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Protein Restriction Without Strong Caloric Restriction Decreases Mitochondrial Oxygen Radical Production and Oxidative DNA Damage in Rat Liver

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Previous studies have shown that caloric restriction decreases mitochondrial oxygen radical production and oxidative DNA damage in rat organs, which can be linked to the slowing of aging rate induced by this regime. These two characteristics are also typical of long-lived animals. However, it has never been investigated if those decreases are linked to the decrease in the intake of calories themselves or to decreases in specific dietary components. In this study the possible role of the dietary protein was investigated. Using semipurified diets, the ingestion of proteins of Wistar rats was decreased by 40% below that of controls while the other dietary components were ingested at the same level as in animals fed ad libitum. After seven weeks in this regime the liver of the protein restricted animals showed 30–40% decreases in mitochondrial production of reactive oxygen species (ROS) and in oxidative damage to nuclear and mitochondrial DNA. The decreases in ROS generation occurred specifically at complex I. They also occurred without changes in mitochondrial oxygen consumption. Instead, there was a decrease in the percent free radical leak (the percentage of total electron flow leading to ROS generation in the respiratory chain). These results are strikingly similar to those previously obtained after 40% caloric restriction in the liver of Wistar rats. Thus, the results suggest that part of the decrease in aging rate induced by caloric restriction can be due to the decreased intake of proteins acting through decreases in mitochondrial ROS production and oxidative DNA damage. Interestingly, these tissue oxidative stress-linked parameters can be lowered by restricting only the intake of dietary protein, probably a more feasible option than caloric restriction for adult humans.

KEY WORDS: Mitochondria; free radicals; oxygen radicals; DNA damage; protein restriction; caloric restriction; 8-hydroxydeoxyguanosine; mitochondrial DNA; nuclear DNA.

INTRODUCTION

Among theories of aging, the mitochondrial free radical theory is currently receiving considerable support from comparative and experimental studies (Barja 1999, 2002a; Barja and Herrero, 2000; Beckman and Ames, 1998; Dröge, 2001). It is well known that caloric restriction slows down the rate of aging of animals including mammals like laboratory rodents, increases both their mean and maximum life spans, and delays the occurrence of many degenerative diseases (Barger *et al*., 2003; Wang *et al*., 2004). Recent studies suggest that the same can be true in primates (Mattison *et al*., 2003) and thus possibly in human beings. However, the fundamental mechanisms underlying the effects of caloric restriction on aging and longevity are still uncertain. Unveiling these mechanisms would be important to understand the aging process and its future possible slowing in humans.

We have previously shown that caloric restriction decreases mitochondrial free radical production and oxidative DNA damage (Gredilla *et al*., 2001a,b; Gredilla and Barja, 2003; López-Torres *et al*., 2002). Low levels of these two characteristics are also constitutively exhibited by long-lived species when compared to short-lived

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ones (Barja, 2004; Barja and Herrero, 2000). These results connect caloric restriction with the mitochondrial free radical theory of aging. They are interesting since they offer a plausible mechanism by which caloric restriction could slow down the rate of aging, by decreasing oxidative damage and long-term accumulation of mutations in mitochondrial DNA (Barja, 2002a, 2004). Implication of mitochondria in the effects of caloric restriction has been also recently described by others (Armeni *et al*., 2003). However, it has never been investigated if the decreases in mitochondrial production of reactive oxygen species (ROS) and oxidative DNA damage during caloric restriction are due to the reduction in calories themselves or to specific dietary components. We have thus initiated a series of systematic studies to clarify this relevant issue.

In the present investigation we test the possibility that the restriction of protein is responsible at least in part for the two main effects of caloric restriction related to oxidative stress described above. Although a general consensus was reached in the last decade that the life extension effect of caloric restriction is related to the reduction in calories themselves, variations in the proportions of the main dietary components could also modulate longevity (Archer, 2003; Mark *et al*., 1995), and experimental studies exist that describe significant increases in mean and maximum life span of Fisher 344 (Horakova *et al*., 1988; Yu *et al*., 1985) and Wistar rats (Barrows and Kokkonen, 1975) after restricting only the dietary intake of proteins. We have used a dietary protocol in which protein ingestion is reduced while the intake of carbohydrates and fat is maintained at the same level as in control animals. This avoids confusing the effects of protein restriction with those of increasing the percentage of other dietary components, mainly carbohydrates (Horakova *et al*., 1988; Youngman *et al*., 1992).

