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Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging

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Navarro, Ana, and Alberto Boveris. Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging. *Am J Physiol Regul Integr Comp Physiol* 287: R1244–R1249, 2004. First published July 22, 2004; doi:10.1152/ajpregu.00226.2004.—The mitochondrial mass of rat brain and liver remained unchanged on aging in young adults, old adults, and senescent animals (28, 60, and 92 wk of age); the values were 15–17 and 29–31 mg protein/g for brain and liver, respectively. The whole aging process was associated with an increased content of the oxidation products, thiobarbituric acid-reactive substances and protein carbonyls, by 61–69% in brain and 36–45% in liver, respectively. The activities of critical enzymes for mitochondrial function, mitochondrial nitric oxide synthase, Mn-superoxide dismutase, complex I, and complex IV, decreased progressively during aging with activity losses of 73, 37, 29, and 28%, respectively, in the brain and 47, 46, 30, and 24% in the liver of senescent rats compared with young adults. Brain mitochondria isolated from aged rats showed increased mitochondrial fragility, as assayed by mitochondrial marker enzyme activities in the postmitochondrial supernatant, and increased volume and water permeability, as assayed by light scattering. Liver mitochondria isolated from young and old rats did not show differences in fragility and water permeability. A subpopulation of brain mitochondria with increased size and fragility was differentiated in aging rats, whereas liver showed a homogeneous mitochondrial population.

mitochondrial nitric oxide synthase; manganese-superoxide dismutase; NADH-cytochrome *c* reductase; cytochrome oxidase; mitochondrial fragility; oxidative stress

THE MAINTENANCE OR LOSS of mitochondrial function during aging is both an interesting and a controversial issue. On the one hand, mitochondria are brought to attention in aging biology due to *1*) the central role of mitochondria in producing chemical energy (ATP) to meet cellular requirements, and *2*) the declines of metabolic rate and of physical performance in energy-requiring tasks that are associated with the aging process (29). Mitochondrial function includes the conversion of the chemical redox energy of substrate oxidation: first to a proton electrochemical gradient and then to high-energy phosphate chemical bonds (ATP) in a highly organized and coupled process that requires the physical integrity of the mitochondrial inner membrane. On the other hand, the mitochondrial hypothesis of aging considers mitochondria as the pacemaker of tissue aging due to continuous mitochondrial production of reactive oxygen and nitrogen species ($O_2^{-}\cdot$, H_2O_2, NO, $ONOO^{-}$, HO·, ROO·, and $^1O_2$). It is understood that the reactive species, although kept in low steady-state concentrations by antioxidant enzymes, reductants, and quenchers, are able to react and damage biomolecules leading to cumulative oxidative damage (1, 10).

Mitochondria are the main intracellular source of oxidizing free radicals; two free radicals, $O_2^{-}\cdot$ and NO, are primarily and continuously produced in these organelles. Other reactive species such as H_2O_2, $ONOO^{-}$, HO·, ROO·, and $^1O_2$, derive from the primary production of $O_2^{-}\cdot$ and NO. $O_2^{-}\cdot$ is generated as a by-product of the respiratory chain electron transfer, mainly by the autoxidation of ubisemiquinone (3, 8). NO is the product of the enzymatic action of mitochondrial NO synthase (mtNOS) (16, 17), a specialized NOS (13) that carries out a classical NOS reaction, requiring NADPH, arginine, O_2, and Ca^{2+}/calmodulin for enzyme activity (47).

The free radical chain reactions are started by $O_2^{-}\cdot$ and NO and involve a series of reactive oxygen and nitrogen species that are capable of damaging mitochondrial membranes, proteins, and DNA. Mitochondria, the main cellular site of oxygen uptake and oxyradical generation, are also the main target of oxyradical-mediated damage. Cumulative free radical damage contributes to cell and tissue senescence and leads mitochondria to a state of mitochondrial dysfunction with decreased organelle ability to synthesize ATP and to adapt themselves to the destabilizing effects of cellular stress (1, 44). Dysfunctional mitochondria show decreased state 3 oxygen uptake (32) and inner membrane potential (40), and increased $O_2^{-}\cdot$ and H_2O_2 production (45), as well as increased size (40). Dysfunctional mitochondria generate intracellular signals for lysosomal digestion and for apoptosis.

