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Mitochondrial Dysfunctions During Aging: Vitamin E Deficiency or Caloric Restriction—Two Different Ways of Modulating Stress

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Caloric restriction (CR), which has been demonstrated to offset the age-associated accrual of oxidative injury, involves a reduction in calory intake while maintaining adequate nutrition, preserves the activities of antioxidant enzymes in postmitotic tissues, maintains organ function, opposes the development of spontaneous diseases, and prolongs maximum life span in laboratory rodents. It has been proposed that reductions in Reactive Oxygen Species (ROS) production and cellular oxidative injury are central to the positive effects of CR. In the present investigation we studied the effect of CR and of a vitamin E deprived diet on mitochondrial structure and features in the liver of rats during aging, in order to ascertain the extent of modifications induced by these experimental conditions. CR rats displayed structural and functional mitochondrial properties (fatty acid pattern, respiratory chain activities, antioxidant levels, and hydroperoxide contents) similar to those of younger rats whilst vitamin E deficient rats appeared older than their own age. The mitochondria of the former, together with those of young rats, possessed the lowest Coenzyme Q_9, hydroperoxide, and cytochrome contents as well as a suitable fatty acid membrane composition. Our study confirms that CR is a valuable tool in limiting aging-related free-radical damage also at mitochondrial liver level.

KEY WORDS: Coenzyme Q; respiratory chain activities; hydroperoxides; polyunsaturated fatty acids.

INTRODUCTION

Although mechanisms underlying age-related alterations are not well defined, growing evidence supports the validity of the oxidative stress hypothesis of aging, which suggests that continuous exposition of cell to oxidative injury contributes to the lowered functional capacity associated to age. One proposed mechanism for this observation is that the increase in oxidative stress and subsequent biomolecular damage associated with aging are the result of an enhanced rate of reactive oxygen species (ROS) generation and a greater susceptibility of tissues and the organism to oxidative injury (Ames *et al.*, 1993).

Currently, the underlying mechanisms through which cellular oxidative lesions increase with age remain unknown. Previous studies showed that the activities of one or more antioxidant enzymes, e.g., Cu/Zn-superoxide dismutase (SOD), Mn-SOD, catalase, and glutathione peroxidases (Gpx), declined with age in different tissues such as liver, kidney, and heart of mice and rats (Chen and Snyder, 1995; Xia *et al.*, 1995). These tissues also showed increased lipid peroxidation (Davis *et al.*, 1993; Pieri *et al.*, 1992), protein oxidation (Youngman *et al.*, 1992), and DNA oxidation (Anson *et al.*, 1999) with age. The age-related increase in oxidative lesions was reduced by caloric

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restriction (CR) (Davis *et al.* 1993; Djuric *et al.*, 1993; Youngman *et al.*, 1992), which was found to increase life span of rodents by delaying aging (Masoro, 1994).

In the aging process, mitochondria play a crucial role because they are not only the main generators of the primary ROS namely, superoxide anion radical and hydrogen peroxide, but also perhaps the most immediate targets of the oxidative damage inflicted by the ROS (Shigenaga *et al.*, 1994). Age-associated increase in the rate of mitochondrial $O_2^{\bullet-}/H_2O_2$ generation (Sohal and Dubey, 1994), and the amount of oxidative damage to mitochondrial proteins and DNA have been observed to increase in mammalian (Sohal *et al.*, 1994a,b) and insect tissue (Agarwal and Sohal, 1994). Experimentally induced oxidative damage to mitochondria has also been demonstrated to increase their rate of H_2O_2 generation (Sohal and Sohal, 1991). In addition, the rates of mitochondrial $O_2^{\bullet-}/H_2O_2$ generation exhibit an inverse correlation with the life span of different mammalian (Ku *et al.*, 1993) and insect (Sohal *et al.*, 1995) species. A variety of decrements in mitochondrial respiratory activities, including the overall measurement of performance, namely, the maximal rate of ADP-stimulated oxygen consumption, have also been detected during aging (Darnold *et al.*, 1990). Together, results of such studies raise the possibility that mitochondrial damage may play a critical role in the senescence of tissue.

Many mitochondrial functions are also linked directly to the structural integrity of its membranes, which is dependent primarily on the interaction of lipids and proteins within the membranes (Gabbita *et al.*, 1997). The components of the electron transport chain can, under certain abnormal conditions, initiate free-radical-mediated peroxidation of mitochondrial lipid membrane system (Sohal and Dubey, 1994). Rigidization of the membrane with advancing aging has been correlated to the biochemical interaction between the changing phospholipid composition and increasing cholesterol content (Naeim and Walford, 1985). Decreased membrane fluidity is now speculated to be a secondary event to two age-dependent processes: (1) the lipid peroxidation process, and (2) increase in accumulation of lipids with a higher degree of unsaturation more prone to oxidation (Yu *et al.*, 1992). Because 80–90% of cellular oxygen is normally consumed by the activity of the mitochondrial respiratory chain, mitochondria represent the main site of cellular oxygen activation. Although, the mechanism underlying the age-related increases in mitochondrial production of $O_2^{\bullet-}$ and H_2O_2 is unknown, it has been well established that the mitochondrial macromolecules undergo damage by self-generated ROS (Sohal and Dubey, 1994).

On intervention that offsets the age-associated accrual of oxidative injury is CR (Bartke *et al.*, 2001; Sohal and Weindruch, 1996). CR, which involves a reduction in calory intake while maintaining adequate nutrition, preserves the activities of antioxidant enzymes in postmitotic tissues, maintains organ function, opposes the development of spontaneous diseases, and prolongs maximum life span in laboratory rodents (Bartke *et al.*, 2001; Yu *et al.*, 1992). It has been proposed that reductions in ROS production and cellular oxidative injury are central to the positive effects of CR.

The liver plays a key role in mammalian metabolism and is very susceptible to diet variation. For this reason, the effect of CR and of a vitamin E deprived diet on mitochondrial structure and features in the liver of rats during aging was studied in the present investigation, in order to ascertain the extent of modifications induced by these experimental conditions.

