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Dietary Restriction at Old Age Lowers Mitochondrial Oxygen Radical Production and Leak at Complex I and Oxidative DNA Damage in Rat Brain

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Previous studies in mammalian models indicate that the rate of mitochondrial reactive oxygen species ROS production and the ensuing modification of mitochondrial DNA (mtDNA) link oxidative stress to aging rate. However, there is scarce information concerning this in relation to caloric restriction (CR) in the brain, an organ of maximum relevance for ageing. Furthermore, it has never been studied if CR started late in life can improve those oxidative stress-related parameters. In this investigation, rats were subjected during 1 year to 40% CR starting at 24 months of age. This protocol of CR significantly decreased the rate of mitochondrial H_2O_2 production (by 24%) and oxidative damage to mtDNA (by 23%) in the brain below the level of both old and young ad libitum-fed animals. In agreement with the progressive character of aging, the rate of H_2O_2 production of brain mitochondria stayed constant with age. Oxidative damage to nuclear DNA increased with age and this increase was fully reversed by CR to the level of the young controls. The decrease in ROS production induced by CR was localized at Complex I and occurred without changes in oxygen consumption. Instead, the efficiency of brain mitochondria to avoid electron leak to oxygen at Complex I was increased by CR. The mechanism involved in that increase in efficiency was related to the degree of electronic reduction of the Complex I generator. The results agree with the idea that CR decreases aging rate in part by lowering the rate of free radical generation of mitochondria in the brain.

KEY WORDS: Caloric restriction; brain mitochondria; free radical generation; aging; old age; oxygen radicals; Complex I; oxidative DNA damage; 8-hydroxydeoxyguanosine; mitochondrial DNA.

INTRODUCTION

Caloric restriction (CR) is the only known experimental manipulation that decreases the rate of aging and increases maximum life span in mammals. The beneficial effects of CR have been demonstrated in the brain, an organ of maximum relevance for the aging process. In laboratory rodents CR opposes the development of age-associated deficits in psychomotor and spatial memory tasks (Ingram *et al.*, 1987) and dendritic spine loss (Moroi-Fetters, 1989), bolsters neuroprotective mechanisms (Mattson *et al.*, 2002), reduces neuronal damage improving behavioral outcome (Mattson, 2003), and retards age-related motoneuronal death (Kanda, 2002). However, the molecular mechanisms responsible for these protective effects is still largely unknown.

Many lines of evidence currently support the mitochondrial free radical theory of aging (Beckman and Ames, 1998; Edwards *et al.*, 2003; Barja, 2004a; Sanz *et al.*, 2005). The mitochondrial rate of reactive oxygen species (ROS) generation is a key trait connecting aging

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Key to abbreviations: dG, deoxyguanosine; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; 8-oxodG, 8-oxo,7,8-dihydro-2′-deoxyguanosine; ROS, reactive oxygen species.

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and oxidative stress. Both long-lived animal species and caloric restricted animals have low rates of mitochondrial ROS production and low steady-state levels of oxidative damage in mitochondrial DNA (mtDNA). These traits can be responsible for their slower rate of accumulation of mtDNA mutations and lower aging rate (Barja, 2004a). Detailed studies concerning the effect of CR on mitochondrial ROS production as well as its localization and the mechanisms involved have been performed in liver (López-Torres *et al.*, 2002), a tissue containing cells with mitotic capacity. However, no similar studies have been performed in the brain. Furthermore, although some studies concerning the effects of long-term CR on oxidative damage to brain nuclear DNA (nDNA) are available (Hamilton *et al.*, 2001) information concerning brain mtDNA is lacking.

On the other hand the age at which CR is started can be also important. Some investigations have recently raised the question that the beneficial effects of CR in rodents could occur only if CR is started early in life (Forster *et al.*, 2003). According to these results, implementation of CR in animals of advanced age could give no benefits or would be even harmful. In contrast, other studies indicate that CR beyond middle age is still beneficial (Takahashi and Goto, 2002). This problem has not been studied concerning mitochondrial ROS production and oxidative damage to mtDNA.

This investigation was designed to clarify if CR decreases the rate of ROS generation of functional rat brain mitochondria, to localize the site in the respiratory chain where such putative decrease occurs and to study the mechanisms responsible for it. It was also studied whether such decrease is associated or not with a lowering of oxidative damage in mtDNA and nDNA. CR was started in old animals to clarify if these protective changes can occur during the last segment of the animal life span. The study design allowed to simultaneously investigate the effects of CR and aging on the parameters measured.