In this study we characterized the dysfunctional state of brain and liver mitochondria associated with normal aging by assaying oxidative stress indicators, mitochondrial enzyme activities that behaved as aging indicators, and mitochondrial size, fragility, and passive water permeability.

MATERIALS AND METHODS

Animals. Male Wistar rats in the stages of young adults, aged adults and senescent, 28, 60, and 92 wk of age, were used ($n = 20$ in each group). The animals were siblings grown and aged at the Department of Experimental Animals of the University of Cádiz, housed in groups of five animals and kept at 24 ± 1°C with 12:12-h light-dark cycles, with full access to water and food. Animal experiments were carried out in accordance with the 86/609/CEE European Community regulations and the "Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society.

Isolation of mitochondria. Brain and liver mitochondria were isolated from the whole organs homogenized in 0.23 M mannitol, 0.07 M sucrose, 15 mM MOPS-KOH (pH 7.2) at a ratio of 9 ml of

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homogenization medium/1 g of tissue in a Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 700 *g* for 10 min and the supernatant at 8,000 *g* for 10 min to precipitate mitochondria that were washed in the same conditions (34, 35). Mitochondrial suspensions, containing ~20 mg protein/ml, were used immediately after isolation for swelling studies or frozen in liquid N_2 and kept at −80°C. Mitochondrial samples, twice thawed and frozen, were homogenized by passage through 15/10 tuberculin needles; the resulting submitochondrial membranes were used for the determination of enzyme activities and oxidative stress markers. Protein content of samples was determined using the Folin reagent and BSA as standard.

Mitochondrial electron transfer activities. The electron transfer activities of complexes I-III, II-III, and IV were determined spectrophotometrically at 30°C in 100 mM phosphate buffer (pH 7.4) (34). For NADH-cytochrome *c* reductase (complexes I-III) and succinate-cytochrome *c* reductase (complexes II-III) activities, samples were added with 0.2 mM NADH or 20 mM succinate as substrates, 0.1 mM cytochrome c^{3+}, and 1 mM KCN, and the enzymatic activity was determined at 550 nm ($\epsilon = 19\ mM^{-1}\cdot cm^{-1}$) and expressed as nanomoles cytochrome *c* reduced per milligram protein. Cytochrome oxidase (complex IV) activity was determined in the same phosphate buffer added with 0.1 mM cytochrome c^{2+}, which was prepared by reduction with $NaBH_4$ and HCl. The rate of cytochrome *c* oxidation was calculated as the first-order reaction constant per milligram protein and expressed as nanomoles cytochrome *c* oxidized at 20 μM cytochrome *c* and at 1 mg protein/ml, which gives rates of the order of physiological activities (34, 35).

Mitochondrial superoxide dismutase activity (Mn-superoxide dismutase). This enzymatic activity was determined in tissue homogenates and isolated mitochondria by the adrenochrome assay in a reaction medium containing 1 mM epinephrine, 1 mM KCN, and 50 mM glycine/KOH (pH 10.0). One Misra-Fridovich unit of enzyme activity, 50% inhibition of the rate of adrenochrome formation, is equivalent to 11 pmol of superoxide dismutase (SOD) active center (18, 30).

mtNOS activity. Mitochondrial NO production was determined by the oxyhemoglobin (HbO_2) oxidation assay as described elsewhere (2). The reaction medium consisted of 0.1 mM NADPH, 0.2 mM arginine, 1 mM $CaCl_2$, 4 μM Cu,Zn-SOD, 0.1 μM catalase, and 25 μM HbO_2 heme, in 50 mM phosphate buffer at pH 7.2 (liver) and at pH 5.8 (brain). A diode array sensitive spectrophotometer (Agilent, model 8453) was used to follow the absorbance change at 577 nm with a reference wavelength at the isosbestic point of 591 nm ($\epsilon_{577-591} = 11.2\ mM^{-1}\cdot cm^{-1}$). NO production was calculated from the absorbance change that was inhibited by 2 mM N^G-methyl-L-arginine, usually 86–94%, and expressed in nanomoles NO per minutes per milligram protein.