MATERIALS AND METHODS

Experimental Protocol

The entire experimental protocol had been approved by the local ethical committee. Female Wistar rats from our breeding colony were used. They were randomly allocated into three groups and subjected to different dietary treatment: ad libitum (AL), caloric restricted (CR), or a vitamin E depleted diet (−Vit. E). Two groups had free access to water and diet (young and old), one group was fed on an every-other-day schedule (CR) from the age of 3.5 months with the same diet given to the control group, and one group was fed a commercially available vitamin E depleted diet beginning at 1 month of age (−Vit. E). As previously reported in detail (Pieri *et al.*, 1990) these treatments were able to prolong or to shorten the mean, median, and maximum life span of the respectively CR and −Vit. E animals. The survival curves of AL, −Vit. E, and CR fed animals in our breeding colony were as previously indicated (Armeni *et al.*, 1998). The chow (Nossan, Italy) contained 41% carbohydrate, 21% protein, and 6% fat. For the CR animals, food was provided in the morning hours and removed the following morning; the animals had free access to water and were kept in threes per cage in plastic cages. The young and old rats were killed when they were 6 and 28 months old. The CR rats were killed at 28 months of age after 24-h feeding. The −Vit. E rats were killed at 24 months of age. Each group consisted of 10 animals.

Chemicals

All chemicals used were purchased from Boehringer Mannheim (GmSH, Germany) and Sigma (St. Louis,

MO) and were of the highest purity available. *S*-D-lactoylglutathione was synthesized and purified as described (Ball and Vander Jagt, 1979). Methylglyoxal was purified by fractional distillation and standardised by endpoint enzymatic assay with glyoxalase I (McLellan *et al.*, 1992).

Preparation of Mitochondrial and Cytosolic Fractions

The animals were killed by cervical dislocation. The purification of mitochondria from rat liver was carried as follows: aliquots of liver tissue (4–5 g) were homogenised [1:10 (w/v)] in ice-cold buffer, pH 7.5, containing 75 mM sucrose, 225 mM mannitol, 1 mM EDTA, 5 mM HEPES, and 0.5 mg/mL fatty-acid free bovine serum albumin. The homogenate was centrifuged for 10 min at 600 *g* at 4°C. Sediment was discarded and supernatant was centrifuged for 20 min at 12,000 *g* at 4°C, obtaining a mitochondrial pellet and a supernatant. This latter was centrifuged for 30 min at 55,000 *g* at 4°C and the supernatant (soluble cytosolic fraction) was divided into 1-mL aliquots. The mitochondrial pellet was washed two times and purified mitochondria were carefully resuspended in 1 mL of ice-cold homogenisation buffer.

Enzymatic Assays

Samples were aliquots of cytosolic and mitochondrial fractions kept frozen at −80°C until use. Mitochondrial pellets were dissolved in buffer containing 0.5% (v/v) Triton X-100. Determinations of enzymatic activities were carried out at a constant temperature of 30 ± 1°C.

Glyoxalase I (GI, EC4.4.1.5) was determined at 240 nm (Ekwall and Mannervik, 1970) using 1.0 mM GSH/methylglyoxal hemithioacetal as substrate in 100 mM sodium phosphate buffer, pH 6.8. The hemithioacetal is generated in situ by preincubation of methyglyoxal with GSH in sodium phosphate buffer at 37°C and this step is essential to avoid condition where the formation of the hemithioacetal is rate limiting (Vander Jagt *et al.*, 1975).

Glyoxalase II (GII, EC3.1.2.6) was determined at 412 nm using 0.9 mM *S*-D-lactoylglutathione as substrate in 100 mM MOPS buffer, pH 7.2, containing 0.2 mM 5,5′-dithio-bis-nitrobenzoic acid (Principato *et al.*, 1987).

Glutathione reductase (GR, EC1.6.4.2) activity was measured by following the oxidation of NADPH at 340 nm during the reduction of GSSG ($\varepsilon = -6.22$ mM^{-1} cm^{-1}) (Ramos-Martinez *et al.*, 1983). Assay conditions were 100 mM Na-phosphate buffer, pH 7.0, 1 mM GSSG, 60 μM NADPH.

Glutathione *S*-transferase (GST, EC2.5.1.18) activity was assayed with 1-chloro-2,4-dinitrobenzene as substrate (Habig and Jakoby, 1981). GST activity was monitored at 340 nm ($\varepsilon = 9.6$ mM^{-1} cm^{-1}) and a final volume of 1 mL contained 100 mM Na-phosphate buffer, pH 6.5, 1 mM CDNB, 1 mM GSH, and 100–150 μL sample or blank.

Glutathione peroxidase (GPx, EC1.11.1.9) activities were assayed in a coupled enzyme system where the formed GSSG is reduced to GSH from excess glutathione reductase (Lawrence and Burk, 1976). The consumption of NADPH was monitored at 340 nm ($\varepsilon = -6.22$ mM^{-1} cm^{-1}) and activities were measured using H_2O_2 as substrate. A final volume of 1 mL contained 100 mM Na-phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM NaN_3, 2 mM GSH, 1 U glutathione reductase, 0.24 mM NADPH, 100–150 sample or blank, and 0.6 mM H_2O_2. The rate of blank reaction was subtracted from the total rate.

Catalase (EC1.11.1.6) activity was measured by the decrease in absorbance at 240 nm due to H_2O_2 consumption ($\varepsilon = 0.04$ mM^{-1} cm^{-1}) (Claiborne, 1985). A final volume of 1 mL contained 100 mM Na-phosphate buffer, pH 7.0, 12 mM H_2O_2, and 100 or 150 μL sample or blank.

Glutamate dehydrogenase (GDH, EC1.4.1.2), activity was assayed spectrophotometrically at 340 nm by monitoring oxidation of NADH ($\varepsilon = -6.22$ mM^{-1} cm^{-1}) (Wrzeszczynski and Colman, 1994). Aspartate aminotransferase (AST, EC2.6.1.1) activity was assayed by the method of Reitman and Frankel (1957).

