in the initial part of their profile but differ in the shape of their middle portion where the titration curve of the aged group presents a less steep bending, leading to a lower slope with a rotenone concentration for half-inhibition (I_{50}) significantly higher (Table 1). The final extent of inhibition at high rotenone titer (see inset in Fig. 1) is the same in both groups of animals (approx. 93%).

Fig. 2 shows the experimental data and the fitted titration curves of Complex I activity and NADH aerobic oxidation in young (A) and old animals (B). In both cases, the curves of the two activities exhibited a similar hyperbolic profile and were almost superimposable in their initial slopes. This property, which can be explained in the framework of the metabolic control theory, accounts for the high coefficients obtained for Complex I flux control over NADH oxidase activity, both in young and old animals (Table 2).

Much lower coefficients were measured for Complex I control over substrate-driven mitochondrial respiration in intact mitochondria in the two groups of animals (Table 2). Values were extremely low when respiration was not stimulated by ADP (i.e. state 4 respiration). Moreover, in the presence of ADP, the effect of rotenone inhibition on the whole flux (i.e. state 3 respiration) was weaker than that on Complex I activity alone at the same concentrations of the inhibitor, as can be also deduced from the shape of the inhibition curves in Fig. 3. It can be observed by inspection of the plots that the slopes of the entire curves diverge much more strongly in the young than in the old animals; nevertheless, the initial slopes are not dramatically higher in the old rats, thus producing only a slight rise of the flux

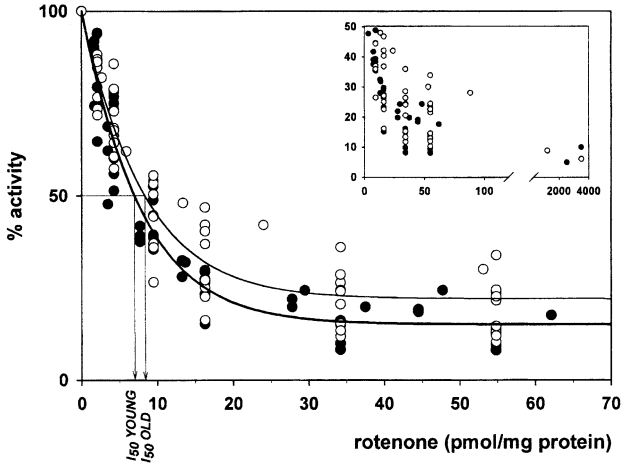

Fig. 1. Comparison between the rotenone titrations of Complex I activity in liver mitochondria from young (●) and old (○) rats. Activity rates are expressed as percentage of the control which had no inhibitor present. The final extent of inhibition at high rotenone titer is the same in both groups of animals (cf. inset). All activities were measured spectrophotometrically following oxidation of 75 μM NADH at 340–380 nm.