Biochemical markers of oxidative stress. The mitochondrial content of the oxidation products, thiobarbituric acid-reactive substances (TBARS) and protein carbonyls, was determined as previously described (15, 34, 38).

Light scattering and passive mitochondrial swelling. Light scattering and hypotonically induced mitochondrial swelling were determined in 90 mM KCl, 20 mM MOPS-KOH (pH 7.2) at 1 mg protein/ml by the absorbance changes that were followed spectrophotometrically at 540 nm. The absorption change (ΔA) after the addition of mitochondria to the suspension medium and the initial rate (1 min) of mitochondrial swelling (ΔA/min) were recorded.

Mitochondrial mass in the tissue. The content of mitochondria (mitochondrial mass) of the whole organs was calculated from the ratios of cytochrome oxidase and Mn-SOD activities in organ homogenates and in isolated mitochondria (41).

Fraction of marker mitochondrial activities isolated in the mitochondrial fraction. The mitochondrial electron transfer activities, NADH-cytochrome *c* reductase, succinate-cytochrome *c* reductase and cytochrome oxidase, were determined, as described before, in the whole organ homogenate, in the isolated mitochondrial fraction, and in the postmitochondrial supernatant in each individual animal ($n = 8$). The percentage of organ mitochondria isolated by the utilized isolation procedure was calculated as the fraction of homogenate activity centrifuged down in the isolated mitochondrial fraction (see list in Table 5). The sum of the marker enzyme activities in isolated mitochondria and in the postmitochondrial supernatant accounted for the following percentages of homogenate enzyme activities: NADH-cytochrome *c* reductase, 96–114%; succinate-cytochrome *c* reductase, 90–95%; and cytochrome oxidase, 94–98%. The fraction of mitochondrial activity in the postmitochondrial supernatant is a direct indication of mitochondrial fragility and rupture during the homogenization procedure.

Statistics. The numbers in tables and figures indicate mean values ± SE. Differences between groups were analyzed by Dunnett's bilateral post hoc test after significant one-way ANOVA. A P value of <0.05 was considered to be biologically significant. Statistical analyses were carried out using a statistical package (SPSS 11.5 for Windows).

RESULTS

Aging and mitochondrial mass in brain and liver. The content of mitochondria in brain and liver, calculated from the activities of the mitochondrial marker enzymes cytochrome oxidase and Mn-SOD, was 15.2–17.0 and 29.2–31.0 mg mitochondrial protein/g of organ for brain and liver and did not show differences in the aging animals (Table 1). The mitochondrial mass was markedly lower (~52%) in the brain compared with the liver.

Aging and oxidative stress markers. Protein carbonyls and TBARS increased significantly in brain and liver mitochondria on rat aging from 28 to 92 wk of age as an indication of progressive mitochondrial oxidative stress (Table 2). In brain mitochondria, protein carbonyls increased by 42 and 69% at 60 and 92 wk of age and in liver mitochondria by 26 and 36% at the same ages. TBARS were a similarly sensitive indicator of mitochondrial oxidative stress: in brain, they augmented by 47 and 61% at 60 and 92 wk of age, and in liver by 24 and 45% at the same ages.

Aging, mtNOS, and Mn-SOD activities. The activities of these two physiologically important mitochondrial enzymes markedly and progressively decreased on aging (Table 3). In brain, mtNOS activity decreased to 27% and Mn-SOD decreased to 62% at 92 wk of age. In liver, the same activities were similarly affected by aging; mtNOS and Mn-SOD activities decreased to 53% and to 50% at 92 wk of age. A significant positive correlation was found between the activities of these two enzymes in brain ($r^2 = 0.99$, $P < 0.05$) and liver ($r^2 = 0.99$, $P < 0.05$).

