J Bioenerg Biomembr (2006) 38:327–333
DOI 10.1007/s10863-006-9051-0

ORIGINAL PAPER

Carbohydrate restriction does not change mitochondrial free radical generation and oxidative DNA damage

A. Sanz · J. Gómez · P. Caro · G. Barja

Received: 25 April 2006 / Accepted: 30 May 2006 / Published online: 30 November 2006

Abstract Many previous investigations have consistently reported that caloric restriction (40%), which increases maximum longevity, decreases mitochondrial reactive species (ROS) generation and oxidative damage to mitochondrial DNA (mtDNA) in laboratory rodents. These decreases take place in rat liver after only seven weeks of caloric restriction. Moreover, it has been found that seven weeks of 40% protein restriction, independently of caloric restriction, also decreases these two parameters, whereas they are not changed after seven weeks of 40% lipid restriction. This is interesting since it is known that protein restriction can extend longevity in rodents, whereas lipid restriction does not have such effect. However, before concluding that the ameliorating effects of caloric restriction on mitochondrial oxidative stress are due to restriction in protein intake, studies on the third energetic component of the diet, carbohydrates, are needed. In the present study, using semipurified diets, the carbohydrate ingestion of male Wistar rats was decreased by 40% below controls without changing the level of intake of the other dietary components. After seven weeks of treatment the liver mitochondria of the carbohydrate restricted animals did not show changes in the rate of mitochondrial ROS production, mitochondrial oxygen consumption or percent free radical leak with any substrate (complex I- or complex II-linked) studied. In agreement with this, the levels of oxidative damage in hepatic mtDNA and nuclear DNA were not modified in carbohydrate restricted animals. Oxidative damage in mtDNA was one order of magnitude higher than that in nuclear DNA in both dietary groups. These results, together with previous ones, discard lipids and carbohydrates, and indicate that the lowered ingestion of dietary proteins is responsible for the decrease in mitochondrial ROS production and oxidative damage in mtDNA that occurs during caloric restriction.

Keywords Carbohydrates · Dietary restriction · Free radicals · Mitochondria · DNA damage · Aging · Oxidative stress

Abbreviations
8-oxodG: 8-oxo-7,8-dihydro-2'-deoxyguanosine · CR: caloric restriction · mtDNA: mitochondrial DNA · nDNA: nuclear DNA · ROS: reactive oxygen species

Introduction

Caloric restriction (CR) slows down the aging rate and increases maximum longevity in laboratory rodents and other animals (Barger et al., 2003). Nevertheless, the basic mechanisms underlying the effects of CR on aging and longevity are still unclear. The oxygen free radical mitochondrial theory of aging is currently receiving considerable support both from comparative and caloric restriction studies (Beckman and Ames, 1998; Barja, 2004a; Ramsey et al., 2004). Many investigations have consistently found that CR decreases the rate of production of reactive oxygen species (ROS) at mitochondria and the steady-state level of oxidative damage to mitochondrial DNA (mtDNA) in rodent tissues (Gredilla and Barja, 2005). Low levels of these two characteristics are also constitutively exhibited by long-lived species when compared to short-lived ones

A. Sanz· J. Gómez · P. Caro · G. Barja
Department of Animal Physiology-II, Faculty of Biological Sciences, Complutense University, Madrid 28040, Spain

G. Barja (✉)
Departamento de Fisiología Animal-II, Facultad de Ciencias Biológicas, Universidad Complutense, c/Antonio Novais-2, Madrid 28040, Spain
e-mail: gbarja@bio.ucm.es

(Barja, 2004b). These results offer a plausible mechanism by which CR could slow down the rate of aging, by decreasing oxidative damage and long-term accumulation of mutations in mitochondrial DNA (Barja, 2004a). However, it has not been clarified if the decreases in mitochondrial ROS production and oxidative DNA damage during CR are due to the reduction in calories themselves or are specifically related to the decreased ingestion of any of the three main energetic dietary components, proteins, lipids or carbohydrates.

Although the general consensus was reached in the last decade that the life extension effect of CR is related to the reduced ingestion of calories themselves, recent studies and revision of old data suggest that variations in the main individual dietary components can also modulate longevity in rodents (Archer, 2003; Pamplona and Barja, 2006) and insects (Mair et al., 2005, Piper et al., 2005). We have thus initiated a series of studies to clarify the possible effect of restriction of those dietary components on mitochondrial oxidative stress. Two reports on the separate effects of protein and lipid restriction without changing the rest of the dietary components have been already published (Sanz et al., 2004, 2006a).