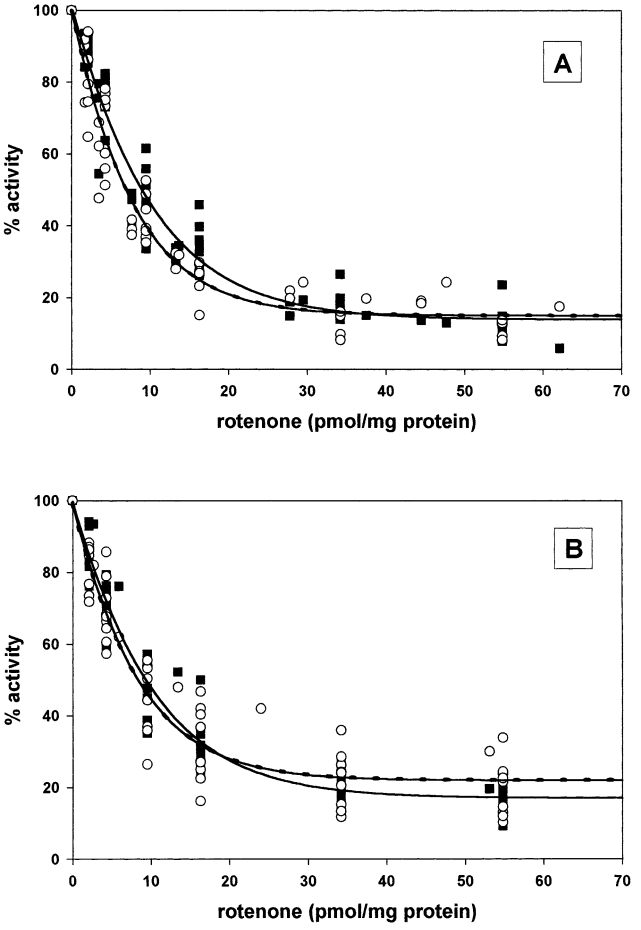

Fig. 2. Flux control of NADH oxidase activity in liver mitochondria from young (A) and old (B) rats. The figure shows the experimental data and the fitted titration curves of Complex I activity (○) and of NADH aerobic oxidation (■).

control coefficients, which are measured on the initial slopes (Table 2).

As a control experiment, the flux control exerted by Complex III was also determined in both groups of rats on succinate-driven state 3 respiration using mucidin to progressively inhibit Complex III activity. Succinate oxidase activity was only slightly decreased in the old. The flux control coefficients in both groups were less than 0.1, thus indicating that Complex III exerts only little control on respiration, and that this control is not affected by aging.

3.3. Threshold curves and threshold values

The examined activities (NADH aerobic oxidation, state 3 and state 4 respiration) showed a trend

Table 2
Flux control coefficients for Complex I over glutamate–malate oxidation and over NADH oxidation in rat liver mitochondria from young and old animals[a]

	Young	Old
State 4 respiration	0.07	0.23
State 3 respiration	0.34	0.38
NADH oxidase activity	0.78	0.87

[a]Values were calculated using the whole set of experimental data obtained from each group of animals, according to the analytical procedure described in Section 2. Owing to that kind of mathematical analysis, the flux control coefficients shown in the table could not be in the form of mean values with standard errors but, as a gauge of the accuracy of the data, it can be emphasized that the correlation coefficient (r^2) of each fitted inhibition curve was higher than 0.9.