Aging and the mitochondrial electron transfer activities. Aging decreased NADH-cytochrome *c* reductase activities (complexes I-III) in brain and liver. The age-dependent loss in activity was 29–30% for brain and liver at 92 wk of age (Table 4). Cytochrome oxidase (complex IV) activity was also progressively lost on aging; activities diminished by 28% in brain and by 43% in liver at 92 wk of rat age (Table 4). At variance, aging did not modify succinate-cytochrome *c* reductase activities (complexes II-III) in both organs (Table 4), indicating a selective effect of aging on mitochondrial electron transfer activities.

Aging and mitochondrial fragility. In brain, the percentage of organ mitochondrial marker enzyme activities (NADH → cytochrome c^{3+}; succinate → cytochrome c^{3+}; and cyto-

Table 1. *Mitochondrial content in the brain and liver of aging rats*

Marker/Organ/Age	Homogenate Organ Activity, nmol·min^{-1}·g organ^{-1}	Mitochondria Specific Activity, nmol·min^{-1}·mg protein^{-1}	Mitochondrial Mass, mg protein/g organ)
Cytochrome oxidase			
Brain			
28 wk	2,205±93	139±8	15.9±1.3
60 wk	2,074±83	122±7	17.0±0.8
92 wk	1,532±62‡	100±7†	15.3±1.0
Liver			
28 wk	4,031±179	138±6	29.2±1.4
60 wk	3,720±107*	124±6	30.0±1.1
92 wk	2,449±94‡	79±5‡	31.0±0.8
Marker/Organ/Age	**Homogenate Organ Activity, pmol/g organ**	**Mitochondria Specific Activity, pmol/mg protein**	**Mitochondrial Mass, mg protein/g organ**
Mn-SOD			
Brain			
28 wk	272±22	16±2	17.0±1.1
60 wk	198±17*	13±2	15.2±1.0
92 wk	161±14‡	10±2	16.1±1.5
Liver			
28 wk	861±76	28±3	30.7±2.2
60 wk	642±46*	22±3	29.2±1.4
92 wk	433±38‡	14±2*	28.9±1.2

Values are means ± SE. *1*) Brain cytochrome oxidase: homogenate $F(2,27) = 21.2$, $P < 0.001$; mitochondria $F(2,27) = 6.7$, $P < 0.01$; mitochondrial mass $F(2,27) = 0.8$, not significant (NS). *2*) Liver cytochrome oxidase: homogenate $F(2,27) = 40.2$, $P < 0.001$; mitochondria $F(2,27) = 29.7$, $P < 0.001$; mitochondrial mass $F(2,27) = 0.8$, NS. *3*) Brain Mn-superoxide dismutase (SOD): homogenate $F(2,27) = 9.7$, $P < 0.01$; mitochondria $F(2,27) = 1.8$, NS; mitochondrial mass $F(2,27) = 1.4$, NS. *4*) Liver Mn-SOD: homogenate $F(2,27) = 14.2$, $P < 0.001$; mitochondria $F(2,27) = 4.9$, $P < 0.05$; mitochondrial mass $F(2,27) = 0.3$, NS. *$P < 0.05$; †$P < 0.01$; and ‡$P < 0.001$ vs. 28-wk-old rats.

chrome $c^{2+} \rightarrow O_2$) isolated in the mitochondrial fraction was significantly lower in senescent rats (18–19%) compared with young adults (28–29%) and with old adults (21–23%) (Table 5). The whole age-dependent loss in marker activities in isolated brain mitochondria was ~35%. In liver, at variance, the percentages of the same marker activities isolated in the mitochondrial fraction were similar, in the 35–38% range, for young and old adult animals and for senescent rats (Table 5).

A direct estimation of mitochondrial size by light scattering indicated an increased size (less light scattering and absorption) in brain mitochondria isolated from senescent rats (ΔA = 0.751) compared with the ones isolated from young adults (ΔA = 0.948), with intermediate sizes in old adults (ΔA = 0.864) (Fig. 1). At variance, there were no significant changes in light scattering associated with aging in liver mitochondria (ΔA = 0.810–0.848).