The decreases in mitochondrial ROS production and oxidative DNA damage in caloric restriction have been demonstrated previously by us both in liver (Gredilla *et al*., 2001a; López-Torres *et al*., 2002) and heart (Gredilla *et al*., 2001b) of Wistar rats. However, while detection of these decreases in the heart usually needs long-term restriction (Gredilla *et al*., 2002), in the liver the effect is quicker and can be detected already after only 6 weeks of treatment (Gredilla *et al*., 2001a). We have thus selected this rat organ for the present study of protein restriction because it allows performing the experiment (which needs the use of semipurified diets) in a much shorter time. The experiments were designed in such a way that could allow us also to identify the sites and mechanisms involved in putative decreases in mitochondrial ROS production and oxidative DNA damage in protein restricted animals, and to compare them with those found previously in caloric restriction (Gredilla *et al*., 2001a).

MATERIALS AND METHODS

Animals and Diets

Male Wistar rats of 250 g of body weight were obtained from Iffa-Creddo (Lyon, France). The animals were caged individually and maintained in a 12:12 (light:dark) cycle at $22 \pm 2^{\circ}$C and $50 \pm 10\%$ relative humidity. Control animals were fed ad libitum the semipurified American Institute of Nutrition diet AIN-93G: 39.7486% cornstarch, dextrinized cornstarch 13.20%, sucrose 10.00%, casein 20.00%, soybean oil 7.00%, alphacel (nonnutritive bulk) 5%, mineral mix 3.5%, vitamin mix 1.0%, L-cystine 0.3%, choline bitartrate 0.25% and tert-butylhydroquinone 0.0014%. The diet given to the protein restricted animals was a modified AIN-93G diet. Its protein content was reduced while its content in sucrose, in soybean oil, and in all the rest of its components was appropriately increased. Its composition was: cornstarch 39.7486%, dextrinized cornstarch 13.20%, sucrose 15.4719%, casein 13.04%, soybean oil 7.61%, alphacel 5.44%, mineral mix 3.80%, vitamin mix 1.09%, L-cystine 0.326%, choline bitartrate 0.272%, and tert-butylhydroquinone 0.0015%. This diet was given each day to the protein restricted animals in an amount equal to 92% of the food eaten by the controls. The final result is that protein restricted animals ingested daily 40% less proteins than the controls while the total amount of carbohydrates, fat, and the rest of dietary components eaten was the same in control and protein restricted animals. With this procedure, protein restricted animals ingested 8.5% less calories than the controls. The daily amount of protein casein eaten by the protein restricted animals was substantially higher than the minimum daily requirement (Dibak *et al*., 1984). The mean body weight at the end of the dietary experiment was 354 g in controls and 342 g in protein restricted animals. After 7 weeks of dietary treatment the animals were sacrificed by decapitation. The liver was immediately processed to isolate mitochondria while liver samples were stored at -80°C for the assays of oxidative damage to DNAs.

Mitochondria Isolation

Liver mitochondria were obtained from fresh tissue. The liver was rinsed and homogenized in 60 mL of isolation buffer (210-mM mannitol, 70-mM sucrose, 5-mM Hepes, 1-mM EDTA, pH 7.35). The nuclei and cell debris were removed by centrifugation at $1000 \times g$ for 10 min.

Supernatants were centrifuged at $10,000 \times g$ for 10 min and the resulting supernatants were eliminated. The pellets were resuspended in 40 mL of isolation buffer without EDTA and centrifuged at $1000 \times g$ for 5 min. Mitochondria were obtained after centrifugation of the supernatants at $10,000 \times g$ for 10 min. After each centrifugation step any overlaying layer of fat was eliminated. The mitochondrial pellets were resuspended in 1 mL of isolation buffer without EDTA. All the above procedures were performed at 5°C. Mitochondrial protein was measured by the Biuret method. The final mitochondrial suspensions were maintained over ice and were immediately used for the measurements of oxygen consumption and H_2O_2 production.

