Major changes in Complex I activity in mitochondria from aged rats may not be detected by direct assay of NADH:coenzyme Q reductase

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We have investigated the respiratory activities and the concentrations of respiratory chain components of mitochondria isolated from the livers and hearts of two groups of rats aged 6 and 24 months respectively. In comparison with the adult controls (6 months), in aged rats there was a decline in total aerobic NADH oxidation in both tissues; only minor (non-significant) changes, however, were found in NADH:coenzyme Q reductase and cytochrome oxidase activities, and there was no change in ubiquinol–cytochrome *c* reductase activity. The coenzyme Q levels were slightly decreased in mitochondria from both organs of aged rats. The lowered NADH oxidase activity is not due to the slight decrease observed in the coenzyme Q levels, but is the result of decreased Complex I activity. Since the assay of NADH:coenzyme Q reductase requires quinone analogues, none of which can evoke its maximal turnover [Estornell, Fato, Pallotti and Lenaz (1993) FEBS Lett. **332**, 127–131], its activity has been calculated indirectly by taking advantage of the relationship that exists between NADH oxidation and ubiquinol oxidation through the coenzyme Q pool. The results, expressed in this way, show a drastic loss of activity of Complex I in both the heart and the liver of aged animals in comparison with adult controls.

INTRODUCTION

Mitochondria appear to be intimately involved in the aging process [1,2]. It has been proposed that the accumulation of somatic mutations of mitochondrial DNA (mtDNA) induced by long-term exposure to free radical attack, mainly in post-mitotic cells, leads to errors in the mtDNA-encoded polypeptide chains belonging to the proton-translocating complexes of the mitochondrial inner membrane; the consequence of these alterations would be defective electron transfer and energy conservation [2]. Unlike nuclear DNA, mutations of mtDNA could accumulate because of the intrinsic instability of the mitochondrial genome, as it is not protected by proteins and by efficient DNA repair mechanisms [3].

Indeed, a number of mtDNA deletions [4–7] and point mutations [8] have been described in tissues from aged animals and humans. Functional changes in the activity of respiratory enzymes have also been described, but their presence and extent appear to be extremely variable [5,9–12]; a 'mosaic' loss of cytochrome *c* oxidase, but not of succinate dehydrogenase, in skeletal and heart muscle mitochondria, as detected by histochemistry [13–15], seems to be a consistent bioenergetic change found in aging.

Structurally, mtDNA is a single molecule of circular DNA encoding 13 hydrophobic polypeptide chains of the four H^+-translocating complexes of the inner mitochondrial membrane [16], notably seven chains (the ND subunits) of Complex I [NADH:coenzyme Q (CoQ) oxidoreductase], the cytochrome *b* subunit of Complex III (ubiquinol–cytochrome *c* oxidoreductase), three subunits (COI–III) of Complex IV (cytochrome *c* oxidase) and two subunits of the integral sector of the H^+-ATPase (ATP synthase). Moreover, it also encodes 22 tRNAs and two rRNAs necessary for mitochondrial protein synthesis.

It is predicted, according to the mitochondrial theory of aging, that the highest frequency of mutations would affect Complex I and secondarily Complex IV, for which respectively seven and three mitochondrial genes are present. Moreover, the deletions described to date which increase in aging, such as the 5 kb 'common' deletion, usually encompass a region containing genes for ND subunits [17].

The present study investigates the function of the mitochondrial respiratory chain in the liver and heart of 6- and 24-month-old rats. The results show small decreases in Complex I and Complex IV activity in both organs. Nevertheless, it is shown that the activity of NADH:CoQ reductase, when calculated from the total rate of NADH oxidation and the rate of ubiquinol oxidation according to the so-called 'pool equation' [18], is conspicuously lower in mitochondria from aged rats than in those from adult controls.

EXPERIMENTAL

Materials

All chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and all solvents were pure reagents from Merck (Darmstadt, Germany). Ubiquinone homologues (coenzymes Q_1, Q_9 and Q_{10}) were gifts from Eisai Co. (Tokyo, Japan). Decyl-ubiquinone (DB; from Sigma) was reduced according to Rieske [19].

Animals

Two groups of male albino rats of the Wistar strain, aged 6 and 24 months respectively, purchased from Charles River Italia S.p.A. (Milano, Italy), were kept for 1 week under constant environmental conditions [10] and fed on a normal laboratory diet; each group comprised eight animals. All procedures involving the care of animals were performed according to the

Abbreviations used: CoQ, coenzyme Q (ubiquinone); DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (decyl-ubiquinone); DBH_2, reduced DB (decyl-ubiquinol); mtDNA, mitochondrial DNA.