In agreement with the above-referenced results, the passive rate of mitochondrial swelling in a hypotonic medium was significantly faster in brain mitochondria isolated from 92-wk-old rats (ΔA/min = 0.096) than in the organelles isolated from 28- and 60-wk-old rats (ΔA/min = 0.063 and 0.080) (Fig. 1). Again at variance, in the liver, the rate of passive mitochondrial swelling was similar for mitochondria isolated from senescent animals and from young adult rats (ΔA/min = 0.051–0.055) (Fig. 1).

DISCUSSION

Reduction of the mitochondrial capacity to produce ATP and oxidative stress in the organs and tissues of old mammals are the two underlying concepts of the mitochondrial hypothesis of aging (1, 43, 49). The mentioned reduction in ATP production

Table 2. *Effect of aging on oxidative stress markers of rat brain and liver mitochondria*

Organ Marker	Age 28 wk	60 wk	92 wk
Brain			
Protein carbonyls	48±4	68±6*	81±6‡
TBARS	11.5±1.3	16.9±1.4*	18.5±1.4†
Liver			
Protein carbonyls	115±8	145±8*	157±9†
TBARS	3.3±0.3	4.1±0.4	4.8±0.4*

Values are means ± SE in units of pmol/mg mitochondrial protein. *1*) Brain protein carbonyls: $F(2,42) = 10.8$, $P < 0.001$. *2*) Brain thiobarbituric acid-reactive substances (TBARS): $F(2,42) = 6.7$, $P < 0.01$. *3*) Liver protein carbonyls: $F(2,42) = 6.6$, $P < 0.01$. *4*) Liver TBARS: $F(2,42) = 4$, $P < 0.05$. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ vs. 28-wk-old rats.

Table 3. *NOS and Mn-SOD activities in the brain and liver mitochondria of aging rats*

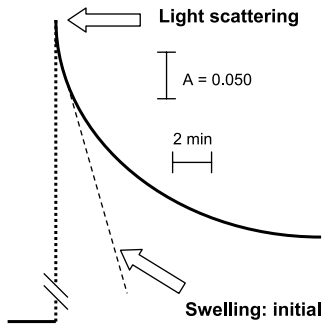
Organ/Age	mtNOS, nmol NO·min^{-1}·g organ^{-1}	Mn-SOD, pmol SOD/mg protein
Brain		
28 wk	0.67±0.04	16±2
60 wk	0.32±0.03‡	13±2
92 wk	0.18±0.02‡	10±2
Liver		
28 wk	0.75±0.05	28±3
60 wk	0.61±0.04*	22±3
92 wk	0.40±0.03‡	14±3*

Values are means ± SE. *1*) Brain mitochondrial nitric oxide synthase (mtNOS): $F(2,27) = 60$, $P < 0.001$. *2*) Liver mtNOS: $F(2,27) = 18.4$, $P < 0.001$. *3*) Brain Mn-SOD: $F(2,27) = 2.3$, NS. *4*) Liver Mn-SOD: $F(2,27) = 4.1$, $P < 0.05$. *$P < 0.05$; ‡$P < 0.001$ vs. 28-wk-old rats. NO, nitric oxide.

Table 4. *Mitochondrial electron transfer activities in the brain and liver mitochondria of aging rats*

Organ/Age	NADH → Cyt c^{3+}	Succ → Cyt c^{3+}	Cyt c^{2+} → O_2
Brain			
28 wk	385±12	121±8	139±8
60 wk	321±12†	121±8	122±7
92 wk	274±12‡	110±9	100±7†
Liver			
28 wk	455±16	126±8	138±6
60 wk	364±14‡	122±9	124±6
92 wk	318±13‡	120±9	79±5‡

