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Effect of Short-Term Caloric Restriction on H_2O_2 Production and Oxidative DNA Damage in Rat Liver Mitochondria and Location of the Free Radical Source[1]

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Oxygen free radicals (ROS) of mitochondrial origin seem to be involved in aging. Whereas in other tissues complexes I or III of the respiratory chain contain the ROS generators, in this study we find that rat liver mitochondria generate oxygen radicals at complexes I, II, and III. Short-term (6 weeks) caloric restriction significantly decreased H_2O_2 production in rat liver mitochondria. This decrease in ROS production was located at complex I because it occurred with complex I-linked substrates (pyruvate/malate), but did not reach statistical significance with the complex II-linked substrate succinate. The mechanism responsible for the lowered ROS production was not a decrease in oxygen consumption. Instead, the mitochondria of caloric-restricted animals released less ROS per unit electron flow. This was due to a decrease in the degree of reduction of the complex I generator. Furthermore, oxidative damage to mitochondrial and nuclear DNA was also decreased in the liver by short-term caloric restriction. The results agree with the idea that caloric restriction delays aging, at least in part, by decreasing the rate of mitochondrial ROS generation and thus the rate of attack to molecules, like DNA, highly relevant for the accumulation of age-dependent changes.

KEY WORDS: Oxidative stress; oxygen radicals; free radical production.

INTRODUCTION

Available information points to reactive oxygen species (ROS) as important determinants of animal aging (Harman, 1956; Sohal and Weindruch, 1996; Barja, 1998; Beckman and Ames, 1998). Mitochondria are considered the main source of ROS production in healthy tissues (Boveris and Chance, 1973). These ROS oxidatively damage cellular proteins, lipids, and DNA. Among those molecular targets, oxidative damage to DNA seems especially important for aging, since repair of the other kinds of macromolecules is finally dependent on the DNA-encoded information.

Location studies have identified the sites of ROS production at complex I (Turrens and Boveris, 1980; Hansford *et al.*, 1997; Herrero and Barja, 1997a,b, 1998; Barja and Herrero, 1998) and complex III (Boveris *et al.*, 1976; Turrens *et al.*, 1985; Herrero and Barja, 1997a,b, 1998) in heart mitochondria. Few investigations have approached the topic in other organs like the brain (Barja and Herrero, 1998) and there is no information on the subject, using specific methods, in the liver.

Caloric restriction is the only known experimental manipulation that prolongs life span and delays the incidence of many age-related diseases (Sohal and Weindruch, 1996). Although the effects of caloric restriction are well established, the mechanisms underlying them are not fully understood, and several hypotheses have been proposed (Sohal and Weindruch, 1996; Wanagat *et al.*, 1999). On the other hand, previous comparative studies have consistently shown that the rate of mitochondrial ROS production is lower in long-lived than in short-lived animals (Sohal *et al.*, 1990; Ku *et al.*, 1993; Barja *et al.*, 1994;

[1] Key to abbreviations: dG, deoxyguanosine; 8-oxodG, 8-oxo-7,8-dihydro-2' deoxyguanosine; ROS, reactive oxygen species; TTFA, thenoyltrifluoroacetone.

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Herrero and Barja, 1997b, 1998; Barja and Herrero, 1998; Barja, 1999a). Therefore, it is interesting to test if a decrease in mitochondrial ROS production is also involved in the life-extension effect of caloric restriction. Only one previous investigation has studied this possibility (Sohal *et al.*, 1994a), it dealt with medium- or long-term restriction, and it did not include liver tissue. The liver plays a central role in mammalian metabolism and is very susceptible to diet variations. Thus, the effect of short-term caloric restriction on the rate of H_2O_2 generation of rat liver mitochondria was studied in the present investigation. In order to ascertain if putative changes in ROS generation affect the genetic material, the marker of oxidative DNA damage 8-oxo-7,8-dihydro-2[1] deoxyguanosine (8-oxodG) was also measured both in mitochondrial and nuclear hepatic DNA.