Mitochondrial H_2O_2 Production

The rate of mitochondrial H_2O_2 production 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, 2002b). Reaction conditions were 0.25 mg of mitochondrial protein per mL, 6 U/mL of horseradish peroxidase, 0.1-mM homovanillic acid, 50-U/mL of superoxide dismutase, and 2.5-mM pyruvate/2.5-mM malate, 2.5-mM glutamate/2.5-mM malate, or 5-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. Unless otherwise stated, 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 or Complex III 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 2.0-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^- produced (if any) to H_2O_2, the measurements represent the total (O_2^- plus H_2O_2) rate of mitochondrial ROS production.

Mitochondrial Oxygen Consumption

The oxygen consumption of liver 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- (2.5-mM pyruvate/2.5-mM malate or 2.5-mM glutamate/2.5-mM malate) or complex II-linked (5-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

Liver mitochondrial H_2O_2 production and O_2 consumption were measured in parallel in the same samples 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

Liver nuclear DNA (nDNA) was isolated after homogenization, centrifugation at $1000 \times g$ 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. 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.

8-oxodG HPLC Assays

Steady-state oxidative damage to 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 analyzed by HPLC with online

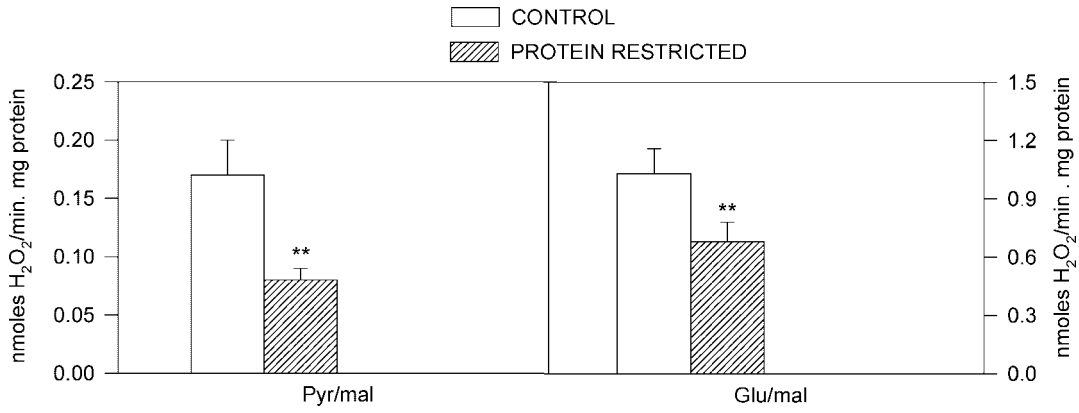

Fig. 1. Rate of H_2O_2 production of liver mitochondria with complex I-linked substrates in ad libitum-fed (control) and protein restricted rats. Pyr/mal = pyruvate/malate; glu/mal = glutamate/malate. Values are means ± SEM from 6 to 10 animals. * = significantly different from controls ($^{**}p < 0.01$).

electrochemical and ultraviolet detection, respectively. The nucleoside mixture was injected into a reverse-phase Beckman Ultrasphere ODS column (5 μM, 4.6 mm × 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 protein restricted 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

The rate of H_2O_2 production of liver mitochondria was measured in control and protein restricted rats using different combinations of substrates and inhibitors of the respiratory chain. Mitochondrial H_2O_2 production was significantly lower in protein restricted animals than in the controls both with pyruvate/malate (by 53%) and with glutamate/malate (by 34%) as substrates (Fig. 1). However, when the substrate used was succinate (plus rotenone, see Materials and Methods), there was no difference in H_2O_2 production between protein restricted and ad libitum-fed rats (Fig. 2, right). Noticeably, when the experiments with succinate were repeated in the absence of rotenone (Fig. 2, left), the lower rate of H_2O_2 production (35% decrease) of the protein restricted animals reappeared.

Respiratory inhibitors were also used to assay maximum rates of H_2O_2 production under full reduction of the complex I or III ROS generators, as opposed to partial reduction (with substrate alone). Addition of rotenone to pyruvate/malate or glutamate/malate (complex I-linked substrates) strongly stimulated (as expected) H_2O_2 production in both animal groups. In the presence of pyruvate/malate plus rotenone, the mitochondria from protein restricted animals showed significantly lower levels of H_2O_2 generation than the controls (Table I). However, with glutamate/malate plus rotenone, although protein restricted mitochondria showed a trend to decreased values of H_2O_2 production (23% decrease) statistical significance was not reached. Antimycin A also strongly stimulated (as expected) the rate of H_2O_2 generation with succinate in both animal groups. In the presence of succinate plus antimycin A no significant differences in the rates of H_2O_2 generation were detected between control and protein restricted animals (Table I).