MATERIALS AND METHODS

Animals and Design

The animals (male Wistar rats) were caged individually and maintained in a 12:12 (light:dark) cycle at $22 \pm 2^{\circ}$C and $55 \pm 10\%$ relative humidity. They were fed a standard rodent diet (B&K, Humberside, UK). The CR rats daily received 60% of the intake of the ad libitum-fed companion control animals of the same age (40% energy restriction). The dietary restriction experiment was initiated at 24 months of age and was continued during 1 year until sacrifice. At that time two groups of animals of 36 months of age were obtained: OC (old controls fed ad libitum) and OR (old restricted). An additional group of ad libitum-fed animals was maintained in parallel under the same dietary conditions than OC animals during the last months of the long-term dietary restriction experiment. These animals had 6 months of age at time of sacrifice, were classified as (young controls YC) and were used for aging-related comparisons between YC and OC animals. Thus, three groups of animals were available: mature adult young controls (YC), old controls (OC), and old restricted (OR). All the animals were killed at the laboratory by decapitation. Whole brains were sagitally divided in two equal parts. Those used to isolate functional mitochondria were immediately processed from fresh tissue while the rest were quickly frozen in liquid nitrogen and stored at -80°C for the assays of oxidative damage to DNAs.

Preparation and Purification of Functional Brain Mitochondria

The brains were rinsed several times, chopped, and manually homogenized with a loose fitting pestle in 17.5 mL of isolation medium (250 mM sucrose, 0.5 mM K^+-EDTA, 10 mM Tris–HCl, pH 7.4). The homogenates were centrifuged at 2,000 $\times g$ for 3 min and this centrifugation protocol was repeated in the first supernatant. The second supernatant was centrifuged at 12,500 $\times g$ for 8 min to obtain the crude mitochondrial pellet. Non-synaptic free mitochondria were obtained from this pellet by centrifugation in Ficoll gradients according to the procedure of Lai and Clark (1979).

Mitochondrial H_2O_2 Production

Just after their isolation the rate of H_2O_2 production of brain mitochondria was assayed by measuring the increase in fluorescence (excitation at 312 nm, emission at 420 nm) due to oxidation of homovanillic acid by H_2O_2 in the presence of horseradish peroxidase (Barja, 2002). Reaction conditions were 0.39 mg of mitochondrial protein per milliliter, 6 U/mL of horseradish peroxidase, 0.1 mM homovanillic acid, 50 U/mL of superoxide dismutase, and 5 mM pyruvate/2.5 mM malate or 10 mM succinate + 2 μM rotenone as substrates, added at the end to start the reaction to the incubation buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% albumin, pH 7.4) at 37°C, in a total volume of 1.5 mL. All the assays with succinate as substrate were performed in the presence of rotenone in order to avoid the backwards flow of electrons to Complex I. In some experiments rotenone (2 μM) or antimycin A (2 μM) were

additionally included in the reaction mixture to assay maximum rates of Complex I (pyr/mal + rotenone) or Complex III (succinate + antimycin A) H_2O_2 generation. Duplicated samples were incubated for 15 min at 37°C, the reaction was stopped transferring the samples to a cold bath and adding 0.5 mL of 2.2 M glycine-NaOH containing 50 mM EDTA, and the fluorescence was read in a LS50B Perkin-Elmer fluorometer. Known amounts of H_2O_2 generated in parallel by glucose oxidase with glucose as substrate were used as standards. Since the SOD added in excess converts all $O_2^{\cdot-}$ produced (if any) to H_2O_2, the measurements represent the total ($O_2^{\cdot-}$ plus H_2O_2) rate of mitochondrial ROS production.

Mitochondrial Oxygen Consumption

The oxygen consumption of brain mitochondria was measured at 37°C in a water-thermostatized incubation chamber with a computer-controlled Clark-type O_2 electrode (Oxygraph, Hansatech, UK) in 0.5 mL of the same incubation buffer used for H_2O_2 measurements. The substrates used were Complex I-linked (5 mM pyruvate/2.5 mM malate) or Complex II-linked (10 mM succinate + 2 μM rotenone). The assays were performed in the absence (State 4-resting) and in the presence (State 3-phosphorylating) of 500 μM ADP.

Mitochondrial Free Radical Leak

H_2O_2 production and O_2 consumption were measured in parallel in the same brain mitochondria under similar experimental conditions. This allowed the calculation of the fraction of electrons out of sequence which reduce O_2 to ROS at the respiratory chain (the percent free radical leak) instead of reaching cytochrome oxidase to reduce O_2 to water. Since two electrons are needed to reduce 1 mole of O_2 to H_2O_2 whereas four electrons are transferred in the reduction of 1 mole of O_2 to water, the percent free radical leak was calculated as the rate of H_2O_2 production divided by two times the rate of O_2 consumption, and the result was multiplied by 100.