MATERIALS AND METHODS

Animals and Treatments

Nine-week-old male Wistar rats were obtained from Iffa-Creddo (Lyon, France). They were caged individually and were maintained at $22 \pm 2^{\circ}$C, 12:12 (light:dark) cycle and $50 \pm 10\%$ relative humidity. Control animals were fed *ad libitum.* Restricted animals received daily 60% of the food intake of the controls (40% energy restriction). After 6 weeks of treatment, animals were sacrificed by decapitation. Liver samples were stored at -80°C for later 8-oxodG analyses. For liver mitochondrial H_2O_2 production determinations, the fresh organ was directly processed.

Isolation of Liver Mitochondria

After decapitation the liver was immediately processed to obtain functional mitochondria. The liver was rinsed and fat was removed before homogenization 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 then centrifuged at $10{,}000 \times g$ for 10 min and supernatants and fat eliminated. Pellets were resuspended in 40 ml of isolation buffer without EDTA and centrifuged at $1000 \times g$ for 5 min. Liver mitochondria were obtained after centrifugation of supernatants at $10{,}000 \times g$ for 10 min. 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.

Mitochondrial Oxygen Consumption

Oxygen consumption was measured at 37°C in a water-thermostated incubation chamber with a computer-controlled Clark-type 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) with 2.5 mM pyruvate/2.5 mM malate or 5 mM succinate ($+0.2$ μM rotenone) as substrates, in the absence (State 4) and in the presence (State 3) of 500 μM ADP and at 2.5 mg mitochondrial protein in the electrode chamber. The respiratory control ratio with succinate as substrate was 3.6 ± 0.17 in control and 3.8 ± 0.17 in restricted mitochondria ($n = 7$ per group).

Mitochondrial H_2O_2 Production

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 (Ruch *et al.*, 1983; Barja, 1999b). Reaction conditions were 0.25 mg of mitochondrial protein per ml, 6 U/ml of horse radish peroxidase, 0.1 mM homovanillic acid, 50 U/ml of superoxide dismutase, and 2.5 mM pyruvate/2.5 mM malate or 5 mM succinate ($+0.2$ μM rotenone) as substrates, which were added at the end to start the reaction to the same incubation buffer was used for oxygen consumption, at 37°C, total volume 1.5 ml. All the assays with succinate as substrate were performed in the presence of rotenone in order to avoid backward flow of electrons to complex I. In some experiments, rotenone (2 μM), antimycin A (2 μM) or thenoyltrifluoroacetone (TTFA; 11 μM) were additionally included in the reaction mixture to assay maximum rates of complex I, complex II + III, and complex II H_2O_2 generation, respectively. After 15 min of incubation the reaction was stopped by transferring the samples to a cold bath; 0.5 ml of 0.1 M glycine–25 mM EDTA–NaOH, pH 12 were added (Ruch *et al.*, 1983), 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.

Mitochondrial Free Radical Leak

Mitochondrial H_2O_2 production and oxygen consumption were measured in parallel in the same samples under the same experimental conditions. This allowed the calculation of the fraction of electrons out of sequence, which reduce O_2 to oxygen radicals at the respiratory chain

(the free radical leak) instead of reaching complex IV to reduce O_2 to water. Since two electrons are needed to reduce one molecule of O_2 to H_2O_2, whereas four electrons are transferred in the reduction of one molecule of O_2 to water, the percentage free radical leak was calculated as the rate of H_2O_2 production divided by two times the rate of O_2 consumption; the result was multiplied by 100.

Isolation and Digestion of Mitochondrial and Nuclear DNA

Nuclear DNA was isolated after homogenization, resuspension of nuclear pellets of liver samples, and SDS treatment, by chloroform extraction and ethanol precipitation (Loft and Poulsen, 1999). Mitochondrial DNA, free of nuclear DNA, was isolated by the method of Latorre *et al.* (1986) with some modifications (Asunción *et al.*, 1996; Barja and Herrero, 2000). All aqueous solutions used for DNA isolation, digestion, and chromatographic separation were prepared in HPLC-grade water (Fischer Chemicals, Loughborough, UK). Isolated nuclear and mitochondrial DNA were digested to deoxynucleoside level by incubation at 37°C with 5 units of nuclease P1 (in 20 μl of 20 mM sodium acetate, 10 mM $ZnCl_2$, 15% glycerol, pH 4.8) during 30 minutes and with 1 unit of alkaline phosphatase (in 20 μl of 1 M Tris–HCl, pH 8.0) for 1 h (Loft and Poulsen, 1999).