In the present study we investigate whether restriction of the third energetic component of the diet, the carbohydrates, can be responsible for the two main effects of CR related to mitochondrial oxidative stress described above. In our dietary protocol carbohydrate ingestion is decreased while the intake of proteins, lipids and other dietary components is maintained at the same level as in control animals. This avoids confusing the effects of carbohydrate restriction with those of increasing the percentage of other dietary components.

In previous studies we have found that CR decreases mitochondrial ROS production and oxidative DNA damage in liver (Gredilla et al., 2001a), heart (Gredilla et al., 2001b), and brain (Sanz et al., 2005a) of Wistar rats. However, while detection of these decreases usually needs long-term restriction in other tissues (Gredilla and Barja, 2005), in the liver the effects are quicker and can be detected after only seven weeks CR (Gredilla et al., 2001a). We have thus selected this rat organ and implementation time for the present study of carbohydrate restriction because it allows performing the experiment (which needs the use of semipurified diets) in a much shorter time. The results obtained are discussed together with those previously obtained after seven weeks of protein (Sanz et al., 2004) or lipid (Sanz et al., 2006a) restriction also in the liver of Wistar rats, as well as with the available results on the effects on protein, lipid or carbohydrate restriction on animal longevity.

Materials and methods

A total number of 14 male Wistar rats of 250 g of body weight were obtained from the Complutense University Animal Facility and were caged individually and maintained in a 12:12 (light:dark) cycle at 22 ± 2°C. Control animals were fed ad libitum a semipurified diet prepared by MP Biomedicals (Irvine, CA, USA) based on the American Institute of Nutrition AIN-93G diet: 39.7486% cornstarch, dextrinized cornstarch 13.20%, sucrose 10.00%, soy protein 20.00%, soybean oil 7.00%, alphacel (non-nutritive 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 carbohydrate restricted animals was a modified AIN-93G diet. Its carbohydrate content was reduced while its content in proteins, in fats and in all the rest of its components was appropriately increased. Its composition was: cornstarch 31.8%, dextrinized cornstarch 10.56%, sucrose 8%, soy protein 26.67%, soybean oil 9.33%, alphacel 6.91%, mineral mix 4.67%, vitamin mix 1.33%, L-cystine 0.4%, choline bitartrate 0.33%, and tert-butylhydroquinone 0.0019%. This diet was given each day to the carbohydrate restricted animals in an amount equal to 75% of the food eaten by the controls. The final result was that carbohydrate restricted animals ingested daily 40% less carbohydrates than the controls while the total amount of protein, fat and the rest of dietary components eaten was the same in control and carbohydrate restricted animals. After seven 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 in mtDNA and nuclear DNA (nDNA).

Isolation of mitochondria

Just after decapitation 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 1,000 x *g* for 10 min. Supernatants were centrifuged at 10,000 x *g* for 10 min and the resulting supernatants were discarded. The pellets were resuspended in 40 mL of isolation buffer without EDTA and were centrifuged at 1,000 × *g* for 10 min. Mitochondria were obtained after centrifugation of the supernatants at 10,000 × *g* for 10 min. After each centrifugation step any overlaying layer of fat was removed. 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 measurement of oxygen consumption and H_2O_2 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 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). The substrates used were complex I- (2.5 mM pyruvate/2.5 mM malate or 2.5 mM glutatamete/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 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 essentially as described (Barja, 2002). 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 gluatamate/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. 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 (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 stop solution (2.0 M glycine, 2.2 M NaOH, 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 superoxide dismutase added in excess converts all $O_2^{\cdot -}$ excreted by mitochondria (if any) to H_2O_2, the measurements represent the total ($O_2^{\bullet -}$ plus H_2O_2) rate of mitochondrial ROS production.

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 the electrons out of the sequence which reduce O_2 to ROS at the respiratory chain (the percent free radical leak, FRL) instead of reaching cytochrome oxidase to reduce O_2 to water. Since two electrons are needed to reduce one mole of O_2 to H_2O_2 whereas four electrons are transferred in the reduction of one 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.