The rate of oxygen consumption was measured without (State 4) and with (State 3) ADP in mitochondria from both kinds of animals in the presence of the same three different substrates used for the H_2O_2 measurements. In all cases the addition of ADP strongly increased oxygen consumption from resting (State 4) to phosphorylating (State 3) rates, indicating the good quality

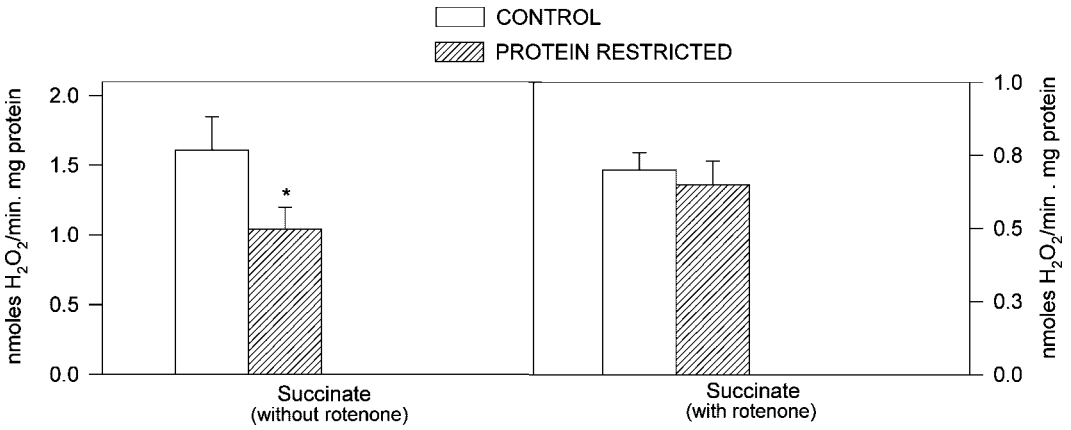

Fig. 2. Rate of H_2O_2 production of liver mitochondria with succinate as substrate in the absence (left) and presence (right) of rotenone in ad libitum-fed (control) and protein restricted rats. Values are means ± SEM from 6 to 10 animals. * = significantly different from controls ($^*p < 0.05$).

of the mitochondrial preparations in both animal groups (Table II). No significant differences between protein restricted and control animals were observed with any substrate either in State 4 or in State 3 (Table II).

The assay of H_2O_2 production and oxygen consumption in the same mitochondrial preparations under similar conditions allowed the calculation of the free radical leak (Table III). This leak represents the percentage of total electron flow leading to ROS production instead of reaching the end of the respiratory chain to tetravalently reduce oxygen to water (see Material and Methods for calculations). The free radical leak was significantly lower (Table III) in mitochondria from protein restricted animals than in controls with pyruvate/malate and with glutamate/malate (both complex I-linked substrates). With succinate, however, no significant differences between both groups of animals were observed (Table III).

Oxidative damage to DNA was estimated by measuring the levels of 8-oxodG in the mitochondrial and nuclear DNA (Fig. 3). Protein restriction significantly decreased the liver steady-state level of 8-oxodG both in mtDNA (by 34%) and in nDNA (by 39%) below those of ad libitum-fed animals.

Table I. Maximum Rates of H_2O_2 Production (Nanomoles H_2O_2/min. mg Protein) of Liver Mitochondria in the Presence of Substrates Plus Complex I and III Specific Inhibitors in ad Libitum-fed and Protein Restricted Rats

	Control	Protein restricted
Pyr/mal+rotenone	0.96 ± 0.13	0.58 ± 0.11**
Glu/mal+rotenone	1.70 ± 0.34	1.31 ± 0.17
Succinate+Antimycin A	5.14 ± 0.92	3.80 ± 0.45

Note. Values are means ± SEM from 6 to 10 different animals. Pyr/mal = pyruvate/malate. Glu/mal = glutamate/malate.
*Significantly different from controls ($^{**}p < 0.01$).