Protein content was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumine as standard.

Respiratory Chain Activities

Mitochondria were assayed for respiratory chain activities at 25°C in a 25 mM K-phosphate buffer, pH 7.5, under quasi-saturating substrate concentrations in a Beckman DU 640 spectrophotometer, as previously described in detail by Battino *et al.* (1991). Briefly, complex II (succinate–ubiquinone oxidoreductase; EC 1.3.5.1) activity was measured as a rate of reduction of ubiquinone-2 by succinate followed by the secondary reduction of 2,6-dichlorophenolindophenol (DCPIP) by the ubiquinol formed. The decrease in absorbance was measured at 600 nm after the addition of DCPIP, ubiquinone, and the mitochondrial fraction. Complex III (ubiquinol–cytochrome *c* oxidoreductase; EC 1.10.2.2) activity was assayed by monitoring the rate of reduction of cytochrome *c* by ubiquinol-2. The reaction was started by adding of ubiquinol-2 to cytochrome *c* in a Tris-HCl buffer containing potassium cyanide. Reduced cytochrome *c* was

measured as an increase in absorbance at 550 nm. Complex IV (cytochrome *c* oxidase; EC 1.9.3.1) activity was measured as a rate of oxidation of reduced cytochrome *c*. The decrease in reduced cytochrome *c* was monitored at 550 nm.

Cytochrome Contents

Cytochrome contents were evaluated by the differential spectra (dithionite reduced minus ferricyanide oxidized) in the presence of 1% deoxycholate in a Beckman (DU-640) spectrophotometer as previously described (Battino *et al.*, 1991; Quiles *et al.*, 2001).

Coenzyme Q_n (CoQ_n) Assays

CoQ was assayed by HPLC with a method that we successfully developed in the last years (Battino *et al.*, 2001). The HPLC system consisted of a Beckman Model 126 pump, a Rheodyne model 7125 valve fitted with a 20-μL loop, a stainless steel column 15-cm long 4.6-mm i.d. packed with 3-μm ODS Supelcosil from Supelchem, an ESA Coulochem model 5100 A electrochemical detector and a model 5011 Analytical cell. Chromatograms were integrated with Model 4290 Varian integrator. Mobile phase consisted of lithium 20 mM perchlorate, 10 mM perchloric acid, 20% ethanol, 80% methanol; electrode 1 was set at -0.5 V, electrode 2 was set at $+0.35$ V. Retention time $=$ 12 min. Briefly, 50 μL of the sample were precipitated with 150 μL of isopropanol and vortexed for 60 s. After centrifugation at 11200 *g* for 10 min in a bench top centrifuge for eppendorf vials, 20 μL of supernatant was injected into the HPLC.

Fatty Acid Composition

Mitochondrial fatty acid composition was measured by gas liquid chromatography as described by Lepage and Roy (1986). A gas–liquid chromatograph Model HP-5890 Series II (Hewlett Packard, Palo Alto, CA) equipped with a flame ionization detector was used to analyse fatty acids as methyl esters. Chromatography was performed using a 60-m long capillary column; 32-mm i.d. and 20-mm thickness impregnated with Sp 2330™ FS (Supelco Inc., Bellefonte, Palo Alto, CA) as already described (Battino *et al.*, 2000, 2002). Plasma fatty acid patterns were calculated on a percentage basis as well as the relative contents of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and the unsaturation index (UI) that considers all the double bounds existing in 100 fatty acid molecules.

Hydroperoxides (HP)

The ferrous-oxide xylenol orange (FOX2) method was used for determining HP. HP levels were assayed according to the principle of the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions (Jiang *et al.*, 1992) slightly modified (Nourouz-Zadeh *et al.*, 1994) using tryphenylphosphine (TPP), an agent that avoids artifactual color generation in samples that might contain substantial quantities of loosely available iron. Authentic mitochondrial HP can be determined by this strategy in which the HP reductant, TPP, is used to discriminate between the background signal generated by ferric ions present in the sample and the one generated by HP in the sample. Briefly, mitochondria (0.1 mg) were incubated at 37°C for 30 min with and without 1 mM TPP. Then FOX2 reagent was added to each sample and incubated again at 37°C for 30 min in a water shaking bath. After centrifugation (2000 *g* for 5 min) the supernatants were monitored at 560 nm. Moreover, the samples were also challenged, in vitro, by further peroxidative attack in order to elucidate the peroxidation rate constant for each group of mitochondria. This aim was reached by incubating mitochondria at 37°C for 30, 60, 120, 180, and 240 min in presence of 5 mM 2,2′-azobis(amidinopropane hydrochloride) (AAPH) a chemical free radical initiator.

Statistical Analysis

Results are reported as mean $\pm$ standard deviation (SD). The statistical significance between groups was assessed by computer-assisted analysis of variance (ANOVA). The survival curves were estimated using the Kolmogorof–Smirnov 2-sample test (Mode *et al.*, 1984).

RESULTS

The slope of the survival curve versus age was lower for CR animals than for controls and especially when compared to vitamin E deficient rats, the latter displaying the shortest expectancy of life (Fig. 1).