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guidelines for animal experimentation issued by the University of Bologna.

Preparation of mitochondria

Mitochondria were prepared from the liver and the heart, immediately after killing the animals by decapitation, according to Kun et al. [20] but without digitonin treatment. Mitochondrial protein was determined by a biuret method [21] with the addition of 10% sodium deoxycholate and using BSA as standard.

Enzymic activities

Individual oxidative enzymic activities (NADH:CoQ reductase, ubiquinol–cytochrome *c* reductase and cytochrome *c* oxidase) and integrated aerobic NADH oxidation were assayed at 30 °C after freezing and thawing the isolated mitochondria [10,22]. Ubiquinol–cytochrome *c* reductase and cytochrome oxidase were assayed in a 25 mM potassium phosphate buffer, pH 7.5, with the further addition of 1 mM KCN in the cytochrome *c* reductase assay.

Ubiquinol–cytochrome *c* reductase was assayed using oxidized cytochrome *c* at a quasi-saturating concentration of 48 μM and decyl-ubiquinol (DBH_2) at 9.8 μM; data reported are $V_{max.}$ values. For cytochrome *c* oxidase activity, ferrous cytochrome *c* (40 μM), reduced by dithionite and purified on a Sephadex G-25 column, was used as substrate.

All activities involving cytochrome *c* were evaluated by monitoring the absorbance change of cytochrome *c* upon reduction or oxidation at 550 nm minus 540 nm in a Sigma Biochem ZWS2 double-wavelength spectrophotometer equipped with a rapid mixing apparatus. The molar absorption coefficient used for cytochrome *c* was 19.1 $mM^{-1} \cdot cm^{-1}$.

NADH:CoQ reductase was assayed essentially as described [23,24] in 50 mM KCl, 10 mM Tris/HCl, 1 mM EDTA, pH 7.4, with 1 mM KCN and 1 μM antimycin A. The activity was assayed using 75 μM NADH and 60 μM DB as substrates and by following the decrease in absorbance of NADH at 340 nm minus 380 nm using a molar absorption coefficient of 5.5 $mM^{-1} \cdot cm^{-1}$. Aerobic NADH oxidation was assayed in a similar way, but both DB and inhibitors were omitted from the assay mixture.

CoQ assay

An aliquot of mitochondria (generally corresponding to 1 mg of protein) was used for CoQ determination as previously described [12,25], but we used a Spherisorb S5 ODS1 column in a Waters Data Module M730-Model 721 Programmable System Controller equipped with a Lambda-Max Model 481 LC spectrophotometer.

Cytochrome determination

The content of cytochromes was evaluated by the differential spectra (dithionite-reduced minus ferricyanide-oxidized) of a sample of 3–6 mg of mitochondrial protein diluted with 1 ml of 25 mM KH_2PO_4, 1 mM EDTA, pH 7.4, in the presence of 1% potassium deoxycholate in a Jasco (UVIDEC B 10) double-beam spectrophotometer [26].

Statistics

All data are presented as means ± S.D.; the significance of differences was evaluated by the unpaired *t* test and accepted when $P < 0.05$.

RESULTS

The respiratory activities assayed are reported in Tables 1 and 2 for liver and heart mitochondria respectively. All activities tested were > 90% sensitive to their respective inhibitors, i.e. rotenone for Complex I, antimycin A for Complex III and KCN for cytochrome *c* oxidase. In particular, the rotenone sensitivity of NADH:DB reductase was almost the same as that of NADH oxidase. Preliminary results (not shown) had demonstrated that CoQ_1, although eliciting higher rates in comparison with DB, was reduced in a partially rotenone-insensitive fashion in liver mitochondria.

Comparison of the activities in the adult (6 months) and aged (24 months) animals revealed small, though significant, decreases in the aged group for NADH oxidase in both tissues. On the other hand NADH:CoQ reductase, assayed with DB as acceptor, and the other activities investigated, did not show significant changes.

Determination of cytochrome levels (Table 3) was employed to establish catalytic-centre activities for ubiquinol–cytochrome *c* reductase and cytochrome oxidase; however, no significant differences in activity were apparent between the two groups of animals. The decrease in the cytochrome oxidase specific activity in the livers of aged rats (cf. Table 1) was the result of the decrease in cytochrome aa_3, so that the catalytic-centre activity of the enzyme was unchanged.