8-oxodG and dG Assays in Nuclear and Mitochondrial DNA

The concentrations of 8-oxodG and deoxyguanosine (dG) were measured by HPLC with online electrochemical and ultraviolet detection, respectively. For analyses, the nucleoside mixture was injected into a reverse-phase Beckman Ultrasphere ODS column (5 μm, 4.6 mm × 25 cm), eluted with 2.5% acetonitrile in 50 mM phosphate buffer, pH 5. The amount of deoxynucleosides injected was higher than the minimum needed to avoid potential artifacts due to injection of small quantities of deoxynucleosides in the HPLC system (Beckman and Ames, 1996). A Waters 510 pump at 1 ml/min was used. 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) detected 8-oxodG 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 of 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 analysis.

Table I. Rate of H_2O_2 Generation of Rat Liver Control Mitochondria under Different Conditions[a]

	Mitochondrial H_2O_2 production (nmoles H_2O_2/min mg protein)
Pyruvate/malate	0.22 ± 0.02
Pyruvate/malate + rotenone	$1.03 \pm 0.1^{b,d}$
Succinate	$0.71 \pm 0.1^{b,d}$
Succinate + TTFA	$2.88 \pm 0.39^{c,d}$
Succinate + antimycin A	$3.64 \pm 0.32^{c,d}$

[a] Values are means ± SEM from 6 to 7 animals. TTFA, thenoyltrifluoroacetone.
[b] Significant difference in relation to pyruvate/malate.
[c] Significant difference in relation to succinate.
[d] $p < 0.001$.

Statistical Analyses

Data were statistically analyzed with Student's *t*-tests. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

RESULTS

The rate of H_2O_2 production of control liver mitochondria with pyruvate/malate as substrates was significantly increased by the addition of rotenone (Table I). TTFA addition to succinate-supplemented liver mitochondria significantly increased H_2O_2 generation (Table I). The same was true for antimycin A, which significantly increased H_2O_2 production in relation to that with succinate alone (Table I). The rate of H_2O_2 generation was also significantly higher with succinate than with pyruvate/malate (Table I).

The rate of mitochondrial oxygen consumption was measured with pyruvate/malate or succinate as substrates in States 4 and 3. In any case, no significant differences were observed between control and restricted rats (Table II).

Table II. Oxygen Consumption of Rat Liver Mitochondria Supplemented with Pyruvate/Malate or Succinate in States 4 and 3[a]

	Oxygen consumption (nmoles O_2/min mg protein)			
	Pyruvate/malate		Succinate	
	State 4	State 3	State 4	State 3
Control	9.33 ± 0.71	30.54 ± 2.64	21.63 ± 1.56	77.37 ± 7.29
Restricted	9.67 ± 0.47	33.69 ± 2.22	24.38 ± 1.20	92.44 ± 5.44

[a] Values are means ± SEM from 6 to 7 animals.

The rate of H_2O_2 production with pyruvate/malate as substrates was significantly lower in the restricted animals than in the control ones (Fig. 1a). However, rotenone-inhibited pyruvate/malate-supplemented H_2O_2 generation did not show significant differences between control (1.03 ± 0.1 nmoles H_2O_2/min mg protein) and restricted (0.93 ± 0.1 nmoles H_2O_2/min mg protein) mitochondria. On the other hand, succinate-supported H_2O_2 production did not show significant differences between restricted and control animals (Fig. 2a).

The free radical leak with pyruvate/malate as substrate was significantly decreased by caloric restriction (Fig. 1b). However, no significant differences between control and restricted rats were observed with succinate as substrate (Fig. 2b).