Young control rats showed the highest glutathione reductase (GR), glutathione-*S*-transferase (GST), glutathione peroxidase (GPX), and catalase (CAT) activities (Fig. 2(A)–(D)) and aging provoked a generalized decrease in these activities. Significant differences ($P < 0.01$) were always found in young vs. old rats. In GR, GST,

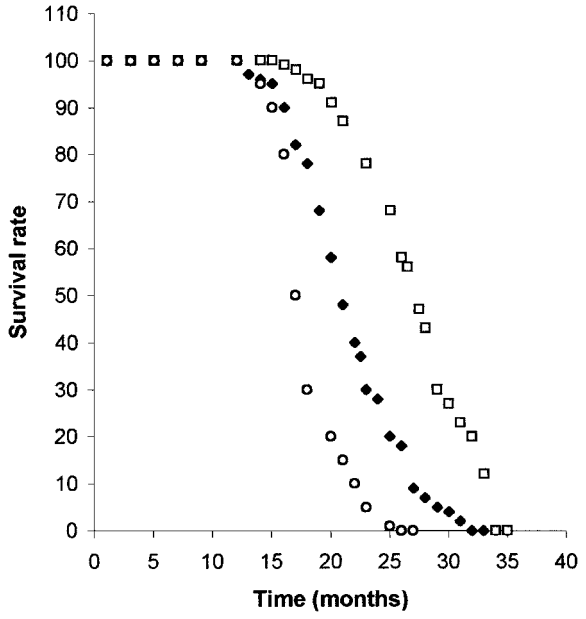

Fig. 1. Survival curves of AL (ad libitum), CR (caloric restricted), and −Vit. E (vitamin E deficient) fed female Wistar rats. Statistical comparison were estimated using the Kolmogorof–Smirnov two-sample test and the difference between the two dietary groups was statistically significant ($P < 0.001$). (○-○) −Vit. E rats, (♦-♦) AL rats and (□-□) CR rats.

and CAT the differences between young and vitamin E deficient rats were also significant, as for GR and GST in CR vs. vitamin E deficient rats. GPX showed generalized significant differences in young vs. all the other aged groups. The results obtained from activity markers of liver status and mitochondrial integrity (i.e., aspartate amino transferase (AST) and mitochondrial glutamate dehydrogenase (GDH), respectively) were more homogeneous (Fig. 3(A) and (B)). In fact, only the assay of AST activity showed higher values for young animals with very few significant differences (i.e., young vs. old), while no statistical relevance was obtained from mitochondrial GDH assay. Also the response of those enzymes differently involved in glutathione use and recycling (glyoxalase I (GI), cytosolic glyoxalase II (GII), and mitochondrial GII) did not show any significant differences (Fig. 4(A)–(C)). Liver mitochondrial Coenzyme Q_9 (CoQ_9) content was higher in old rats than in CR, vitamin E deficient, or young animals (Fig. 5(A)) while these last groups did not present any differences among them. CoQ_{10} content displayed similar levels in old and vitamin E deficient animals and both groups differed significantly from young and CR rats that were similar to each other and lower than the former ones (Fig. 5(B)). The CoQ_9/CoQ_{10} molar ratio partially reflected this behavior (Fig. 5(C)) with old and vitamin E deficient groups characterized by lower values than young

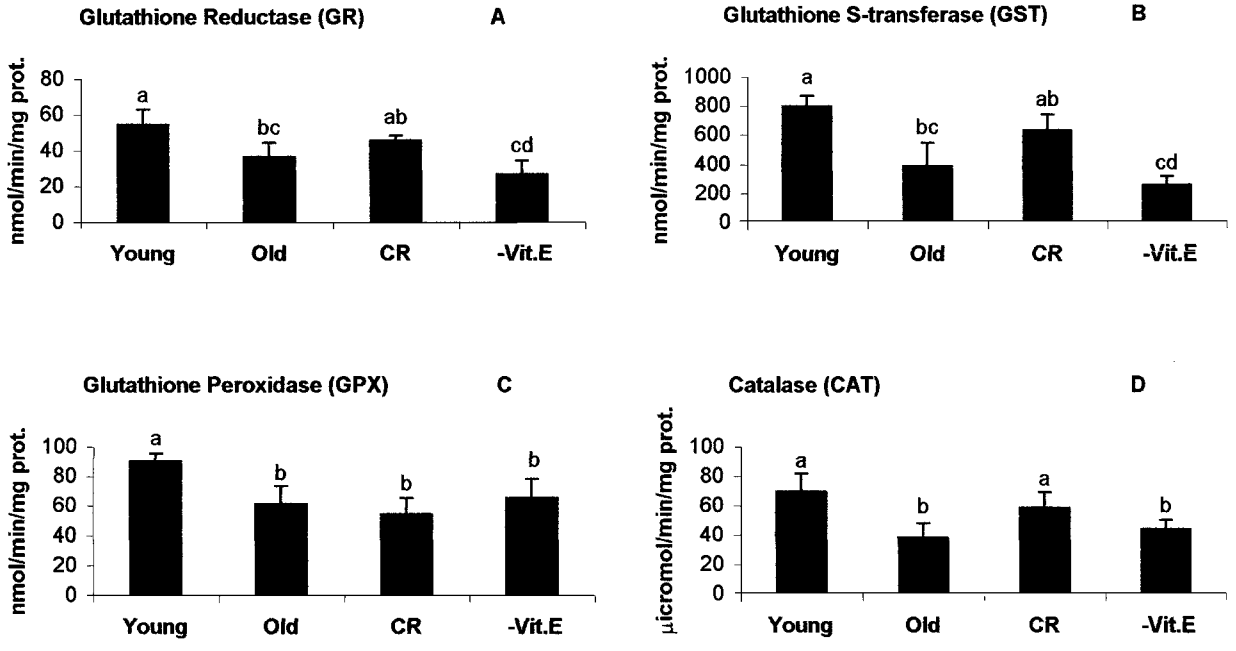

Fig. 2. Levels of Glutathione reductase (GR), Glutathione *S*-transferase (GST), Glutathione Peroxidase (GPx), and Catalase (CAT) activities in the rat liver cytosolic fraction of Young, Old, CR, or −Vit. E rats. Glutathione dependent enzymes are expressed as nmol min^{-1} mg $protein^{-1}$ and catalase activity is expressed as μmol min^{-1} mg $protein^{-1}$. Results are mean values ± SD of $n \geq 6$. The same letter on the top of two or more bars means no statistically significant differences between the corresponding values (ANOVA, $P < 0.01$).