The concentration of CoQ (Table 4) showed a small decrease in both liver and heart mitochondria from aged animals. The

Table 1 Specific respiratory chain activities in liver mitochondria from 6- and 24-month-old rats

The data are means ± S.D. of triplicate determinations from eight different mitochondrial preparations. See the text for details. Abbreviation: cyt. *c*, cytochrome *c*. Significant difference compared with controls is indicated by $^*P < 0.025$.

	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	
Activity	6 months	24 months
NADH:O_2	0.157 ± 0.037	0.101 ± 0.043*
NADH:DB	0.154 ± 0.021	0.120 ± 0.041
DBH_2–cyt. *c*	1.099 ± 0.373	1.168 ± 0.334
Cyt. *c*–O_2	1.263 ± 0.348	0.984 ± 0.377

Table 2 Specific respiratory chain activities in heart mitochondria from 6- and 24-month-old rats

The data are means ± S.D. of triplicate determinations from eight different mitochondrial preparations. See the text for details. Abbreviation: cyt. *c*, cytochrome *c*. Significant difference compared with controls is indicated by $^*P < 0.02$.

	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	
Activity	6 months	24 months
NADH:O_2	0.983 ± 0.203	0.700 ± 0.162*
NADH:DB	0.292 ± 0.081	0.256 ± 0.059
DBH_2–cyt. *c*	2.980 ± 0.783	3.133 ± 0.768
Cyt. *c*–O_2	4.298 ± 1.204	3.853 ± 0.566

Table 3 Cytochrome determination and catalytic-centre activities (CCA) of ubiquinol–cytochrome *c* reductase and cytochrome oxidase in liver and heart mitochondria from 6- and 24-month-old rats

The content of ubiquinol–cytochrome *c* reductase was taken to be one-half that of cytochrome *b*. See the text for further details. The data are means ± S.D. from eight different mitochondrial preparations. Significant difference compared with controls is indicated by $^*P < 0.01$.

	Liver mitochondria		Heart mitochondria	
Component or activity	6 months	24 months	6 months	24 months
Cytochrome aa_3 (nmol · mg^{-1})	0.120 ± 0.020	0.083 ± 0.029*	0.389 ± 0.071	0.428 ± 0.151
Cytochrome *b* (nmol · mg^{-1})	0.145 ± 0.019	0.162 ± 0.034	0.411 ± 0.077	0.385 ± 0.108
Cytochrome $c + c_1$ (nmol · mg^{-1})	0.194 ± 0.025	0.200 ± 0.022	0.458 ± 0.086	0.424 ± 0.094
Cytochrome oxidase CCA (s^{-1})	181 ± 64	209 ± 84	191 ± 65	162 ± 46
Ubiquinol–cytochrome *c* reductase CCA (s^{-1})	254 ± 77	248 ± 96	244 ± 61	283 ± 88

Table 4 CoQ_9 and CoQ_{10} contents of liver and heart mitochondria from 6- and 24-month-old rats

Data are means ± S.D. from eight different mitochondrial preparations.

	CoQ content (nmol · mg^{-1})			
	Liver mitochondria		Heart mitochondria	
	6 months	24 months	6 months	24 months
CoQ_9	1.025 ± 0.162	0.885 ± 0.230	3.603 ± 0.625	2.858 ± 1.048
CoQ_{10}	0.095 ± 0.011	0.103 ± 0.038	0.323 ± 0.087	0.387 ± 0.131

CoQ_9 content exceeded that of CoQ_{10} by about 10-fold in all samples, and no age-linked differences were present in the CoQ_{10}/CoQ_9 ratio.

DISCUSSION

Determination of NADH:CoQ reductase activity by the 'pool equation'

We have previously established [24] that the best electron acceptor for the assay of NADH:CoQ reductase in bovine heart mitochondria is CoQ_1. In the present study, however, we have been unable to successfully employ that short-chain CoQ homologue because its reduction in liver mitochondria has a strong rotenone-insensitive component; since this inconvenience is not encountered using the analogue DB, which is widely employed in the Complex I activity assay, we decided to use DB as the oxidizing substrate for the entire experiment.