Isolation and Digestion of nDNA and mtDNA

Brain nuclear DNA (nDNA) was isolated after homogenization, centrifugation at 1,000 $\times g$ for 10 min, resuspension of nuclear pellets, and SDS treatment, by chloroform extraction and ethanol precipitation following the method of Loft and Poulsen (1999) except that the initial homogenization buffer contained 5 mM EDTA. Brain mitochondrial DNA (mtDNA) was isolated by the method of Latorre *et al.* (1986) adapted to mammals (Asunción *et al.*, 1996). The isolated nuclear and mitochondrial DNAs were digested to deoxynucleoside level by incubation at 37°C with 5 U of nuclease P1 (in 20 μL of 20 mM sodium acetate, 10 mM $ZnCl_2$, 15% glycerol, pH 4.8) for 30 min and 1 U of alkaline phosphatase (in 20 μL of 1 M Tris–HCl, pH 8.0) for 1 h (Loft and Poulsen, 1999). All aqueous solutions used for nDNA and mtDNA isolation, digestion and chromatographic separation were prepared in HPLC-grade water.

Assay of 8-oxodG by High Performance Liquid Chromatography

Oxidative damage to brain nDNA and mtDNA was estimated by measuring the level of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). 8-oxodG and deoxyguanosine (dG) were analysed by HPLC with on line electrochemical and ultraviolet detection respectively. The nucleoside mixture was injected into a reverse-phase Beckman Ultrasphere ODS column (5 μm, 4.6 mm $\times$ 25 cm), and was eluted with a mobile phase containing 2.5% acetonitrile and 50 mM phosphate buffer pH 5.0. A Waters 510 pump at 1 mL/min was used. 8-oxodG was detected with an ESA Coulochem II electrochemical coulometric detector (ESA, Inc. Bedford, MA) with a 5011 analytical cell run in the oxidative mode (225 mV/20 nA), and dG was detected with a Biorad model 1806 UV detector at 254 nm. For quantification peak areas of dG standards and of three level calibration pure 8-oxodG standards (Sigma) were analyzed during each HPLC run. Comparison of areas of 8-oxodG standards injected with and without simultaneous injection of dG standards ensured that no oxidation of dG occurred during the HPLC run.

Statistical Analyses

Comparisons between ad libitum and caloric restricted animals and between young and old ad libitum-fed animals were statistically analyzed with Student's *t*-tests. The minimum level of statistical significance was set at $p < 0.05$ in all the analyses.

RESULTS

One year of caloric restriction initiated at 24 months of age significantly decreased (by 24%) the rate of H_2O_2 production of rat brain mitochondria respiring with

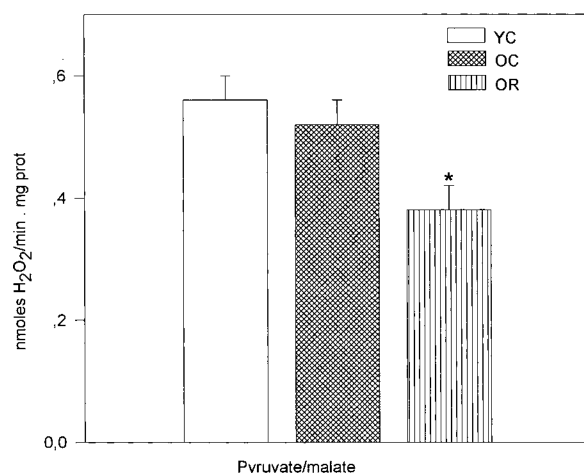

Fig. 1. Effect of age and caloric restriction on rates of H_2O_2 production of rat brain mitochondria with pyruvate/malate as substrates. YC: young controls (6 months of age); OC: old controls (36 months of age); OR: old restricted (36 months of age). Values are means ± SE from 10 (YC and OC) or 9 (OR) different animals. The asterisk (*) denotes significant difference from OC ($p < 0.021$).

pyruvate/malate in relation to that of old ad-libitum fed control animals (Fig. 1). However, when the same mitochondria were supplemented with succinate (+rotenone) as substrate, no significant differences between old caloric restricted and old control animals were observed (Fig. 2). No significant differences in H_2O_2 production between young and old control animals were found with either substrate (Figs. 1 and 2). Maximum rates of ROS generation were assayed in the presence of Complex I-(rotenone) and Complex III-(antimycin A) specific inhibitors of the respiratory chain. H_2O_2 production with succinate plus antimycin A was significantly lower in caloric restricted old rats than in old control animals ($p < 0.027$; Table I). In the presence of pyruvate/malate plus rotenone no significant differences were detected ($p < 0.053$; Table I).