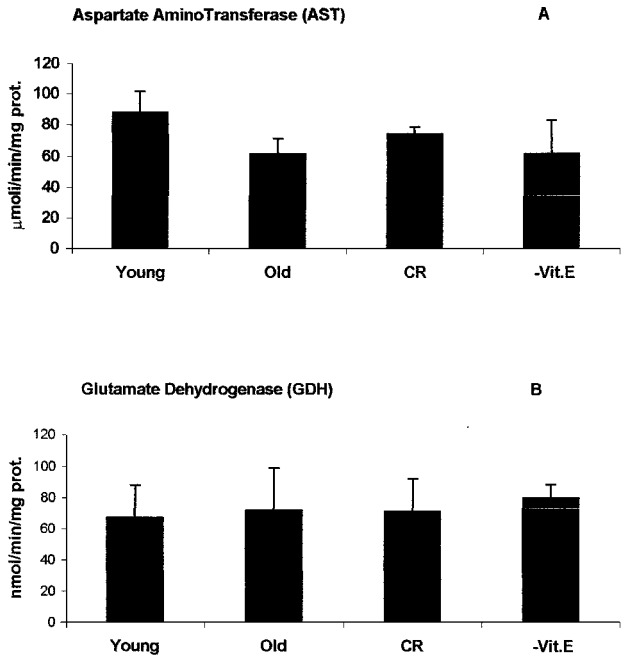

Fig. 3. Levels of cytosolic Aspartate aminotransferase (AST) and mitochondrial Glutamate dehydrogenase (GDH), activities in the rat liver of Young, Old, CR, or −Vit. E rats. The enzymes are expressed as μmol min^{-1} mg $protein^{-1}$. Results are mean values ± SD of $n \geq 6$. The same letter on the top of two or more bars means no statistically significant differences between the corresponding values (ANOVA, $P < 0.01$).

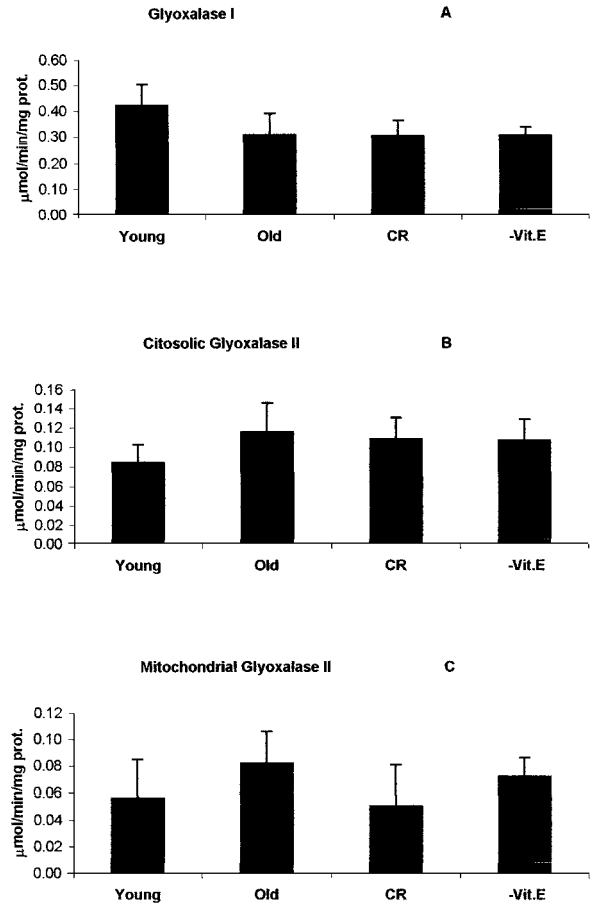

Fig. 4. Levels of cytosolic Glyoxalase I (GI), cytosolic Glyoxalase II (GII), and mitochondrial Glyoxalase II activities in the rat liver of Young, Old, CR, or −Vit. E rats. The enzymes are expressed as μmol min^{-1} mg $protein^{-1}$. Results are mean values ± SD of $n \geq 6$. The same letter on the top of two or more bars means no statistically significant differences between the corresponding values (ANOVA, $P < 0.01$).

and CR groups. Basal (i.e., native) hydroperoxide (HP) contents were the highest in vitamin E deficient group and progressively lower in old, CR, and young animals, the latter possessing the lowest HP presence (Fig. 5(D)). If the formation of HP was elicited by challenging the mitochondrial membranes with 5 mM AAPH (Fig. 6), the increase in HP content was similar in young, vitamin E deficient and CR rats and it was quite constant along the experimental time (240 min) with calculated peroxidation rate constants of 0.46, 0.48, and 0.51 nmol of HP per minute, respectively. The slope completely changed in the case of old rats that had a notably higher peroxidation rate constant of 0.81 nmol of HP per minute. The high peroxidizability of mitochondria from old rats is accompanied by the highest PUFA content ($P < 0.01$) and UI value held by these organelles (Table I). The MUFA:PUFA ratio was unaffected and PUFA enrichment took place at the expense of SFA, that had the lowest value in the old group. MUFA were highest in vitamin E deficient animals which, at the same time, displayed the lowest PUFA content and, consequently the highest MUFA:PUFA ratio. However, vitamin E deficient rats, together with old rats, had very high PUFA(ω-3) contents. The lowest UI values were possessed by young and CR groups with 4.04 and 4.19, respectively, which differed significantly from the remaining two groups.

Finally, in Table II some structural and functional mitochondrial features, other than CoQ_n and HP contents, have been collected. Neither cytochrome $c + c_1$ nor cytochrome b differed among groups and only cytochrome $a + a_3$ levels were significantly higher in old and vitamin E deficient animals with respect to young ones ($P < 0.01$). A similar situation occurred in the case of cytochrome oxidase activity with significantly higher activities in old and vitamin E deficient animals rather than in young and CR ones. The contrary was found in the case of complex III (CoQ_2H_2–cytochrome c) activity where the significantly highest values were possessed by young and CR rats. No significant differences were found in the succinate–ubiquinone oxidoreductase activity.