Perusal of Tables 1 and 2 reveals an inconsistency between the rates of NADH oxidation by molecular oxygen and by the CoQ analogue DB. In fact it appears, especially in the case of heart mitochondria, that NADH oxidation by DB is slower than NADH oxidation by oxygen. Irrespective of the mechanism of electron transfer, it is not possible that the rate of an overall pathway can be higher than that of a partial reaction of the same pathway. If we assume that electron transfer occurs via ubiquinone by a fixed stoichiometry, then the two rates should be at best equal. If we consider (as is generally assumed based on the convincing evidence of Kröger and Klingenberg [18] and widely confirmed thereafter [27,28]) that NADH oxidation occurs through a mobile homogeneous CoQ pool, then the rate of the NADH:CoQ reductase reaction ($V_{red.}$) would be higher than the rate of the NADH oxidase reaction ($V_{obs.}$), and would approach it only for a large rate excess of the portion of the chain that reoxidizes ubiquinone ($V_{ox.}$), as is readily shown by the pool equation [18]:

$$V_{obs.} = V_{red.} V_{ox.}/(V_{red.} + V_{ox.})$$

The reasons for such a discrepancy can be ascribed, at least in part, to the intrinsic difficulties in the assay of NADH:CoQ reductase, as previously shown in our laboratory [24].

In view of the difficulties described above, it is likely that rate differences of NADH:CoQ reductase between young and old individuals may not be adequately revealed by direct assay of the enzyme, particularly when the specific activity is exceedingly high, as in rat heart mitochondria. For this reason we have recalculated the rate of Complex I activity by appropriate rearrangement of the pool equation:

$$V_{red.} = V_{ox.} V_{obs.}/(V_{ox.} - V_{obs.})$$

In our experiments, NADH oxidation by oxygen is taken as $V_{obs.}$; $V_{ox.}$ has been considered to be equal to the rate of ubiquinol–cytochrome *c* reductase (which is the rate-limiting step in $V_{ox.}$ in our experimental condition; cf. Tables 1 and 2), normalized for two-electron transfer by dividing the rate of cytochrome *c* reduction (a one-electron carrier) by two. We have not been able to measure reliable rates of either NADH–cytochrome *c* reductase (due to the high values of the rotenone-insensitive activity, particularly in liver) or ubiquinol oxidase (due to the insufficient amount of sample for the oxygen electrode and to optical interference in the spectrophotometric determination of ubiquinol oxidation). For this reason we have considered ubiquinol–cytochrome *c* reductase to represent the most likely value for $V_{ox.}$. Control experiments in beef heart submitochondrial particles, where ubiquinol oxidase could be measured, strongly support our assumption (the $V_{max.}$ of ubiquinol oxidase is very close to the $V_{max.}$ of ubiquinol–cytochrome *c* reductase; e.g. 1.19 and 1.02 μmol of ubiquinol-2 oxidized/min per mg of protein by oxygen and by cytochrome *c* respectively in one preparation of particles).

By using the pool equation we have recalculated the activities of NADH:CoQ reductase in the young and old rats and obtained the values shown in Table 5. It appears that all NADH:CoQ reductase activities calculated from the pool equation are higher than those obtained by the direct assay of NADH:DB reductase; nevertheless, if we compare the two groups, the results indicate that the activities are now strikingly lower in the aged group than in the controls (59% in liver; 57% in heart).

The rationale of our calculation is independent of the CoQ concentration in the membrane. However, the total rate of NADH oxidation, $V_{obs.}$, is also a function of the total CoQ concentration in the membrane according to a hyperbolic re-

Table 5 NADH:CoQ reductase activities of liver and heart mitochondria from 6- and 24-month-old rats

Activities were individually calculated from the pool equation using the data in Tables 1 and 2 (see the text for details). The corresponding NADH:DB reductase activities (from Tables 1 and 2) are shown for comparison. Significant differences compared with controls are indicated by $^{*}P < 0.05$; $^{**}P < 0.02$.