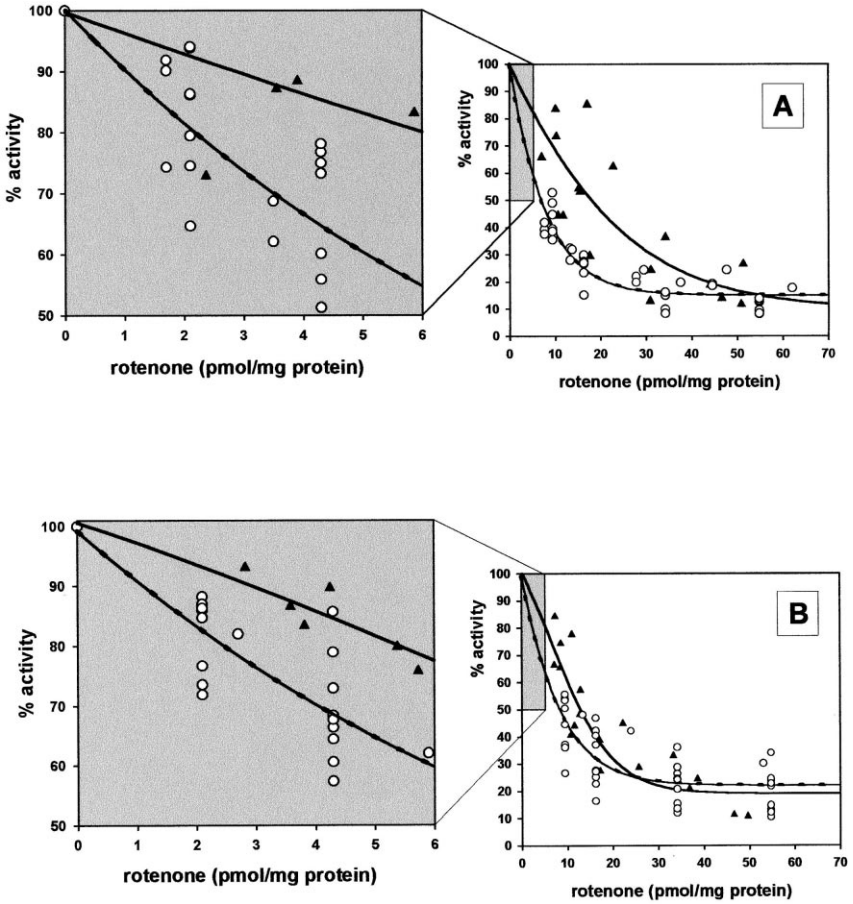

Fig. 3. Flux control of respiration in liver mitochondria from young (A) and old (B) rats. The plots on the right side show the stepwise inhibition by rotenone of NADH-CoQ reductase (○) and of aerobic state 3 oxidation of glutamate plus malate (▲) in mitochondria; the data points are cumulative of 10 and five experiments, respectively, in both groups of animals. Symbols were omitted in the gray area of the plots on the right side and were drawn for clearness in the expanded region between 0 and 6 pmol rotenone/mg protein shown on the left.

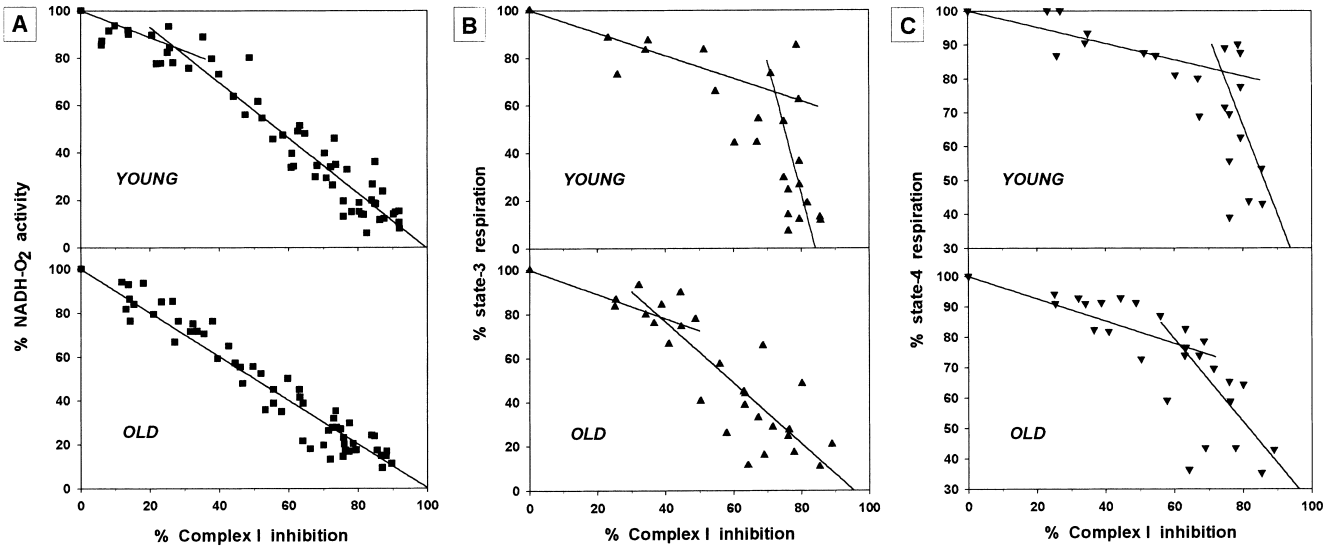

Fig. 4. Threshold curves of NADH oxidase activity (A), state 3 respiration (B) and state 4 respiration (C) in liver mitochondria from young and old rats. Each point comes from the experimental titration curves and represents the percent rate of the examined activity as a function of the percentage of Complex I inhibition for the same rotenone concentration. Freehand threshold plots were drawn through the data points.

wherein the Complex I flux control coefficient is increased during aging (Table 2).

This trend is better pointed out with the construction of threshold effect curves for Complex I over NADH oxidase activity (Fig. 4A) and over NAD-linked respiration (Fig. 4B,C). The profile of the graphs is characterized by a plateau followed by a smooth slope where the break point is indeed not much evident. The threshold value is therefore difficult to determine with precision and the curves are more qualitative than quantitative [41]; nevertheless, the visual comparison of the plots could be as much informative as the analysis of the numerical values [41], showing that in old animals a threshold effect occurs at a much lower percentage of inhibition than in the young animals.

In the case of NADH oxidase (Fig. 4A), it can be emphasized that the threshold value is low in the young group of animals (approx. 30%); nevertheless it decreases with aging to such an extent that the plateau is no longer evident.