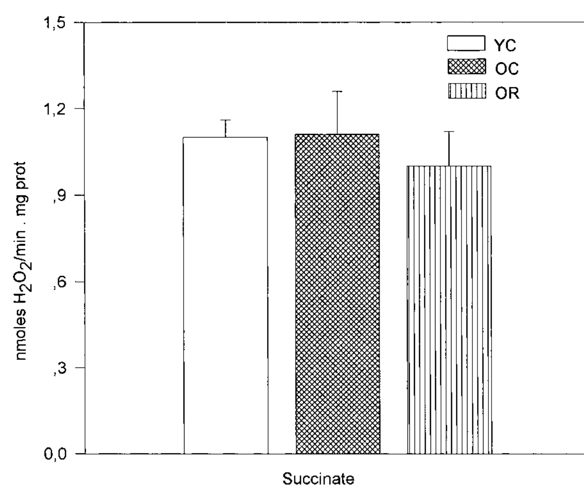

Fig. 2. Effect of age and caloric restriction on rates of H_2O_2 production of rat brain mitochondria with succinate (+rotenone) as substrate. YC: young controls; OC: old controls; OR: old restricted. Values are means ± SE from nine (YC and OC) or eight (OR) different animals.

Table I. Effect of Age and Caloric Restriction on Maximum Rates of H_2O_2 Production (nanomoles H_2O_2/min per mg protein) of Rat Brain Mitochondria in the Presence of Substrates Plus Complex I and III Specific Inhibitors.

	YC	OC	OR
Pyr/mal + ROT	3.23 ± 0.21 (7)	3.16 ± 0.30 (8)	2.38 ± 0.33 (7)
Succinate + AA	6.19 ± 0.47 (6)	6.17 ± 0.67 (7)	4.43 ± 0.37* (6)

Note. Values are means ± SE from the number of different animals shown in parenthesis. YC: young controls; OC: old controls; OR: old restricted. Pyr/mal: pyruvate/malate. ROT: rotenone; AA: antimycin A. *Significantly different from OC ($p < 0.027$).

The rate of oxygen consumption was measured in brain mitochondria with pyruvate/malate and succinate as substrates in the absence (state 4, resting) and in the presence (state 3, active) of ADP. No significant differences in oxygen consumption were found between groups in any of the comparisons with different substrates, states, ages and diets (Table II).

The percent electron flow reducing oxygen to ROS in relation to that reducing O_2 to water at cytochrome oxidase (the free radical leak) was also measured. The mitochondrial free radical leak was similar in young and old control animals with any substrate. The free radical leak with pyruvate/malate of old restricted animals was significantly lower (by 32%) than that of old control animals (Fig. 3). However, the free radical leak with succinate (+rotenone) did not show significant differences between old control (3.3 ± 0.5) and old restricted (3.0 ± 0.4) animals.

Table II. Effect of Age and Caloric Restriction on State 4 and 3 Oxygen Consumption (nanomoles O_2/min per mg protein) of Rat Brain Mitochondria in the Presence of Different Substrates.

	YC	OC	OR
Pyr/mal (state 4)	9.4 ± 0.8 (10)	8.3 ± 0.8 (10)	9.6 ± 1.1 (9)
Succ (state 4)	20.4 ± 2.4 (10)	19.4 ± 2.4 (10)	18.3 ± 1.8 (9)
Pyr/mal (state 3)	38.5 ± 2.6 (10)	34.6 ± 3.3 (10)	35.7 ± 2.6 (9)
Succ (state 3)	58.2 ± 5.5 (10)	49.5 ± 3.8 (10)	44.9 ± 4.8 (9)

Note. Values are means ± SE from the number of different animals shown in parenthesis. YC: young controls; OC: old controls; OR: old restricted. Pyr/mal: pyruvate/malate. Succ: succinate. No significant differences between groups were observed.

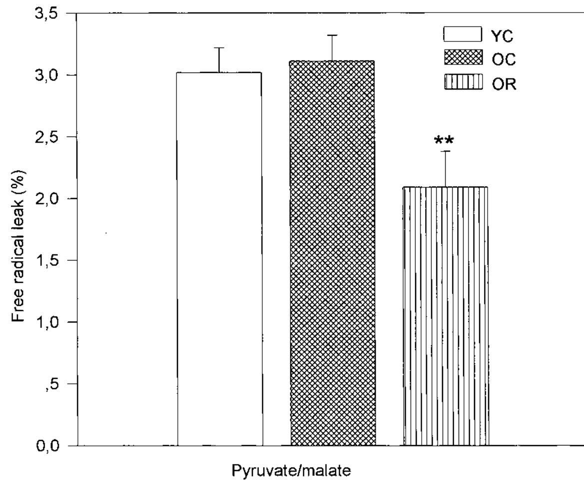

Fig. 3. Effect of age and caloric restriction on the free radical leak (FRL) of rat brain mitochondria with pyruvate/malate as substrates. The FRL is the percentage of total electron flow reducing O_2 to ROS (see Materials and Methods section). YC: young controls; OC: old controls; OR: old restricted. Values are means ± SE from 10 (YC and OC) or 9 (OR) different animals. The asterisks (**) denotes significant difference from OC ($p < 0.005$).