Values are means ± SE. Enzymatic activities (NADH → Cyt c^{3+}, NADH-cytochrome *c* reductase; Succ → Cyt c^{3+}, succinate-cytochrome *c* reductase; Cyt c^{2+} → O_2, cytochrome oxidase) are expressed in nmol cytochrome *c* reduced or oxidized·min^{-1}·mg $protein^{-1}$. *1*) Brain NADH → Cyt c^{3+}: $F(2,27) = 21.7$, $P < 0.001$. *2*) Liver NADH → Cyt c^{3+}: $F(2,27) = 33.7$, $P < 0.001$. *3*) Brain Succ → Cyt c^{3+}: $F(2,27) = 0.5$, NS. *4*) Liver Succ → Cyt c^{3+}: $F(2,27) = 0.1$, NS. *5*) Brain Cyt c^{2+} → O_2: $F(2,27) = 6.7$, $P < 0.01$. *6*) Liver Cyt c^{2+} → O_2: $F(2,27) = 29.7$, $P < 0.001$. †$P < 0.01$; ‡$P < 0.001$ vs. 28-wk-old rats.



Organ/age	Light scattering (ΔA)	Swelling (ΔA/min)
Brain 28 wk	0.948 ± 0.045	0.063 ± 0.006
Brain 60 wk	0.864 ± 0.065	0.080 ± 0.007
Brain 92 wk	0.751 ± 0.042*	0.096 ± 0.008*
Liver 28 wk	0.848 ± 0.044	0.052 ± 0.006
Liver 60 wk	0.824 ± 0.040	0.051 ± 0.005
Liver 92 wk	0.810 ± 0.045	0.055 ± 0.006

Fig. 1. Mitochondrial light scattering and hypotonic swelling. Mitochondria were suspended in 90 mM KCl at 1 mg protein/ml, and light scattering was followed spectrophotometrically at 540 nm. *Left*: the determination procedure; $n = 8$ animals in each group. *A*, absorption; Δ*A*, absorption change. *$P < 0.05$.

is equally possible through a reduction of the mitochondrial mass in the cells or through a decrease in the specific rate of mitochondrial ATP synthesis, referred to a unit of mitochondrial mass (for instance, mg of mitochondrial protein). The data reported here indicate that the first possibility is not supported experimentally, the mitochondrial contents of brain and liver were found not affected by aging, and then the latter possibility seems the right one.

Mitochondria isolated from both organs of aged animals showed an increased content of oxidation products, decreased electron transfer activities, and increased mitochondrial size. Those features, added to decreased rates of O_2 uptake and ATP synthesis and to decreased membrane potential, describe the condition of dysfunctional mitochondria.

Table 5. *Fraction of organ mitochondrial electron transfer activities isolated in the mitochondrial fraction (inverse relationship with mitochondrial fragility)*

Organ (Rat Age)/ Enzyme Activity	Homogenate Total Organ Activity, nmol·min^{-1}·g $organ^{-1}$	Mitochondria: Activity in the mitochondrial fraction, nmol·min^{-1}·g $organ^{-1}$	Mitochondria: Fraction of organ activity in isolated mitochondria, %
Brain (28 wk)			
NADH → Cyt c^{3+}	6,121±349	1,708±121	27.9±1.3
Succ → Cyt c^{3+}	1,924±98	559±35	29.1±1.6
Cyt c^{2+} → O_2	2,205±93	628±48	28.4±1.7
Brain (60 wk)			
NADH → Cyt c^{3+}	5,260±298	1,173±71	22.3±1.5*
Succ → Cyt c^{3+}	2,008±96	464±28*	23.1±1.6*
Cyt c^{2+} → O_2	2,074±83	432±32*	20.8±1.7*
Brain (92 wk)			
NADH → Cyt c^{3+}	4,193±232‡	767±56†	18.3±1.5†
Succ → Cyt c^{3+}	1,783±81‡	329±20*	18.5±1.3†
Cyt c^{2+} → O_2	1,532±62‡	293±16†	19.3±0.9†
Liver (28 wk)			
NADH → Cyt c^{3+}	12,082±635	4,410±194	36.5±1.7
Succ → Cyt c^{3+}	3,345±162	1,221±92	36.1±1.9
Cyt c^{2+} → O_2	4,071±186	1,546±54	38.0±2.0
Liver (60 wk)			
NADH → Cyt c^{3+}	9,696±478*	3,471±187*	35.8±2.0
Succ → Cyt c^{3+}	3,213±167	1,173±83	36.5±2.2
Cyt c^{2+} → O_2	3,320±147*	1,159±51*	34.9±2.1
Liver (92 wk)			
NADH → Cyt c^{3+}	8,586±342†	3,192±126†	37.1±2.1
Succ → Cyt c^{3+}	3,240±154	1,192±66	36.8±1.9
Cyt c^{2+} → O_2	2,449±94†	929±46†	38.1±2.0