Measurement of 8-oxodG in mtDNA and nDNA

Liver nuclear DNA (nDNA) was isolated after homogenization, centrifugation at 1,000 × 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 was isolated by the method of Latorre et al. (1986) adapted to mammals (Asuncion 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 hr (Loft and Poulsen, 1999). All aqueous solutions used for nDNA and mtDNA isolation, digestion and chromatographic separation were prepared in HPLC-grade water. Steady-state oxidative damage to mtDNA was estimated by measuring the level of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) referred to that of the non-oxidized base (deoxyguanosine, dG). 8-oxodG and dG were analyzed 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 × 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 1ml/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

Results were expressed as means ± SEM and were analysed using independent Students t tests. Statistical analyses were carried out using a Graph-pad Prism statistical analysis program (San Diego, CA, USA). The minimum level of statistical significance was set at $p < 0.05$.

Table 1 Body and tissue weight of control and carbohydrate restricted rats

	Control	Carbohydrate restricted
Body weight (g)	425 ± 12	371 ± 3***
Tissue weight (g)		
Heart (g)	1.2 ± 0.1	1.1 ± 0.0
Liver (g)	9.7 ± 0.5	8.7 ± 0.6
Kidney (g)	2.2 ± 0.0	2.0 ± 0.1***
Brain	1.9 ± 0.0	1.9 ± 0.0
Spleen	0.7 ± 0.0	0.6 ± 0.0*

Values are means ± SEM from 7 different animals.
*Significant differences between control and carbohydrate restricted animals.
**$p < 0.01$.
***$p < 0.001$.

Results

The body weight of the carbohydrate restricted animals was significantly lower than that of the ad libitum-fed rats after seven weeks of treatment (Table 1). The weight of the kidneys and spleen were also significantly smaller in carbohydrate restricted animals. These differences were no longer present when the weight of these tissues was referred to body weight. The weight of heart, liver, and brain did not show significant differences between the two groups.

The rate of oxygen consumption of liver mitochondria was measured without (State 4) and with (State 3) ADP in the presence of complex I- (pyruvate/malate or glutamate/malate) and complex II- (succinate) linked substrates. The addition of ADP strongly increased the rate of oxygen consumption in all cases, indicating tight coupling of the mitochondrial preparations (Table 2). No significant differences in oxygen consumption were found between control and carbohydrate restricted groups with any substrate in either state 4 or 3.

The rate of H_2O_2 production of liver mitochondria was measured in control and carbohydrate restricted rats using different combinations of substrates and inhibitors of the respiratory chain (Table 3). Addition of rotenone to pyruvate/malate supplemented mitochondria strongly increased their rates of H_2O_2 generation both in ad libitum-fed and in carbohydrate restricted animals, and the same was observed with glutamate/malate in control animals. Antymicin A strongly stimulated the rate of H_2O_2 generation with succinate as substrate in both control and carbohydrate restricted rats. No significant differences in H_2O_2 production were found between control and carbohydrate-restricted rats with any substrate or substrate plus inhibitor combination (Table 3). The free radical leak of liver mitochondria did not show significant differences between both groups with any substrate (Table 4).

The level of 8-oxodG was significantly higher (7 to 9 fold higher) in mtDNA than in nDNA in the liver of both control and carbohydrate restricted animals (Fig. 1). Similarly to what was observed for the rate of mitochondrial ROS generation, carbohydrate restriction did not significantly change the level of 8-oxodG in either mtDNA or nDNA.

Table 2 Oxygen consumption (nanomoles O_2/min. mg protein) of liver mitochondria from control and carbohydrate restricted rats

Substrate	Control	Carbohydrate restricted
Pyr/mal (State 4)	7.0 ± 0.8	6.4 ± 0.6
Pyr/mal (State 3)	16.8 ± 1.7	17.0 ± 1.2
Glu/mal (State 4)	9.8 ± 1.1	10.3 ± 0.8
Glu/mal (State 3)	56.5 ± 6.1	62.8 ± 3.8
Succinate (State 4)	22.0 ± 1.8	23.2 ± 2.0
Succinate (State 3)	76.7 ± 6.6	80.8 ± 5.4

Values are means ± SEM from 7 different animals. Pyr/mal = pyruvate/malate; Glu/mal = glutamate/malate. Mitochondrial oxygen consumption was measured in the absence (State 4) and in the presence (State 3) of ADP.