	Activity (μmol·min^{-1}·mg^{-1})			
	NADH:CoQ ($V_{red.}$)		NADH:DB	
	6 months	24 months	6 months	24 months
Liver	0.226±0.065	0.134±0.081*	0.154±0.021	0.120±0.041
Heart	2.258±0.691	1.288±0.497**	0.292±0.081	0.256±0.059

lationship [28,29]: since the CoQ concentration in mitochondria is not saturating for yielding $V_{max.}$ [30], changes in CoQ content are bound to determine changes in $V_{obs.}$. We have observed a decrease in the CoQ concentration in liver and heart mitochondria from aged rats (cf. Table 4); nevertheless, assuming the K_m values of NADH oxidation for CoQ found in beef heart mitochondria to be valid also in the mitochondria used in the present study, calculation of the rate ($V_{obs.}$) decrease due to the decrease in CoQ concentration reveals a change not exceeding 15%. It is therefore highly likely that the changes in $V_{obs.}$ that we have found largely reflect a decrease in Complex I activity.

The only possible drawback to the indirect calculation used could be a drastic difference in the K_m of Complex I for the endogenous ubiquinone between the two groups. Although this possibility has not been investigated for lack of sufficient material, and will be the subject of further investigations, we consider it unlikely that so great a change could occur and thus be responsible for the observed differences in $V_{obs.}$.

Enzymic changes in aging

The results of the present investigation strongly support the mitochondrial theory of aging, confirming the predictions of the theory that Complex I and IV activities are likely to be most strongly compromised by mtDNA mutations. In our experiment, however, the change in Complex IV activity was marginal, in contrast with the study of Sugiyama et al. [11] but in accordance with our previous findings in rat heart mitochondria [10]. On the other hand, the decrease in Complex I activity, calculated as discussed in the previous section (see above), was very severe, the activity in 24-month-old rats being of the order of one-half that in adult (6-month-old) controls. Surprisingly, a large decrease with aging was found also in liver mitochondria, where milder effects of aging are expected to occur [3], and where indeed only small changes were observed by Sugiyama et al. in a study on aging in rats and dogs [11]. Nevertheless, a marked decline of state 3 respiration with NAD^+-linked substrates in human liver mitochondria with aging was described in another study [31]. The different results observed may be ascribed to several reasons such as animal species, effective age and level of antioxidants, as well as to assay differences. For such a reason, it is very important to stress that the present study reveals that changes in NADH:CoQ reductase activity may be greatly underestimated and might be revealed only indirectly. This may also be the reason why, in mitochondrial diseases involving Complex I such as Leber's neuropathy, overall NAD^+-linked oxidation was found to be more severely compromised than Complex I activity [32,33].

The lack of observation of dramatic losses of respiratory activities in aged tissues, however, requires a further comment. It is to be noted that a severe loss of mitochondrial bioenergetic function may become incompatible with cell survival [10]; removal of energy-deficient cells by apoptosis has recently been described [34] and may be a prominent factor in aging. The result of cell loss would be to prevent observation of major changes, as analytical procedures would detect only those cells which are still fully functional or not yet severely affected enough to be eliminated.

CoQ in aging

CoQ was found to be only slightly decreased in mitochondria from aged animals. CoQ is synthesized by a complex pathway that does not involve mitochondrial gene products [35]; the lack of any major change in aged rats is in agreement with the hypothesis of a mitochondrial genetic defect [1–3]. Our finding, however, differs from those of Beyer et al. [36] and Appelkvist et al. [37], who reported large decreases in CoQ levels in organs of both rats and humans upon aging.

An explanation for this discrepancy might be found in possible different exposures to free radical damage and in different extents of antioxidant protection under different experimental conditions. We have observed that, in perfused rat livers, oxidative stress results in a decrease in the endogenous CoQ content [38], possibly as the direct result of oxidative damage to the quinone molecule. It may be reasoned that factors indirectly related to aging lead to oxidative destruction of CoQ, aggravating the consequences of hampered electron transfer. It is likely that this extreme stage has not been reached in our 24-month-old animals.

It should be noted that our study involves CoQ levels in isolated mitochondria, whereas those quoted above [36,37] deal with whole tissues. The high content of extramitochondrial CoQ [39] makes it difficult to extrapolate mitochondrial CoQ levels from tissue levels. Nevertheless, as CoQ is a well recognized antioxidant [40,41] which protects endogenous CoQ levels against oxidative stress [38,42], even a small decline in the levels of the quinone in mitochondria of aged animals is likely to enhance the effects of oxidative damage to mtDNA. It has been speculated [43] that higher levels of CoQ may be required in aged animals to counterbalance the progressive accumulation of free radical damage. On the other hand, the decrease in CoQ levels in mitochondria under our experimental conditions appears to be too small to induce a significant decrease in mitochondrial respiration in terms of saturation kinetics [29,30] (see previous section).

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