Values are means ± SE; enzymatic activities are as in Table 4. *1*) Brain NADH → Cyt c^{3+}: organ activity $F(2,27) = 18.9$, $P < 0.001$; mitochondrial activity $F(2,27) = 7.4$ $P < 0.01$; fraction of organ activity in isolated mitochondria $F(2,27) = 8.5$, $P < 0.01$. *2*) Liver NADH → Cyt c^{3+}: organ activity $F(2,27) = 28.7$, $P < 0.001$; mitochondrial activity $F(2,27) = 17.4$, $P < 0.001$; fraction of organ activity in isolated mitochondria $F(2,27) = 0.1$, NS. *3*) Brain Succ → Cyt c^{3+}: organ activity $F(2,27) = 16.6$, $P < 0.001$; mitochondrial activity $F(2,27) = 6.7$, $P < 0.01$; fraction of organ activity in isolated mitochondria $F(2,27) = 10.6$, $P < 0.001$. *4*) Liver Succ → Cyt c^{3+}: organ activity $F(2,27) = 0.9$, NS; mitochondrial activity $F(2,27) = 0.2$, NS; fraction of organ activity in isolated mitochondria $F(2,27) = 0.1$, NS. *5*) Brain Cyt c^{2+} → O_2: organ activity $F(2,27) = 21.2$, $P < 0.001$; mitochondrial activity $F(2,27) = 22.3$, $P < 0.001$; fraction of organ activity in isolated mitochondria $F(2,27) = 8.5$, $P < 0.01$. *6*) Liver Cyt c^{2+} → O_2 in the liver: organ activity $F(2,27) = 40.1$, $P < 0.001$; mitochondrial activity $F(2,27) = 35.7$, $P < 0.001$; fraction of organ activity in isolated mitochondria $F(2,27) = 1.5$, NS. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ vs. 28-wk-old rats.

The observed increase in oxidation products in rat brain and liver mitochondria isolated from aging animals, which describes an oxidative stress condition, agrees with previous reports of increased protein carbonyls (14, 34, 46), TBARS (34), ROOH, and 8-hydroxy-deoxy-guanosine (40, 42). Interestingly, cellular free radicals and oxidants produced at faster rates in the oxidative stress situation exhibit selectivity in damaging mitochondrial macromolecules and impairing membrane functions (43). A series of mitochondrial enzymes, membrane bound or matrix contained, have been consistently reported with lower activities in aging. However, it is not clear if the loss of enzyme activity is due to enzyme inactivation by free radicals and oxidation products or to a decreased expression.

The observed decreased enzyme activities are mtNOS, Mn-SOD, NADH-cytochrome *c* reductase, and cytochrome oxidase. The decrease in brain mtNOS activity associated with aging was the most marked of the observed decreases of mitochondrial enzyme activities, pointing to mtNOS as a marker of tissue aging (33) and suggesting a loss in the regulatory roles of mitochondrial NO as a critical mechanism in aging (5, 7).

The reported effects of aging on Mn-SOD activity have not being consistent. For simplicity we will restrict the consideration to brain, cerebrospinal fluid, and liver. Aging was reported as associated or causing an increased SOD activity (6, 11, 26, 35, 37, 49) and decreased SOD (Table 3 and Refs. 9, 21, 28, 31, 34, 39, 48). Moreover, SOD activity was not affected by aging (23, 36) or was modified in different ways in different tissues (12, 22, 24, 27). Differences may be due in part to the method used for enzyme determination and to interferences. We observed increased Mn-SOD and Cu,Zn-SOD activities in mouse brain and liver in aging (33, 35) using the classical xanthine oxidase/cytochrome *c* assay (43) and decreased Mn-SOD and Cu,Zn-SOD in mouse and rat brain and liver in aging by using the adrenochrome assay (Table 3; Ref. 34).