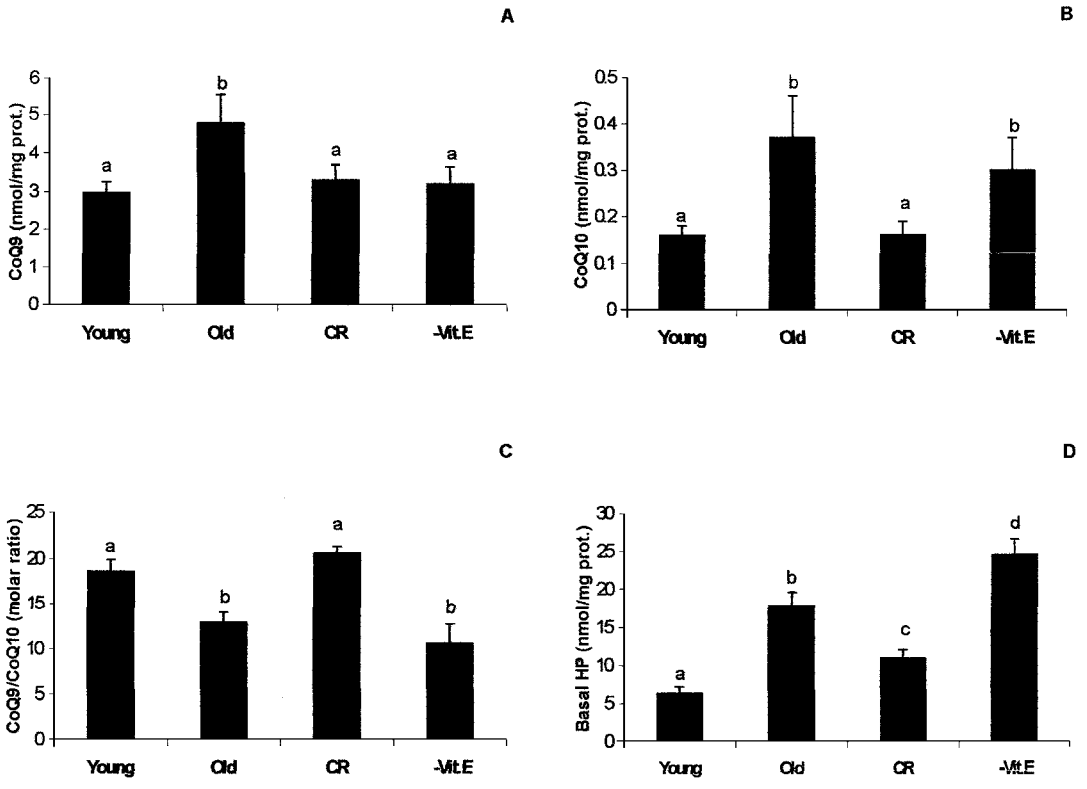

Fig. 5. Levels of mitochondrial coenzyme Q9 (CoQ_9), CoQ_{10}, CoQ_9/CoQ_{10} molar ratio, and basal HP in the rat liver of Young, Old, CR, or −Vit. E rats. The concentrations of both CoQ_n and HP are expressed as nmol mg protein^{-1}. Results are mean values ± SD of $n \geq 6$. The same letter on the top of two or more bars means no statistically significant differences between the corresponding values (ANOVA, $P < 0.01$).

DISCUSSION

The nutrition of mature and aged subjects, mainly in industrialized countries, is often inadequate (because of deficiency or excess), which may lead to premature or pathological senescence. Recent nutritional research on ageing laboratory animals shows that CR may be the most effective procedure to achieve a long and disease-free life span, probably owing to a better protection against mitochondria-linked oxygen stress (Miquel, 2001).

As a paradigm of the life span process, CR possesses the advantage of being easily implemented and its results are rapidly reproduced (Yu, 1996). The hypothesis that CR affects life span through a modification of the metabolism prevails within this paradigm. An important support for the proposition that CR retards aging by slowing oxidative injury of mitochondria comes from Sohal and his group. These investigators observed markedly lower levels of superoxide anion and hydrogen peroxide radicals in mitochondria obtained from different tissues of mice subjected to long-term CR than in normally fed controls (Sohal *et al.*, 1994a,b; Sohal and Weindruch, 1996). Recently, also Barja and coworkers clearly demonstrated that CR decreases H_2O_2 generation and oxidative damage to mitochondrial DNA in rat heart (Gredilla *et al.*, 2001a) and liver (Gredilla *et al.*, 2001b; Lopez-Torres *et al.*, 2002) mitochondria.

CR was effective also in our animals: in fact, the effects on rat survival plot (collected in Fig. 1) of the different diets employed confirmed previous investigations about the increase of CR-induced life span. It is noteworthy that vitamin E deficient animals never reached the average age of control ones. This is the reason why the age at which vitamin E deficient rats had been investigated was lower than for controls and CR animals.

The antioxidant enzyme activities assayed revealed that young control animals always possessed the highest activities although the differences were not always significant. Moreover, a common trend was displayed by all antioxidant enzymes: a decrease in the activities at older ages, the characteristic of CR in limiting this deficiency and in two out of four enzymes (namely glutathione reductase and glutathione-*S*-transferase) the even more negative effect produced by the dietary absence of vitamin