4. Discussion

A special role has been ascribed to ROS-mediated somatic mutations of mitochondrial DNA [2,45] in the energetic failure characterizing senescence. The mitochondrial theory of aging [2,3] predicts that post-mitotic cells are maximally prone to accumulate mtDNA lesions and in fact severe mtDNA deletions [5,46,47] and concomitant respiratory chain activity decreases were described in heart, brain and muscle mitochondria of aged animals [23,25,48,49].

The choice of liver for this study has been dictated by the requirement of large amounts of mitochondrial suspension for a complete analysis. On the other hand this choice is justified in a study on aging since previous investigations have reported a high extent of mtDNA deletions [13,50] and significant respiratory alterations [23,51] in liver mitochondria from aged rats. Yowe and Ames [13] comment that rat liver may behave differently from human liver for chronological and metabolic reasons.

On the other hand, in this study we have had the opportunity to investigate very old rats (30 months) while previous studies, including those from our laboratory, employed 24-month-old animals at most.

The present study shows a statistically significant decline with age of the mitochondrial respiratory chain activity in sonicated liver mitochondria of old rats. Furthermore, a decrease in the respiratory rate is found with aging when glutamate plus malate are used as respiratory substrates in freshly isolated mi-

tochondria. It can also be noted that a significant difference was observed in the specific activity of Complex I between the young and the old group of animals, but no change was found in the NADH-ferricyanide reductase activity. Nor was a significant change observed in the activity of Complex III.

Those observations, while suggesting that the age-related decrease in the experimentally determined NADH-ubiquinone reductase activity does not result from a decrease in the amount of the enzyme protein in the mitochondrial membrane, show that a decrease in its turnover might occur and reveal a defect of Complex I with aging. This is the first demonstration that a decrease in Complex I activity in rat liver mitochondria with aging is able to decrease the rate of coupled NAD-linked respiration in intact mitochondria.

Although the alterations found are compatible with the mitochondrial theory of aging, according to which the postulated increase in somatic mtDNA mutations would affect the hydrophobic subunits of Complex I that are essential for CoQ binding and energy conservation [17], there may be other possible reasons for age-related defects of Complex I such as direct alterations of the protein or of the lipid environment. It is worth noting that the hepatic mitochondrial lipid composition is altered significantly in aged rats and, particularly, that the observed decrement in the cardiolipin content [52] which is required for Complex I activity [53] may play a key role in the age-linked decline of NADH-CoQ reductase.

A defect in Complex I is also indicated, in our group of old animals, by the higher I_{50} for rotenone inhibition corrected for Complex I content, similar to that detected in rat brain mitochondria during aging [25] and in platelet mitochondria both from aged individuals [26] and from patients affected by Leber's hereditary optic neuropathy [54]. Cross-titrations with different inhibitors reveal a rather extended rotenone-binding area [55]: the changed rotenone sensitivity concerns three different forms of Leber's disease, implicating subunits ND1, ND4 and ND6 [54,56]. Since, as already mentioned, rotenone binds the hydrophobic sector of the enzyme that is involved in proton translocation, the altered inhibitor sensitivity may reflect the properties of the membrane part of the enzyme and may be diagnostic of alterations within those subunits which carry the binding site of the electron acceptor, i.e. CoQ, and of the inhibitor itself.

If we examine the problem using the concepts developed in metabolic control analysis [57], the matter can be posed as a quantitative question: How much does the metabolic flux vary as the Complex I activity is changed? In other terms, it can be questioned if the respiratory activity measured in old rat mitochondria is reduced solely in response to the alteration of the NADH-CoQ reductase activity, or other steps in the electron transfer chain may be responsible as well for such a reduction.

Analysis of the literature shows that the control of respiration is shared between different biochemical steps and how the distribution changes according to the rate of phosphorylation that the mitochondria are performing (cf. [44,58]).