In agreement with the lack of changes in mitochondrial ROS generation, young and old control animals did not show differences in 8-oxodG steady-state levels in brain mtDNA (Fig. 4). However, the mtDNA 8-oxodG concentration of caloric restricted old animals was significantly lower (by 23%) than that of old controls fed ad libitum (Fig. 4). This was again consistent with the decrease in H_2O_2 production with pyruvate/malate observed in the brain mitochondria of caloric restricted old animals. Old control animals showed significantly higher levels of 8-oxodG in brain nDNA than young controls (Fig. 5). These increases were totally reversed by caloric restriction (Fig. 5).

DISCUSSION

The results of this study show that CR started in old age decreases the rate of ROS production of brain mitochondria, lowers the steady-state level of oxidative damage to mtDNA, and reverses age-related increases in oxidative damage to nDNA in the rat brain. The decrease in ROS generation takes place in Complex I and is not due to a decrease in mitochondrial oxygen consumption. Instead CR lowers the amount of oxygen radicals released per unit electron flow in the respiratory chain. The mechanism responsible for such change is related to the degree of electronic reduction of the Complex I free radical generator.

Although some studies have described increases in mitochondrial ROS production with age (Sohal *et al.*, 1994a), review of the different available studies (mainly from heart mitochondria) generally indicates a lack of age-related changes (Barja, 1999). In agreement with this, in the present investigation no differences in brain mitochondrial ROS production were found between young and old animals. Such result is the one expected if the rate of ROS generation causally determines the rate of aging. Since ageing is a progressive process, occurring

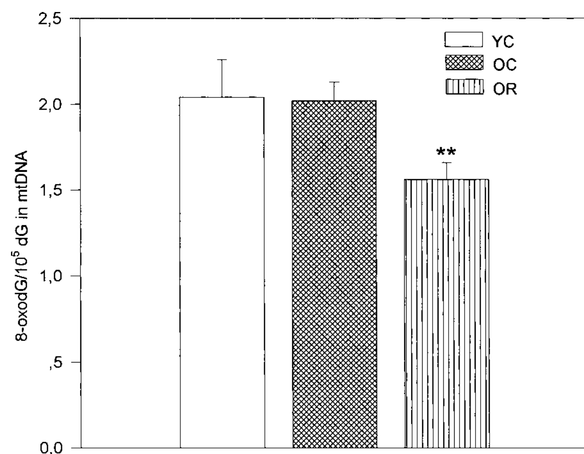

Fig. 4. Effect of age and caloric restriction on steady-state oxidative damage (8-oxodG/10^5dG) to rat brain mitochondrial DNA. YC: young controls; OC: old controls; OR: old restricted. Values are means ± SE from nine (YC and OC) or eight (OR) different animals. The asterisks (**) denotes significant difference from OC ($p < 0.004$).

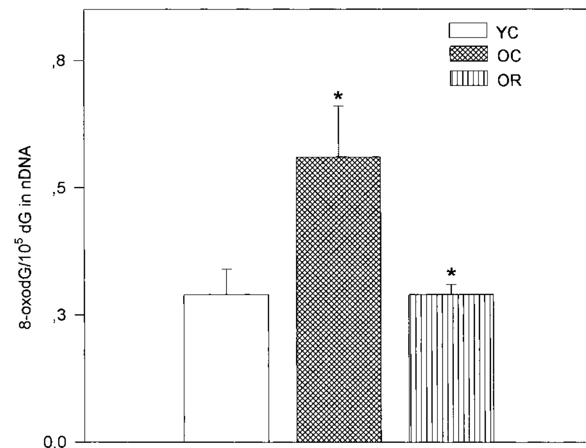

Fig. 5. Effect of age and caloric restriction on steady-state oxidative damage (8-oxodG/10^5dG) to rat brain nuclear DNA. YC: young controls; OC: old controls; OR: old restricted. Values are means ± SE from eight (YC and OC) or seven (OR) different animals. The asterisk (*) denotes significant difference between OC and YC ($p < 0.03$) and between OR and OC ($p < 0.03$).