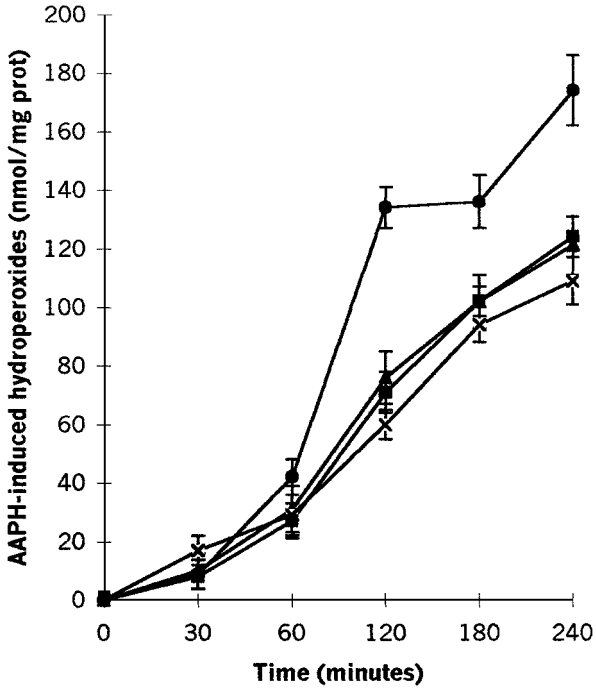

Fig. 6. Mitochondrial rat liver hydroperoxide formation in presence of AAPH 5 mM. See Materials and Methods section for more details. (●-●) Old rats, CR rats (■-■), Young rats (▲-▲), −Vit. E rats (**x**-**x**).

E. On the other hand, the chronic absence of vitamin E did not affect either the liver status (checked as AST activity) or the mitochondrial integrity (through mitochondrial GDH activity) indicating that the influence of vitamin E deficiency is much more subtle and, if exists, its effects lie at a molecular level. Moreover, it seems that neither ageing nor vitamin E deficiency could have any influence on those enzymes that are involved in glutathione use and recycling both at cytosolic and mitochondrial level (i.e., the group of glyoxylase enzymes). These data only apparently contrast with the data regarding respiratory chain enzymes (see below) that appeared to be affected both in their activities and sometimes even quantitatively. In fact, it has already been found that CR effects are not unique, either concerning their localization or concerning their extent, for example, in old rats CR was found to reduce carbonylated proteins only in liver mitochondria but not in skeletal muscle ones or in liver cytosol (Goto *et al.*, 2002) suggesting and supporting that the site of ROS production is a key element in the investigation of ageing-related modifications or dysfunctions.

Specific attention should be devoted to the data about structural and functional features of the mitochondrial respiratory chain. In the last decade we widely discussed the influence of dietary fats (Barzanti *et al.*, 1994; Battino *et al.*, 2002; Mataix *et al.*, 1998; Quiles *et al.*, 1999a, 2001), aging (Battino *et al.*, 1995, 2001), physical exercise (Mataix *et al.*, 1998; Quiles *et al.*, 1999a,b, 2001) and xenobiotics (Huertas *et al.*, 1991a,b, 1992) in modulating the susceptibility of this structure towards oxidative-stress-induced injures. CoQ, because of its pivotal role as both an antioxidant and an essential component of the electron transport chain, is often considered a marker of mitochondrial welfare (Ebadi *et al.*, 2001). Its fluctuations during aging and in some conditions involving oxidative stress have been identified (Battino *et al.*, 1995, 1999); they are bound to affect the respiratory chain activity, but they may also affect its antioxidant efficacy (Kagan and Quinn, 2001). Moreover, the total CoQ content in rodents is peculiar because it depends on the relative amounts of CoQ_9 and CoQ_{10} that are differently distributed in the tissues (Battino, 2001). Several authors found that the amount of CoQ does not decline during aging (Battino *et al.*, 1995; Beyer *et al.*, 1985; Lass *et al.*, 1999) and an increase in the amount of CoQ_9 in skeletal muscle during CR has also been found (Lass *et al.*, 1999). In

Table I. Parameters Obtained From Liver Mitochondrial Fatty Acid Patterns (Data Are Expressed in Percentage of Total Fatty Acids) (Data Are Means ± SD; $n > 6$)

	Young, a	Old, b	CR, c	−Vit. E, d	Statistical analysis ($P < 0.01$)
SFA	47.4 ± 3.1	38.9 ± 2.1	45.9 ± 1.6	47.9 ± 0.9	b vs. a,c,d
UFA	52.6 ± 2.1	61.0 ± 2.9	54.1 ± 1.6	52.1 ± 0.9	b vs. a,c,d
MUFA	8.5 ± 0.6	10.2 ± 1.2	11.4 ± 0.9	15.6 ± 1.4	d vs. a,b,c
PUFA	44.0 ± 2.8	50.8 ± 2.9	42.6 ± 2.5	36.5 ± 2.1	d vs. a,b,c/b vs. a,c
MUFA:PUFA	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	d vs. a,b,c
PUFA (n − 6)	37.2 ± 1.9	36.6 ± 2.2	35.9 ± 1.6	25.3 ± 0.6	d vs. a,b,c
PUFA (n − 3)	3.2 ± 1.5	9.8 ± 0.8	4.7 ± 0.8	8.2 ± 1.4	a vs. b,d/c vs. b,d
UI	4.0 ± 0.12	5.3 ± 0.2	4.2 ± 0.2	4.8 ± 0.2	a vs. b,d/c vs b,d

Note. See text for details about the abbreviated words.

Table II. Liver Mitochondrial Cytochrome Contents (Expressed in nmol/mg Protein) and Respiratory Chain Activities (Expressed in μmol/min/mg Protein) in Rats Underwent Different Dietary Treatment (Data Are Means ± SD; $n > 6$)

	Young, a	Old, b	CR, c	−Vit. E, d	Statistical analysis ($P < 0.01$)
Cytochrome $c + c_1$	0.21 ± 0.02	0.28 ± 0.03	0.22 ± 0.00	0.29 ± 0.02	—
Cytochrome b	0.09 ± 0.01	0.13 ± 0.00	0.10 ± 0.00	0.14 ± 0.00	—
Cytochrome $a + a_3$	0.12 ± 0.00	0.24 ± 0.00	0.16 ± 0.03	0.26 ± 0.04	a vs. b,d
Succinate–DCPIP	0.23 ± 0.04	0.16 ± 0.03	0.19 ± 0.02	0.15 ± 0.04	—
Ubiquinol$_2$–cytochrome c	2.25 ± 0.12	1.68 ± 0.11	1.92 ± 0.11	1.56 ± 0.13	a vs. b,d/c vs. b,d
Cytochrome oxidase	2.05 ± 0.19	2.89 ± 0.21	2.35 ± 0.18	3.08 ± 0.26	a vs. b,d/c vs. b,d