DISCUSSION

In this investigation it is shown for the first time that restricting the dietary intake of protein without strongly decreasing the caloric intake lowers mitochondrial ROS production and oxidative damage to mitochondrial and nuclear DNA in the liver of a mammal. These results are significant since they can contribute to clarify how caloric restriction decreases tissue oxidative stress and slows down aging. They can be also illustrative for future prospects to postpone human aging without the unpleasant and stressful side effects of strongly decreasing the dietary intake of energy.

Table II. Oxygen Consumption (Nanomoles O_2/min. mg Protein) of Liver Mitochondria in ad Libitum-fed and Protein Restricted Rats Under Different Conditions

	Control	Protein restricted
Pyr/mal (State 4)	9.8 ± 0.6	10.5 ± 0.7
Pyr/mal (State 3)	27.4 ± 1.4	25.6 ± 1.0
Glu/mal (State 4)	13.3 ± 0.5	11.9 ± 0.7
Glu/mal (State 3)	64.5 ± 5.0	57.8 ± 3.8
Succinate (State 4)	28.0 ± 1.5	26.9 ± 2.1
Succinate (State 3)	94.7 ± 7.9	81.3 ± 8.0

Note. Values are means ± SEM from 6 to 10 different animals. Pyr/mal = pyruvate/malate. Glu/mal = glutamate/malate. State 4 = O_2 consumption in the absence of ADP. State 3 = O_2 consumption in the presence of ADP. No significant differences were found between control and protein restricted animals.

Table III. Free Radical Leak (%) of Liver Mitochondria in ad Libitum-Fed and Protein Restricted Rats Under Different Conditions

	Control	Protein restricted
Pyr/mal	0.95 ± 0.24	0.37 ± 0.06*
Glu/mal	3.93 ± 0.61	2.79 ± 0.32*
Succinate	1.31 ± 0.16	1.34 ± 0.22

Note. Values are means ± SEM from 6 to 9 different animals. The free radical leak is the percentage of total electron flow reducing O_2 to ROS at the respiratory chain instead of reaching cytochrome oxidase to reduce O_2 to water (see Materials and Methods for calculations). Pyr/mal = pyruvate/malate. Glu/mal = glutamate/malate.
*Significantly different from controls ($^*p < 0.05$).

It is generally believed that the antiaging effect of caloric restriction is due to the decreased intake of calories rather than to specific dietary components, although variations in the proportions of the main dietary constituents seem also to affect longevity (Archer, 2003; Mark *et al*., 1995). Concerning the effects of protein restriction on aging, consideration of published studies performed in mammals shows that increases in longevity have been found much more commonly than no effects or decreases. Thus, some authors found that decreasing the casein content of the diet from 42 to 18% decreased the survival of male rats (Davis *et al*., 1983). However, many other investigators found that low protein diets increase the life span of rats (Barrows and Kokkonen, 1975), increase the mean and maximum life span of Fisher 344 rats (Horakova *et al*., 1988; Yu *et al*., 1985), increase the life span of C57BL/6J and hybrid F1 mice (Goodrick, 1978; Leto *et al*., 1976), prolong life expectancy in BALB/c mice (Stoltzner, 1977), and prolong the life of DBA/2f mice (Fernandes *et al*., 1976). In some of these studies the diet was restricted only with respect to protein (Fernandes *et al*., 1976), like in our experiments, whereas in other cases the decrease in protein was balanced by an increase in dietary carbohydrate (Horakova *et al*., 1988). In some cases it was observed that the life extension effect (15%) of the low protein diets was smaller than that of caloric restricted diets, and it was concluded that the retardation of aging was due to restriction of energy intake rather than to a specific nutrient (Masoro *et al*., 1991). Other authors have found that protein restriction can increase the life span of Wistar rats by as much as 25% (Barrows and Kokkonen, 1975). Taken together, these data suggest that protein restriction can be responsible for part of the life prolonging effects of caloric restriction. This would be also consistent with the observation that methionine or tryptophan restriction increases the maximum life span of rats independently of energy restriction (Richie *et al*., 1994; Zimmerman *et al*., 2003). Caloric and protein restriction share many common effects in addition to life prolongation, including delays in puberty, decreases in growth rate, changes in metabolic rate, boosting of cell-mediated immunity, lowering of cholesterol, or decreases in preneoplastic lesions and tumours (Youngman, 1993; Youngman *et al*., 1992). Low protein diets also decrease IGF-1 levels and decelerate glomerulosclerosis in mice (Doi *et al*., 2001), delay the occurrence of chronic nephropathy and cardiomyopathy in rats (Maeda *et al*., 1985), and protect rat liver against exposure to toxic chemicals (Rodrigues *et al*., 1991). A smaller but significant life extension effect in protein than in caloric restriction would agree with the widely accepted notion that aging has multiple causes. Restriction of protein intake can be responsible for part of the aging-delaying effect of caloric restriction through the decreases in mitochondrial ROS production and oxidative DNA damage that we observe in the present investigation.