at essentially similar rate at all ages, the causes of aging should not increase as a function of age. Otherwise aging will be accelerated with age. What should increase with age is the final consequence of those causes, such as the accumulation of mtDNA mutations with age (Khrapko *et al*., 2003; Barja, 2004a). Thus, if mitochondrial ROS production is a cause of aging it should show a similar rate in young and old animals as it was indeed found in the rat brain in this investigation. Differences in mitochondrial ROS production are known to occur however in animal species with different longevities, in agreement with their different rates of aging (Barja, 2004a). On the other hand, mtDNA is situated very close to the site of ROS generation at the inner mitochondrial membrane. Thus a strong relationship between the values of mitochondrial ROS production and steady-state 8-oxodG levels is expected (Barja, 2000, 2004a,b). In agreement with this notion and with the lack of changes in ROS production, no significant differences in 8-oxodG in mtDNA were found between young and old animals. In the nDNA, however, which is situated further away from the mitochondrial source of ROS, an increase in brain 8-oxodG was found in this investigation. Previous studies concerning variations in 8-oxodG in nDNA with age have yielded controversial results (Herrero and Barja, 2001). However, various previous studies have found that this parameter only increases in the brain at the end of the rat (Kaneko *et al*., 1997) or human (Meccoci *et al*., 1993) life span. This will agree with our results since the age of the old animals used in the present investigation was 36 months.

A previous study suggested that CR can decrease ROS generation in rat brain mitochondria (Baek *et al*., 1999). However, in that report ROS were measured using dichlorofluorescein diacetate. Since this fluorescent probe enters the mitochondrial matrix the decreases observed could be due to decreases in ROS generation, increases in matrix antioxidants or both. Our results show clearly that ROS production of brain mitochondria are decreased by CR. Such decrease does not simply avoid increases in ROS generation with age (Sohal *et al*., 1994a) which would avoid only putative increases in the aging rate taking place at old ages. Instead, the observed lowering of ROS production is more relevant because it decreased mitochondrial H_2O_2 generation below that normally present in control animals of any age. This change is the one expected if a decrease in ROS generation is causally involved in the decrease in ageing rate induced by CR. On the other hand, the decrease in mitochondrial ROS production in the brain of CR rats agrees with previous results obtained in rat liver and heart (López-Torres *et al*., 2002; Gredilla *et al*., 2001).

In agreement with the decrease in mitochondrial ROS generation, 8-oxodG in brain mtDNA was also decreased by CR. Furthermore, the decrease in oxidative damage to mtDNA (23%) was quantitatively almost identical to that found for ROS production (24%). This agrees again with the localization of mtDNA very close to the free radical source, the inner mitochondrial membrane. The increase in maximum life span elicited by CR (around 30%) is also of similar magnitude. Furthermore, recent studies have shown that CR also decreases by 30% base excision repair activity –the one involved in repair of oxidative damage to mtDNA- in the rat brain (Stuart *et al*., 2004). Thus, the cause of the decrease in oxidative damage to brain mtDNA in CR is not an increase in repair of mtDNA but a lower degree of free radical formation. Previous studies have also described CR-induced decreases in 8-oxodG in mtDNA, but these studies (Hamilton *et al*., 2001; López-Torres *et al*., 2002) are mostly limited to liver (a tissue with high mitotic capacity) except for a single study in the heart (Gredilla *et al*., 2001). Concerning nDNA we found that CR restriction reverses age-related increases in 8-oxodG but it does not decrease that value below that of control animals. This profile is different from what was observed both for ROS production and for 8-oxodG in mtDNA. This makes sense since nDNA is situated far away from the sites of ROS production at mitochondria. The lack of decrease in 8-oxodG in nDNA below the value observed in young controls also agrees with the concept that oxidative damage to mtDNA, not to nDNA is the one important factor in relation to ageing. On the other hand, the protection from age-related increases in 8-oxodG in nDNA observed in this investigation agrees with most (Sohal *et al*., 1994b; Kaneko *et al*., 1997; Hamilton *et al*., 2001) although not with all (Greenberg *et al*., 2000) previous reports.