Note. See text for details about the abbreviated words.

the last investigation we obtained confirmatory data that indicate a significant CoQ increment in aged rats for both CoQ homologs. CoQ in CR rats was not increased and this fact deserves specific comments: (i) these are the first available data regarding liver of CR rats, (ii) they agree with the only other existing data, regarding skeletal muscle (Lass *et al.*, 1999), where despite a temporary CoQ increase after 15 months, the CoQ levels in aged rat were similar for control and CR animals, and (iii) the actual amount of both CoQ_9 and CoQ_{10} are the same in young and old CR rats. The CoQ increase during aging can be easily explained through a mechanism that would depend on the side effects produced by the accumulation of oxidative injuries that characterize aging, as already discussed (Battino *et al.*, 1995, 2000). Briefly, while high levels of oxidative stress lead to deep modifications and damage, the effects of constant low levels of oxidative stress (i.e., as in the case of aging) would be paradoxically stimulatory (Burdon *et al.*, 1989). Thus, the dietary absence of vitamin E, which does not dramatically affect the overall antioxidant status of the cell (e.g., see above), would act as an inductory stimulus in the case of CoQ_{10} balance. The CoQ_9/CoQ_{10} molar ratios on one hand confirm the usual values typical for liver mitochondria and on the other reflect the differences emerged with CoQ_{10} contents.

As far as the role played by vitamin E in the stability of mitochondrial membranes is concerned, another proof that the vit. E deficient diet is able to only partially affect the mitochondrial structure is given also by both native HP contents and the peroxidation rate constant of mitochondria challenged in vitro with a free-radical initiator. Native HP in liver mitochondria were the highest in vitamin E deficient rats but were very high also in aged rats. CR treatment was successful in limiting oxidative damage in the lipid domain although HP was twofold higher than the amounts found in the young control animals. However, this was only an index of the accumulated damage at membrane level during the animal life span and it is worthwhile to also consider the membrane potentiality to be peroxidized (i.e., the different susceptibility to undergo peroxidation). The constant rate of HP formation following an AAPH-mediated insult was similar for young, vitamin E deficient, and CR rats but was sharply higher in old ones. A structural feature that can explain the last data was found in the mitochondrial fatty acid profile. Old rats possessed the highest PUFA content, the lowest SFA content and the second lowest MUFA level. This situation is perfectly expressed in these rats by the highest UI value, an index indicating the very high proneness to suffer oxidative injuries of these mitochondria. Venkatraman and Fernandes (1992) demonstrated that CR produces an increase of 18:2, while suppresses 20:4 and 22:4 levels in rat spleen, protecting the membranes from oxidative stress. We found similar results (data not shown) and they also indicate (Table I) that the UI value of CR rats was similar to that of young rats, far below the values of old and vitamin E deficient, animals. In the latter, which membranes are vitamin E impoverished, it is also interesting to note the enhanced amount of MUFA (more than 50% of control value) that is probably due to an attempt of making such membranes less susceptible to oxidative insult. Such kind of rearrangement at membrane levels capable of partially buffering the effects of oxidative stress has already been reported (Mataix *et al.*, 1998).

Respiratory chain activities gave contrasting indications: on one side a decrease in complex III activity for old and vitamin E deficient animals, on another side an increase in complex IV activity in the same animals (accompanied by enhanced cytochrome $a + a_3$ content) and finally the lack of significative variations in complex II activity. An accurate analysis of the data revealed that the age-associated reduction in the V_{max} for CoQ_2H_2–cytochrome c activity in old rats confirms already existing data (Desai *et al.*, 1996; Feuers, 1998). Confirmation of previous data occurred also for CR rats that were able to maintain a complex III activity significantly similar to young rats. The new comparison is with the animals lacking vitamin E in their diet that displayed the lowest

complex III activity. By contrast, complex II did not show significant changes in any of the groups investigated according with other investigations (Feuers, 1998). All these results should be interpreted in the light of cytochrome contents that in the case of both cytochrome *b* and cytochrome $c + c_1$ did not vary significantly, suggesting that the values observed for the above mentioned enzymes did not depend on possible fluctuations in the total amount of the same enzymes but only on their actual catalytic properties. As far as cytochrome oxidase activity is concerned, significantly higher activities in old and vitamin E deficient rats were found together with higher amounts of cytochrome $a + a_3$ in these groups. These findings mean that the reaction rate of the oxidase, as defined by its turnover numbers (TN, in per second) did not increase at all but, on the contrary, young and CR groups held the highest TN values (17 and 14.7, respectively) and old and vitamin E deficient groups the lowest ones (12 and 11.8, respectively). These data agree with both the authors who identified a decrease of complex IV activity during aging and opposing behavior in the CR group (Desai *et al.*, 1996; Feuers, 1998) and with previous indications that suggested possible induction phenomena in cytochrome oxidase synthesis during oxidative stress events (Asson-Batres and Hare, 1991; Huertas *et al.*, 1991b; Quiles *et al.*, 2001). As already hypothised by Feuers (1998) who was also able to identify critical alterations in the number of high affinity binding sites per complex IV, these data suggest that there could be an obstruction of electron flow through some respiratory complexes (namely complex III and complex IV). This would build something like a dam against electron flow. It has been suggested (Feuers, 1998) that due to these problems, a greater number of free radicals could be generated at these sites along the electron transfer chain. One can envision that as oxidative lesions accumulate and the chemical–physical structures of mitochondrial membrane change (e.g., the enhanced HP level could be a marker of the situation), a mechanism exists where a single electron is passed, and due to poor binding, a premature free-radical product would be released prior to the second electron passage. CR seems to partially resolve some of these qualitative and quantitative aging problems associated with respiratory complexes and this may be one mechanism through which CR limits free-radical generation, leading to an extension of maximum achievable life span.

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