Table 3 Rates of H_2O_2 production (nanomoles H_2O_2/min. mg protein) of liver mitochondria from control and carbohydrate restricted rats

Substrate	Control	Carbohydrate restricted
Pyr/mal	0.11 ± 0.03	0.14 ± 0.04
Pyr/mal + rotenone	0.91 ± 0.10***	0.95 ± 0.10***
Glu/mal	0.70 ± 0.20	1.11 ± 0.20
Glu/mal + rotenone	1.25 ± 0.20*	1.12 ± 0.20
Succinate	0.99 ± 0.10	0.83 ± 0.10
Succinate + AA	5.09 ± 0.7***	4.21 ± 0.5***

Values are means ± SEM from 6–7 different animals. Pyr/mal = pyruvate/malate; Glu/mal = glutamate/malate. AA = antimycin A.
*Significant differences between stimulated and basal ROS production (pyr/mal+rotenone vs. pyr/mal; glu/mal+rotenone vs. glu/mal; succinate+AA vs. succinate).
*$p < 0.05$.
***$p < 0.001$.

Discussion

In this investigation the carbohydrate ingestion of male Wistar rats was restricted by 40% during 7 weeks without

Table 4 Free radical leak (%) of liver mitochondria from control and carbohydrate restricted rats

Substrate	Control	Carbohydrate restricted
Pyr/mal	0.90 ± 0.20	1.21 ± 0.40
Glu/mal	3.98 ± 1.50	5.39 ± 1.30
Succinate	2.44 ± 0.50	1.87 ± 0.30

Values are means ± SEM from 6–7 different animals. The FRL is the percentage of the total electron flow in the respiratory chain directed to oxygen radical generation (see Materials and Methods for details). Pyr/mal = pyruvate/malate; Glu/mal = glutamate/malate.

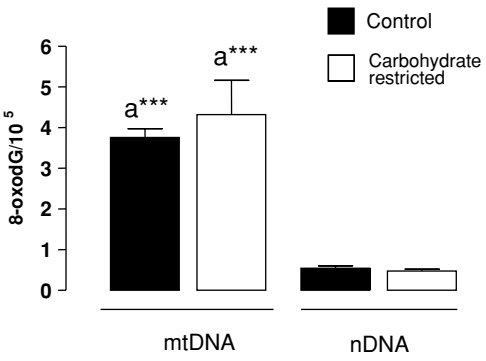

Fig. 1 Oxidative damage to mitochondrial and nuclear DNA, estimated as the level of 8-oxodG, in the liver of control and carbohydrate restricted rats. a***: significantly higher in mtDNA than in nDNA in the same dietary group ($p < 0.001$)

changing the intake of the rest of the dietary components. The dietary treatment affected the animals, since body weight was lower in carbohydrate restricted than in ad libitum fed rats. All the studied organs except the brain showed a trend to lower tissue weight in carbohydrate restricted animals which reached statistical significance in the case of kidney and spleen. Studies with respiratory inhibitors showed that the liver mitochondria of carbohydrate restricted animals, similarly to what happens in ad libitum and CR rodents (Gredilla et al., 2001b) produce ROS both at complex I and complex III of the respiratory chain, since rotenone and antimicyn A strongly stimulate ROS production with pyruvate/malate and succinate as substrates respectively. Carbohydrate restricted animals also showed around one order of magnitude higher oxidative damage in liver mtDNA than in nDNA, similarly to what occurs in ad libitum and CR rats (Gredilla et al., 2001a; López-Torres et al., 2002). However, 7 weeks of carbohydrate restriction did not change the rates of mitochondrial ROS production, mitochondrial oxygen consumption, mitochondrial free radical leak, and oxidative damage to mtDNA (Table 5). In contrast, all these parameters except oxygen consumption decrease in rat liver after 7 weeks of 40% CR (Gredilla et al., 2001a), as well as after 7 weeks of 40% protein restriction without changing the ingestion of the rest of the dietary components (Sanz et al., 2004; Table 5). The decreases observed in protein restriction strongly mimicked those found in caloric restriction quantitatively and also qualitatively, since the decreases in mitochondrial ROS production occurred at complex I in both cases. On the other hand, a recent study found that 7 weeks of 40% lipid restriction without changing the ingestion of the rest of the dietary components, similarly to carbohydrate restriction, did not change either the rate of mitochondrial ROS production or the level of 8-oxodG in mtDNA and nDNA in rat liver (Sanz et al., 2006a; Table 5). These results, taken together, indicate that protein restriction, and not lipid or carbohydrate restriction, is responsible for the lowering of mitochondrial ROS production and oxidative damage to mtDNA that occurs during CR (see Gredilla and Barja, 2005 for review).