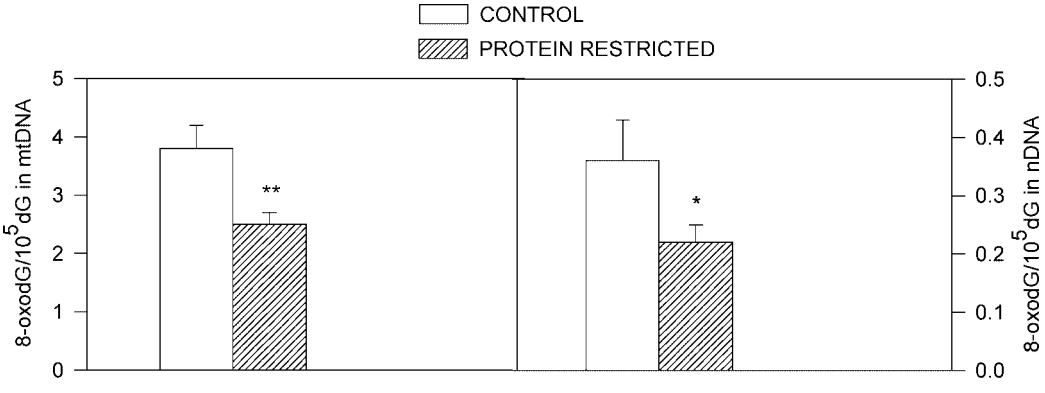

Fig. 3. Oxidative damage to mitochondrial (left) and nuclear (right) DNA, measured as 8-oxodG levels, in the liver of ad linitum-fed (control) and protein restricted rats. Values are means ± SEM from 6 to 10 animals. * = significantly different from controls ($^*p < 0.05$; $^{**}p < 0.01$).

The remaining effects of caloric restriction on aging rate could be related to decreases in other dietary components or in the calories themselves through different additional mechanisms.

Although the large majority of the studies involving caloric restriction use 40% restriction in energy intake, some investigations have shown that 25–35% reduction in energy is still able to increase the survival of rats (Hubert *et al*., 2000; Keenan *et al*., 1997). On the other hand, graded dose studies indicate that at least 20% or higher energy restriction is necessary to decrease oxidative DNA damage (Djuric *et al*., 2002). These minimum degrees of energy restriction are much higher than the one present in the protein restricted rats in our investigation (8.5%). Thus, the results obtained in the present investigation are related to the restriction in proteins themselves and not to the marginal decrease in energy intake (8.5%). We observed that 40% protein restriction during 7 weeks decreased mitochondrial ROS production and oxidative DNA damage to both mitochondrial and nuclear DNA in the liver of male Wistar rats. We have also previously observed these three changes in the liver of male Wistar rats after 6 weeks of 40% caloric restriction (Gredilla *et al*., 2001a). Those decreases in oxidative stress are maintained after 1 year of caloric restriction (López-Torres *et al*., 2002). In both the protein and caloric restriction studies the magnitude of the decreases in mitochondrial ROS production and oxidative DNA damage were around 30–40%. All this suggests that the decrease in protein intake causes those changes during caloric restriction. Since mitochondrial DNA is situated very close to the source of ROS (the inner mitochondrial membrane), the decrease in ROS generation would easily translate into a similar decrease in oxidative attack to mtDNA. This could possibly decrease the long-term accumulation of mtDNA mutations which could be involved in the slowing of aging (Barja, 2002a, 2004) in both restriction paradigms. The nDNA is situated further away from the free radical source and other mechanisms should be involved in its decrease in 8-oxodG levels. These could include increases in turnover of hepatic cells induced by the dietary restrictions. Studies in postmitotic tissues like heart have shown that caloric restriction also decreases oxidative damage to mtDNA but not to nDNA (Gredilla *et al*., 2001b). Previous studies have shown that protein restriction decreases protein carbonyls in rat liver (Youngman *et al*., 1992) and lowers lipid peroxidation in rat kidney (Sambuichi *et al*., 1991). Thus, protein restriction seems to be capable of decreasing oxidative stress to the three main kinds of cellular macromolecules at least in tissues containing mitotic cells, generally agreeing with the predictions of the free radical theory of aging.