In our study the site in the respiratory chain where ROS production decreases in the brain mitochondria of CR animals was also localized for the first time. It is well known that mitochondria produce ROS at two respiratory complexes, Complex I and Complex III. We have measured ROS production in the presence of pyruvate/malate or succinate(+rotenone) as substrates. With pyruvate/malate electrons flow through Complexes I and III, whereas with succinate(+rotenone) they flow only through Complex III because rotenone interrupts reverse electron flow from ubiquinone to Complex I. We found that CR lowers H_2O_2 generation of brain mitochondria with pyruvate/malate but not with succinate (+rotenone). Therefore, the decrease in ROS production induced by CR occurs only at the Complex I not at the Complex III ROS generator. Strikingly, previous studies have shown that Complex I is also responsible for the lower ROS generation of long-lived compared to short-lived animal species (Barja, 2004a,b). An important role of Complex I in ROS

production is expected from the stoichiometric proportions between respiratory complexes, that progressively increase from the NADH side to the oxygen side of the respiratory chain (Tyler, 1992). The relatively low abundance of Complex I will increase its degree of electronic reduction in the steady state and thus its rate of ROS generation. Oxidative stress and Complex I seem also specially relevant concerning important neurodegenerative diseases. The pathogenesis of Parkinson's disease includes impairment of mitochondrial function and deficiency of Complex I activity (Beal, 2003). Furthermore MPP^+, which induces a Parkinson's disease-like syndrome, is a Complex I inhibitor that increases ROS generation in this complex (Hoglinger *et al*., 2003). Increase in neuromelanin associated redox active iron and ROS production by microgial NADPH oxidase seem also to be involved in this disease, and many lines of evidence indicate that oxidative stress is also one of the earliest events in the genesis of Alzheimer's disease (Honda, 2004). Brain mitochondrial ROS production at Complex I has been also recently implicated in the etiology of neural cell death (Starkov and Fiskum, 2003).

Concerning the mechanism responsible for the decrease in ROS production in CR, we have found that it is not due to putative decreases in oxygen consumption. We found that the rates of mitochondrial oxygen consumption stayed constant with CR both in states 4 and 3. What decreased in CR was the percent release of ROS per total electron flow in the respiratory chain (the free radical leak). Thus, CR intrinsically modifies the mitochondria increasing their efficiency to avoid the electron leaks to oxygen that generate ROS. One mechanism responsible for this seems to be related to the degree of reduction of the Complex I generator because the decrease in ROS production with pyruvate malate in CR was no longer significant after addition of rotenone although a non-significant trend to decrease was apparent. With pyruvate/malate alone Complex I is partially reduced whereas with pyruvate/malate plus rotenone is fully reduced. Thus, the difference in ROS production between ad libitum-fed and CR animals is evident only when Complex I is partially reduced. A different picture emerges concerning succinate. In this case, addition of antimycin A to succinate (+rotenone) supplemented mitochondria (full Complex III reduction) leads to the appearance of a decrease in ROS production in CR. Therefore, although Complex III does not contribute to decrease ROS production in CR under normal conditions (substrate alone), in pathological situations in which the respiratory chain could be strongly inhibited Complex III will also contribute to lower ROS generation. A lower Complex III concentration in CR mitochondria could be responsible for such results.

Although caloric restriction is a well recognized antiaging manipulation, recent studies raised the possibility that CR could be ineffective or even detrimental if started late in life (Forster *et al*., 2003). The results obtained in this investigation however show that CR is able to lower mitochondrial ROS production and DNA oxidative damage even when started in 24-month old rats and sustained until 36 months of age. In agreement with these protective effects, various studies have shown positive effects of CR at various levels also when started at middle age or later. Thus CR, initiated at 12 months of age, increases average and maximum life span by 10–20% in two long-lived strains of mice (Wanagat *et al*., 1999). CR, when started at 14 months of age and continued until 30 months, also prevented 75% of the age-related changes in gene expression in $B6C3F_1$ mice (Lee *et al*., 2002) and even 4 weeks of CR in 34-month old mice can reproduce the majority of the effects of dietary restriction from weaning (Cao *et al*., 2001). CR initiated at 23,5–26,5 months also decreases the amount of altered and oxidized proteins and restores proteasome activities (Takahashi and Goto, 2002). A review of available studies (Takahashi and Goto, 2002) shows that, although the results vary between laboratories, most studies find that CR started at middle age or later in mice, rats and hamsters increases life span. Our results, together with that information, suggest that CR can be beneficial even in old subjects because it lowers mitochondrial oxidative stress and oxidative damage to relevant protein and DNA targets during aging.