ROS have mutagenic capacity, the source of ROS at mitochondria is situated very near or even in contact with mtDNA, and increases in mtDNA mutations seem to accelerate aging in rodents (Kujoth et al., 2005). Thus, the decreases in ROS production and oxidative damage to mtDNA induced by CR and protein restriction can be involved at least in part in the well known increase in maximum longevity that occurs in CR animals. In order to clarify this, it is necessary to consider previous studies which investigated the effect of the separate restriction of the three main dietary components, carbohydrates, lipids, and proteins, on rodent longevity.

Concerning the effects of protein restriction on aging rate, the large majority of the studies performed in rodents found increases in maximum longevity. Ten out of eleven published studies (and 16 out of 18 different life-long survival experiments in these studies) in rats or mice found that protein restriction increases maximum life span (Table 5; Reviewed in Pamplona and Barja, 2006), although the magnitude of the increase (ca. 20% increase in maximum longevity taking into account all the 16 positive studies) was usually lower than that typically found in CR (ca., 40%). These investigations, taken together, suggest that protein restriction can be responsible for around 50% of the life-prolonging effects of CR. Recent studies in *Drosophila* also indicate that the life-extension effect of calorie restriction in these insects is not due to the calories themselves (Mair et al., 2005), while they are compatible with a specific role of decreased protein intake in life-extension. This possibility would be also consistent with the well-described fact that methionine restriction also increases maximum longevity in rats and mice independently of energy restriction (Orentreich et al., 1993; Richie et al., 1994; Zimmerman et al., 2003). In relation with this, we have

Table 5 Summary of changes in oxidative stress-related parameters and maximum life span in caloric, protein, lipid and carbohydrate restricted rodents

Caloric restriction	Protein restriction	Lipid restriction	Carbohydrate	
Mitochondrial oxygen consumption	=	=	↑	=
Mitochondrial ROS production	↓	↓	=	=
Free radical leak	↓	↓	=	=
Oxidative damage to mtDNA	↓	↓	=	=
Maximum life span (number of studies)	↑↑(many)	↑(16)	= (2)	=*

= No change; ↑ increase; ↓ decrease; * no consistent evidence of change: see text for details.

recently found that methionine restriction without caloric restriction also decreases mitochondrial ROS production and oxidative damage to mtDNA in rat liver and heart (Sanz et al., 2006b), thus further supporting the possibility that protein and methionine restriction increases rodent longevity through decreases in mitochondrial oxidative stress.

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 levels, or decreases in preneoplastic lesions and tumours and lowering of protein oxidation (Youngman et al., 1992). Low protein diets also 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 lower (but significant) life extension effect in protein restriction than in CR would agree with the widely held notion that aging has multiple causes. CR could decrease aging rate through decreases in mitochondrial oxidative stress (due to protein restriction) as well as through other mechanisms like, e.g., lowering insulin/IGF-1 signalling (Richardson et al., 2004). This would be consistent with recent findings of a lack of relationship between insulin/IGF-1 signalling and mitochondrial ROS generation (Sanz et al., 2005b), which could be two independent mechanisms lowering aging rate in parallel during CR.

What are the effects of lipid restriction on longevity? Various investigations have discussed whether or not lipids are involved in CR effects, some supporting and some rejecting this idea based on different kinds of end point biochemical measurements and experiments (Masoro, 2000; Barzilai and Gabriely, 2001; Muurling et al., 2002). But very few studies have directly tested the effect of lipid restriction on longevity. An investigation in Fisher rats did not found changes in longevity after lipid or mineral restriction without CR (Iwasaki et al., 1988). Another long-term study performed also in Fisher 344 rats found increases in maximum and medium life span after 40% CR but not after 40% lipid restriction (Shimokawa et al., 1996). These and other investigations led to the conclusion that restriction of calories, but not of fats, slows down the primary aging process (Masoro, 1990). Thus, although available direct information is scarce and mainly limited to a particular rat strain, it seems safe to conclude that lipid restriction does not delay aging (Table 5). If that is indeed the case, it will fit well with the finding that lipid restriction without CR does not decrease mitochondrial ROS production and oxidative damage to mtDNA or nDNA (Sanz et al., 2006a).