Concerning the electron transfer activities of the mitochondrial respiratory chain, complexes I and IV (NADH-ubiquinone reductase and cytochrome oxidase) were found with reduced activity (Table 4) in aged animals, in agreement with previous observations (25, 34, 35). The decreased activity of NADH-dehydrogenase in brain and liver of old rats, by ~30%, is close to the limit of a tolerable functional damage in terms of minimal energy production. In the liver of young rats and under physiological conditions, ATP is provided by the respiration of ~36% of the mitochondrial mass in metabolic state 3 (4). Under conditions of increased ATP demand, liver mitochondria will be able to increase ATP synthesis by ~2.8 times by switching more mitochondria from the resting state 4 to the active state 3. Aged hepatocytes, with a 30% reduction in NADH-dehydrogenase activity, will be able to increase ATP production only by 1.9 times by switching mitochondria from state 4 to state 3, due to the decreased NADH-dehydrogenase activity. The situation is clearly worst in the brain due to the lower mitochondrial mass; aged neurons may be close to being unable to respond to any increased ATP demands.

Light scattering studies indicated that brain mitochondria isolated from aged animals had a larger volume, in agreement with the report by Sastre et al. (40), and an increased water permeability when challenged by an hypotonic medium. Instead, liver mitochondria did not show such difference between young and old animals. A partial loss of the selective permeability of the inner membrane of brain mitochondria in old animals, as indicated by the increase in water permeability, is consistent with an increased H^+ permeability, in turn associated with uncoupling of oxidative phosphorylation, increase in state 4 respiration, and decreases in respiratory control and membrane potential, as reported in old animals (20, 32, 40).

The relative fragility of tissue mitochondria was estimated by comparing the fraction of mitochondrial marker enzymes recovered in the mitochondrial fraction after similarly processing (equal tissue disruption procedure and equal shearing forces in the homogenization procedure) tissues from young adults, old adults, and senescent animals. The greater the percentage of marker enzyme activity recovered in isolated mitochondria, the less the mitochondrial fragility or rupture during homogenization. The observed age-dependent decrease in marker activities in isolated brain mitochondria (35%) can be understood as a subpopulation of one-third of brain mitochondrial mass (mg mitochondrial protein/g of brain tissue) with increased fragility, a result in quantitative agreement with the data of Sastre et al. (40) that reported an increased mitochondrial size with age. Most brain cells age at the same time as the animal ages, and the persistent neurogenesis is spatially restricted and quantitatively negligible (19). Thus most neurons are long-living cells and exhibit a slow turnover time for their mitochondria and mitochondrial components, two factors that contribute to the cellular accumulation of dysfunctional mitochondria.

Liver mitochondria isolated from young and old animals did not exhibit a significant difference in fragility. In hepatocytes, mitochondria are continuously subjected to protein and phospholipid turnover and to elimination of the dysfunctional organelles. The cellular population of damaged mitochondria is likely to become determinant in the signaling for apoptosis as hepatocytes reach their lifespan limit.

An estimation of half-life ($t_{1/2}$) of several mitochondrial proteins or mitochondrial fractions gives an estimation of 7–10 days for liver mitochondria but of 4–6 wk for brain mitochondria. This slower turnover time of brain mitochondria makes possible the detection of a fraction of dysfunctional mitochondria with increased fragility, which may correspond to subpopulations of enlarged mitochondria in morphological or flow cytometry studies.

The data reported here show that mitochondria isolated from the organs of aged animals are also aged in terms of cytosolic and mitochondrial oxidative stress and losses of enzymatic activities. The observation recalls the challenging question of why the differentiated cells of systemic organs are subjected to aging whereas the cells of the germ line and the continuously proliferative stem cells, which sustain a population turnover in most tissues (except brain), are free from the aging process.

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