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REFERENCES

Asunción, J. G., Millan, A., Pla, R., Bruseghini, I., Esteras, A., Pallardo, F. V., Sastre, J., and Viña, J. (1996). *FASEB J.* **10**, 333–338.
Baek, B. S., Kwon, H. J., Lee, K. H., Yoo, M. A., Kim, K. W., Ikeno, Y., Yu, B. P., and Chung, H. Y. (1999). *Arch. Pharm. Res.* **22**, 361–366.
Barja, G. (1999). *J. Bioenerg. Biomembr.* **31**, 347–366.
Barja, G. (2000). *Aging Clin. Exp. Res.* **12**, 342–355.
Barja, G. (2002). *J. Bioenerg. Biomembr.* **34**, 227–233.
Barja, G. (2004a). *Trends Neurosci.* **27**, 595–600.
Barja, G. (2004b). *Biol. Rev.* **79**, 235–251.
Beal, M. F. (2003). *Ann. N.Y. Acad. Sci.* **991**, 120–131.
Beckman, K. B., and Ames, B. (1998). *Physiol. Rev.* **78**, 547–581.
Cao, S. X., Dhabi, J. M., Mote, P. L., and Spindler, S. R. (2001). *PNAS.* **98**, 10630–10635.
Edwards, M. G., Sarkar, D., Klopp, R., Morrow, J. D., Weindruch, R. D., and Prolla, T. A. (2003). *Physiol. Genomics.* **13**, 119–127.

Forster, M. J., Morris, P., and Sohal, R. S. (2003). *FASEB J.* **17**, 690–692.
Gredilla, R., Sanz, A., López-Torres, M., and Barja, G. (2001). *FASEB J.* **15**, 1589–1591.
Greenberg, J. A., Wei, H., Ward, K., and Boozer, C. N. (2000). *Mech. Ageing Dev.* **115**, 107–117.
Hamilton, M. L., Van Remmen, H. V., Drake, J. A., Yang, H., Guo, Z. M., Kewitt, K., Walter, C. A., and Richardson, A. (2001). *PNAS* **98**, 10469–10474.
Herrero, A., and Barja, G. (2001). *J. Am. Aging Assoc.* **24**, 45–50.
Hoglinger, G. U., Carrad, G., Michel, P. P., Medja, F., Lombes, A., Ruberg, M., Friguet, B., and Hirsch, E. C. (2003). *J. Neurochem.* **86**, 1297–1307.
Honda, K. (2004). *Ann. N.Y. Acad. Sci.* **1012**, 179–182.
Ingram, D. K., Weindruch, R., Spangler, E. L., Freeman, J. R., and Walford, R. L. (1987). *J. Gerontol.* **42**, 78–81.
Kanda, K. (2002). *Micros Res. Tech.* **59**, 301–305.
Kaneko, T., Tahara, S., and Matsuo, M. (1997). *Free Radical. Biol. Med.* **23**, 76–81.
Khrapko, K., Nekhaeva, E., Kraytsberg, Y., and Kunz, W. (2003). *Mutat. Res.* **522**, 13–19.
Lai, C. K., and Clark, J. B. (1979). *Methods Enzymol.* **55**, 51–60.
Latorre, A., Moya, A., and Ayala, A. (1986). *PNAS USA* **83**, 8649–8653.
Lee, C. K., Allison, D. B., Brand, J., Weindruch, R., and Prolla, T. A. (2002). *PNAS* **99**, 14988–14993.
López-Torres, M., Gredilla, R., Sanz, A., and Barja, G. (2002). *Free Radical. Biol. Med.* **32**, 882–889.
Loft, S., and Poulsen, H. E. (1999). *Methods Enzymol.* **300**, 166–184.
Mattson, M. P., Chan, S. L., and Duan, W. (2002). *Physiol. Rev.* **82**, 637–672.
Mattson, M. P. (2003). *Neurology* **60**, 690–695.
Meccoci, P., MacGarvey, U., Kaufman, A. E., Koontz, D., Shoffner, J. M., Wallace, D. C., and Beal, F. (1993). *Ann. Neurol.* **34**, 609–616.
Moroi-Fetters, S. E. (1989). *Neurobiol. Aging* **10**, 317–322.
Sanz, A., Gredilla, R., Pamplona, R., Portero-Otín, M., Vara, E., Tresguerres, J. A. F., and Barja, G. (2005). *Biogerontol.* **6**, 15–26.
Sohal, R. S., Ku, H. H., Agarwal, S., Forster, M. J., and Lal, H. (1994a). *Mech. Ageing Dev.* **74**, 121–133.
Sohal, R. S., Agarwal, S., Candas, M., Forster, M. J., and Lal, H. (1994b). *Mech. Ageing Dev.* **76**, 215–224.
Starkov, A. A., and Fiskum, G. (2003). *J. Neurochem.* **86**, 1101–1107.
Stuart, J. A., Karahalil, B., Hogue, B. A., Souza-Pinto, N. C., and Bohr, V. A. (2004). *FASEB J.* **18**, 595–597.
Takahashi, R., and Goto, S. (2002). *Micros Res. Tech.* **59**, 278–281.
Tyler, D. D. (1992). *The Mitochondria in Health and Disease*, VCH Publishers, New York.
Wanagat, J., Allison, D. B., and Weindruch, R. (1999). *Toxicol. Sci.* **52S**, 35–40.