Concerning carbohydrates, most of the available investigations have studied the effect on rodent survival after changing the kind of carbohydrate rather than restricting total carbohydrates in the diet. Although information is scarce, it seems that simple carbohydrates like sucrose, glucose or fructose shorten longevity compared to diets containing complex carbohydrates (Archer, 2003). Thus, it has been reported that carbohydrate given either as sucrose or as glucose, compared to starch, decreased the life span of rats (Dalderup and Viser, 1969) and mice (Mlekusch et al., 1996), whereas changing the source of complex carbohydrate from corn to rice did not modify maximum longevity in mice (Yamaki et al., 2005). However, the situation can be more complex since studies in Fisher 344 rats have found that a cornstarch compared to a sucrose-based diet increases both mean and maximum life span when the experiment is performed in ad libitum-fed animals, whereas sucrose is better for mean life span and cornstarch is better for maximum life span when the experiment is performed in CR animals (Murtagh-Mark et al., 1995). Concerning the total amount of carbohydrate in the diet the available information is even more limited and contradictory. One study reported an inverse relationship between total carbohydrate intake and life span in experimental animals (Ross, 1976). However, another study found that increasing the carbohydrate (dextrin) proportion in the diet of male Fisher 344 rats increased their 10th percentile survival by 41 days, a much smaller effect than that of CR which increased the 10th percentile survival in this experiment by 396 days (Khorakova et al., 1990). In any case, such effect would be incompatible with the possibility that carbohydrate restriction increases maximum longevity. Moreover, in several of the 16 studies described above in which protein restriction increased maximum longevity (reviewed in Pamplona and Barja, 2006) the decrease in dietary protein was compensated by corresponding increases in dietary carbohydrate. This is also strongly contradictory with the possibility that carbohydrate restriction increases longevity. Another study did not find changes in the longevity of short-lived autoimmune-prone mice after increasing total dietary carbohydrate under ad libitum feeding conditions (Kubo et al., 1987). Thus, it seems safe to conclude that most of the available information suggests that carbohydrate restriction does not increase rodent longevity (Table 5). This would agree again with the lack of decrease in mitochondrial ROS production and oxidative damage to mtDNA and nDNA observed in our study.

In summary, both caloric restriction and protein restriction increase maximum longevity (by different amounts) and both decrease the rate of mitochondrial ROS production and oxidative damage to mtDNA in rats. In contrast, neither carbohydrate nor lipid restriction seem to increase maximum longevity and neither of them decrease mitochondrial ROS production and oxidative damage to mtDNA. These results, taken together, indicate that the decrease in mitochondrial ROS production and mtDNA oxidative damage observed in caloric restriction is due to the reduced intake of proteins that occurs during this dietary manipulation, and not to

the decreased ingestion of lipids or carbohydrates. This conclusion is also consistent with the recent observation that methionine restriction (which also increases maximum longevity) also decreases mitochondrial ROS generation and oxidative damage to mtDNA.

Acknowledgements The authors are grateful to Tim Hofer and Christiaan Leeuwenburgh for their generous gift of Nuclease P1. This study was supported by a grant SAF 2002-01635 from the Spanish Ministry of Science and Technology to G. Barja. A. Sanz and P. Caro received a predoctoral fellowship from Complutense University and the Spanish Ministry of Science and Education respectively.