In our study, the theory of flux control was applied to describe the control of oxidative phosphorylation by Complex I with aging. Our experimental approach allowed to determine the value of the control coefficient for Complex I over the respiratory chain activity and showed that the enzyme is strongly rate controlling upon the whole respiratory chain in rat liver mitochondria. A very high control is maintained during aging (flux control coefficient: 0.87 vs. 0.78) when both aerobic NADH oxidation and Complex I activity are significantly decreased. Thus, it can be postulated that a mutation giving a defect of this enzyme will necessarily lead to a significant decrease in aerobic NADH oxidation. Conversely, when measuring the control over oxygen consumption in freshly isolated mitochondria using glutamate and malate as respiratory substrates, lower values of the coefficient were obtained.

This indication is better pointed out by plotting the percentage of activity against the percentage of Complex I inhibition: if a threshold effect occurs at low percentages of inhibition then Complex I can be considered rate controlling.

Our results indicate that, in state 3 respiration, Complex I shares the control with other steps in the metabolic pathway. Inomoto et al. [28] found similar values in rabbit liver mitochondria, with flux control coefficients of 0.3 for Complex I, 0.2 for cytochrome oxidase, and 0.5 for other steps not including respiratory chain, ATP synthase and ade-

nylate or P_i carriers. The substrate carriers, the glutamate dehydrogenase and the oxidation steps from α-ketoglutarate to oxaloacetate in the citric acid cycle [28] are probably involved in controlling the rate of respiration. In our study, comparable results were obtained using carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)-uncoupled mitochondria (data not shown), demonstrating that in our system a strong contribution to flux control by ATPase and the proton gradient over ADP-stimulated respiration can be excluded. The fact that flux control by Complex I raises from 0.34 to 0.78 considering NADH oxidation in sonicated particles is in line with the observation of Inomoto et al. [28] and suggests that the remaining control is exerted by cytochrome oxidase.

On the contrary, under state 4 respiration the dissipation of the proton gradient represents the main rate-controlling step upon the whole respiration.

This information is in line with the results reported by other authors [44] suggesting that the control over oxygen consumption in mitochondria is broadly distributed among several steps and that the activity of the components of the respiratory chain is in relative excess over the amount required to support the endogenous respiration rate [59].

At the same time, we observed a trend wherein the Complex I flux control coefficient is increased during aging. Examination of the rotenone inhibition threshold values also pointed out a decrease in the old animals indicating that Complex I becomes more rate controlling with age. It can be postulated that the coefficients are changed during aging because the respiratory steady state is modified in old rat mitochondria and this may play a role in the distribution of the control of the mitochondrial oxidative phosphorylation among several steps, including Complex I [28]. The increase in the I_{50} for rotenone inhibition cannot be responsible for the increased flux control coefficient in the old, since the latter parameter is obviously independent of the power of the inhibitor used. Anyway, Fig. 3B clearly shows that the curves of the state 3 respiration and Complex I activity (from the slope of which the coefficients are calculated) tend to overlap in the old as a result of the increased slope of the former rather than of the decreased slope of the latter.

There is a risk of damaging mitochondria during their isolation and thus modifying the threshold. In our experimental condition, temperature, pH and buffer composition were controlled; thus, only the nature of the mitochondria of the two groups of animals is supposed to be responsible for any variation detected in the threshold values. The RCR was decreased in the preparations of old rat mitochondria. Since this decrease is due to decreased state 3 respiration while the proton leak remains unchanged, we can conclude that in the old mitochondria less control is exerted by the dissipation of the proton gradient and, as a consequence, a relatively higher control is shared by Complex I. However, this conclusion may be justified only for state 4 respiration, since rotenone titration of FCCP-uncoupled mitochondria was undistinguishable from that of state 3 respiration (see above).

This study illustrates the value of metabolic control analysis in the understanding not only of the normal or pathological behavior of metabolic pathways, but also of the consequences of metabolic alterations during aging. Our work shows Complex I to be involved in aging not by simply comparing the enzyme activity in mitochondria of young and old rats, but also by applying the metabolic control analysis which, in our case, means correlating the alterations observed in the specific activity of Complex I both with the integrated NADH oxidase activity of the respiratory chain and with the total respiration of intact mitochondria. According to our data, the threshold effect in the expression of a defect of Complex I [15] can be characterized by the fact that a decrease in the enzyme activity, even if not able to make the respiration collapse abruptly, may lead to a sensible decrease of the energetic function of mitochondria, all the same. In other terms, our novel results suggest that the 25% decrease in Complex I activity observed during aging contributes high percentages to the decrease of mitochondrial respiration.

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