Localization of the site where protein restriction lowers ROS production was performed in our investigation. Protein restriction decreased ROS generation with pyruvate/malate. With these complex I-linked substrates electrons flow through the two well known free radical generators of the respiratory chain: complexes I and III. Confirmation of this finding was obtained with other complex I-linked substrates, since ROS production was also decreased by protein restriction with glutamate/malate. However, when succinate (a complex-II liked substrate) was used in the presence of rotenone, no significant differences between protein restricted and control animals were found. Under these conditions electrons flow from complex II to complex III, thus bypassing the complex I ROS generator. Since the decrease in ROS production occurred when there was electron flow through both the complex I and complex III generators, but not when electrons only flowed through complex III, those experiments localize such decrease at complex I. Furthermore, when the experiments with succinate as substrate were repeated in the absence of rotenone, the decrease in ROS production of the protein restricted group reappeared. Elimination of the rotenone block in the presence of succinate allows part of the electrons to flow back from complex II to complex I. This shows again that the difference in ROS production between protein restricted and control animals becomes apparent only when the electrons flow through the complex I ROS generator. In summary, all the experiments consistently demonstrate that the decrease in mitochondrial ROS generation in protein restriction occurs at complex I. Interestingly, the same was also observed in previous investigations after caloric restriction in rat liver and heart (Gredilla *et al*., 2001a,b).

Concerning the mechanisms involved in the decrease in ROS generation, it was not due to a simple decrease in oxygen consumption since it did not change either in States 4 or 3. This agrees with previous studies in the liver of protein restricted (Ramanadham and Kaplay, 1979) and caloric restricted (Gredilla *et al*., 2001a) rats. What decreased in our protein restricted animals was the free radical leak, the percent of total electron flow directed to ROS generation. Thus the mitochondria of protein restricted animals have a respiratory chain more efficient in avoiding ROS generation since they produce a smaller amount of ROS per unit electron flow. Strikingly, the same was also observed in our previous study in the liver of caloric restricted rats (Gredilla *et al*., 2001a). In that study it was also observed that the degree of electronic reduction of the complex I ROS generator is involved in the mechanism that minimizes ROS production, since caloric restricted rats showed lower rates of mitochondrial ROS generation with pyruvate/malate (partial reduction of the complex

I generator) but not with pyruvate/malate plus rotenone (full reduction). In protein restricted rats the decrease in ROS production also disappeared with glutamate/malate plus rotenone but not with pyruvate/malate plus rotenone which avoids reaching a firm conclusion in this aspect. This is the only result that differed between the protein restriction and caloric restriction experiments.

In summary, both caloric restriction and protein restriction decrease mitochondrial ROS production, oxidative damage to mitochondrial DNA, and oxidative damage to nuclear DNA in rat liver. The decreases in ROS generation occur at complex I and the mechanism implicates an increase in the efficiency of the respiratory chain in avoiding free radical production in both models. Furthermore, the magnitude of the decreases observed are also similar in both experimental manipulations for all the parameters measured. All this suggests that part of the decrease in aging rate induced by caloric restriction can be due to the decreased intake of proteins acting through decreases in mitochondrial ROS production and oxidative DNA damage. In any case, the results show that endogenous liver oxidative stress can be diminished by decreasing the dietary intake of protein. This is interesting because this dietary manipulation does not imply the strong behavioral stress of caloric restriction and is thus possibly a more feasible option for adult human beings.

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