References

Archer VE (2003) Med Hypoteses 60:924–929
Asunción JG, Millan A, Pla R, Bruseghini I, Esteras A, Pallardo FV, Sastre J, Viña J (1996) FASEB J 10:333–338
Barger JL, Walford RL, Weindruch R (2003) Exp Gerontol 38:1343–1351
Barja G (2002) J Bioenerg Biomembr 34:227–233
Barja G (2004a) Free radicals and aging. Trends Neurosci 27:595–600
Barja G (2004b) Biol Rev 79:235–251
Barzilai N, Gabriely I (2001) J Nutr 131:903S–906S
Beckman KB, Ames BN (1998) Physiol Rev 78:547–581
Dalderup LM, Visser W (1969) Nature 222:1050–1052
Doi SQ, Rasaiah S, Tack I, Mysore J, Kopchick JJ, Moore J, Hirszel P, Striker LJ, Striker GE (2001) Am J Nephrol 21:331–339
Gredilla R, Barja G, López-Torres M (2001a) J Bioenerg Biomembr 33:279–287
Gredilla R, Sanz A, López-Torres ML, Barja G (2001b) FASEB J. 15, 1589–1591
Gredilla G, Barja G (2005) The role of oxidative stress in relation to caloric restriction and longevity. Endocrinol 146:3713–3717
Iwasaki K, Gleiser CA, Masoro EJ, McMahan CA, Seo EJ, Yu BP (1988) J Gerontol 43:B13–B21
Khorakova M, Deil Z, Khausman D, Matsek K (1990) Fiziol Zh 36:16–21
Kubo C, Johnson BC, Gajjar A, Good RA (1987) J Nutr 117:1129–1135
Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, Hofer T, Seo AY, Sullivan R, Jobling WA, Morrow JD, Van Remmen H, Sedivy JM, Yamasoba T, Tanokura M, Weindruch R, Leeuwenburgh C, Prolla T (2005) Science 5:71–79
Latorre A, Moya A, Ayala A (1986) PNAS USA 83:8649–8653
Loft S, Poulsen HE (1999) Methods Enzymol 300:166–184
López-Torres M, Gredilla R, Sanz A, Barja G (2002) Free Rad Biol Med 32:882–889
Maeda H, Gleiser CA, Masoro EJ, Murata I, maman CA, Yu BP (1985) J Gerontol 40:671–688
Mair W, Piper MD, Partridge L (2005) PloS Biol 3:1305–1311
Masoro EJ (1990) Proc Soc Exp Biol Med 193:31–34
Masoro EJ (2000) Caloric restriction and aging: an update. Exper Gerontol 35:299–305
Mlekusch W, Lamprecht M, Ottl, K, Tillian M, Reibnegger G (1996) Mech Ageing Dev 92:43–51
Murtagh-Mark CM, Reiser KM, Harris R Jr, McDonald RB (1995) J Gerontol 50A:B148–B154
Muurling M, Jong MC, Mensink RP, Hornstra G, Dahlmans VEH, Hanno P, Voshol PH, Havekes LM (2002) Metabolism 51:695–701
Orentreich N, Matias JR, DeFelice A, Zimmerman JA (1993) J Nutr 123:269–274
Pamplona R, Barja G (2006) Biochim Biophys Acta Bioenerg (In press)
Piper MD, Mair W, Partridge L (2005) J Gerontol A 60:549–555
Ramsey JJ, Hagopian K, Kenny TM, Koomson EK, Bevilacqua L, Weindruch R, Harper ME (2004) Am J Physiol 286:E31–E40
Richardson A, Liu F, Adamo ML, Van Remmen H, Nelson JF (2004) Best Pract Res Clin Endocrinol Metab 18:393–406
Richie JP Jr, Leutzinger Y, Parthasarathy S, Malloy V, Orentreich N, Zimmerman JA (1994) FASEB J 8:1302–1307
Rodrigues MAM, Sanchez-Negrette M, Mantovani MS, Santana LS, Angeleli AYO, Montenegro MR, de Camargo JLV (1991) Food Chem Toxicol 29:757–764
Ross MH (1976) In: Winick M (ed) Nutrition and Aging. Wiley, New York, pp 43–57
Sanz A, Caro P, Barja G (2004) J Bioenerg Biomembr 36:545–552
Sanz A, Caro P, Ibañez J, Gómez J, Gredilla R, Barja G (2005a) J Bioenerg Biomembr 37:83–90
Sanz A, Gredilla R, Pamplona R, Portero-Otín M, Vara E, Tresguerres JAF., Barja G (2005b) Biogerontol 6:15–26
Sanz A, Caro P, Gómez J, Barja G (2006a) Ann NY Acad Sci (In press)
Sanz A, Caro P, Ayala V, Portero-Otin M, Pamplona R, Barja G (2006b) FASEB J (In press)
Shimokawa I, Higami Y, Yu BP, Masoro EJ, Ikeda T (1996) Aging Clin Exp Res 8:254–262
Yamaki K, Ide T, Takano-Ishikawa Y, Shinohara K (2005) Biosci Biotechnol Biochem 69:13–18
Youngman LD, Park JYK, Ames BN (1992) PNAS USA 89:9112–9116
Zimmerman JA, Malloy V, Krajcik R, Orentreich N (2003) Exp Gerontol 38:47–52