Restricted animals showed significantly lower levels of 8-oxodG in liver nuclear DNA than control animals (Fig. 3a). Levels of 8-oxodG were also measured in the mitochondrial DNA and short-term caloric restriction also significantly decreased the levels of this oxidative damage marker in rat liver (Fig. 3b).

DISCUSSION

The results of this investigation show that short-term caloric restriction decreases mitochondrial ROS production and oxidative damage to mitochondrial and nuclear DNA in rat liver. They also identify the site of the respiratory chain and the mechanism responsible for the decrease in free radical generation.

Various respiratory chain inhibitors were used in this study to locate the free radical generation site on rat liver mitochondria. The rate of free radical production increases as a function of the degree of reduction of the autoxidizable electron carriers (Boveris and Chance, 1973). When the respiratory chain is blocked with an inhibitor, the degree of reduction of the electron carriers situated on the substrate side of the inhibitor strongly increases, whereas those in the oxygen side become oxidized. Thus, an increase in mitochondrial ROS production after addition of an inhibitor indicates that the free radical generation site is located on the substrate side in relation to the inhibitor. On the other hand, a decrease in ROS production after addition of an inhibitor means that the free radical generator is situated on the oxygen side.

In this work, TTFA addition to succinate-supplemented rat liver mitochondria significantly increased mitochondrial H_2O_2 production. Thus, in rat liver mitochondria, complex II seems to be an important free radical generator, in agreement with studies in purified complexes (Zhang *et al.*, 1998). This is described here for the first time in intact mitochondria. The increase brought about by rotenone on H_2O_2 production with pyruvate/malate as substrate indicates that complex I is also involved in oxygen free radical generation in the rat liver mitochondria. Antimycin A addition to succinate-supplemented mitochondria strongly increased H_2O_2 production to a greater extend than TTFA, pointing also to complex III as another mitochondrial ROS generator in this tissue. The observation of higher H_2O_2 production with succinate than with pyruvate/malate also indicates that complex II is involved in ROS production because with succinate electrons flow through complexes II and III whereas with pyruvate/malate they flow through complexes I and III. Thus, the results obtained in this work indicate that complexes I, II, and III contribute to H_2O_2 production in rat liver mitochondria. Previous results have shown that heart mitochondria produce ROS at complex I (Takeshigue and Minakami, 1979; Turrens and Boveris, 1980; Herrero and Barja, 1997a, b, 1998; Hansford *et al.*, 1997) and at complex III (Boveris *et al.*, 1976; Turrens *et al.*, 1985; Nohl *et al.*, 1996; Herrero and Barja, 1997b, 1998), whereas nonsynaptic brain mitochondria produce them only at complex I (Barja and Herrero, 1998). Therefore, the situation of the ROS generator in the respiratory chain varies depending on the tissue used as mitochondrial source.

Short-term caloric restriction (6 weeks) significantly decreased ROS production of rat liver mitochondria. This result agrees with the hypothesis that caloric restriction prolongs life span and delays the rate of aging by reducing the rate of oxidative attack to tissue macromolecules. The theoretical role of ROS production in the free radical theory of aging is that of a cause of aging. On the other hand, the magnitude of the life-extension effect is proportional to the time of restriction and aging is a progressive and accumulative process. Thus, the mechanism causing the caloric restriction effect should be a rather early change. This agrees with our observation that ROS production decreases in only 6 weeks in the restricted animals. Besides, when oxygen free radical production was comparatively analyzed in different animals, it was always lower in long-lived than in short-lived species (Sohal *et al.*, 1990; Ku *et al.*, 1993; Herrero and Barja, 1997a,b, 1998; Barja and Herrero, 1998; Barja, 1999a). Thus, a low rate of ROS production can be involved both in the life-extension effect of caloric restriction and in the determination of the slow aging rate of long-lived animals. There is only one previous work specifically measuring mitochondrial H_2O_2 production after caloric restriction (Sohal *et al.*, 1994a). This study was performed in brain, heart, and kidney mitochondria from mice and also found decreases in ROS generation after 8 months of caloric restriction in the three organs.

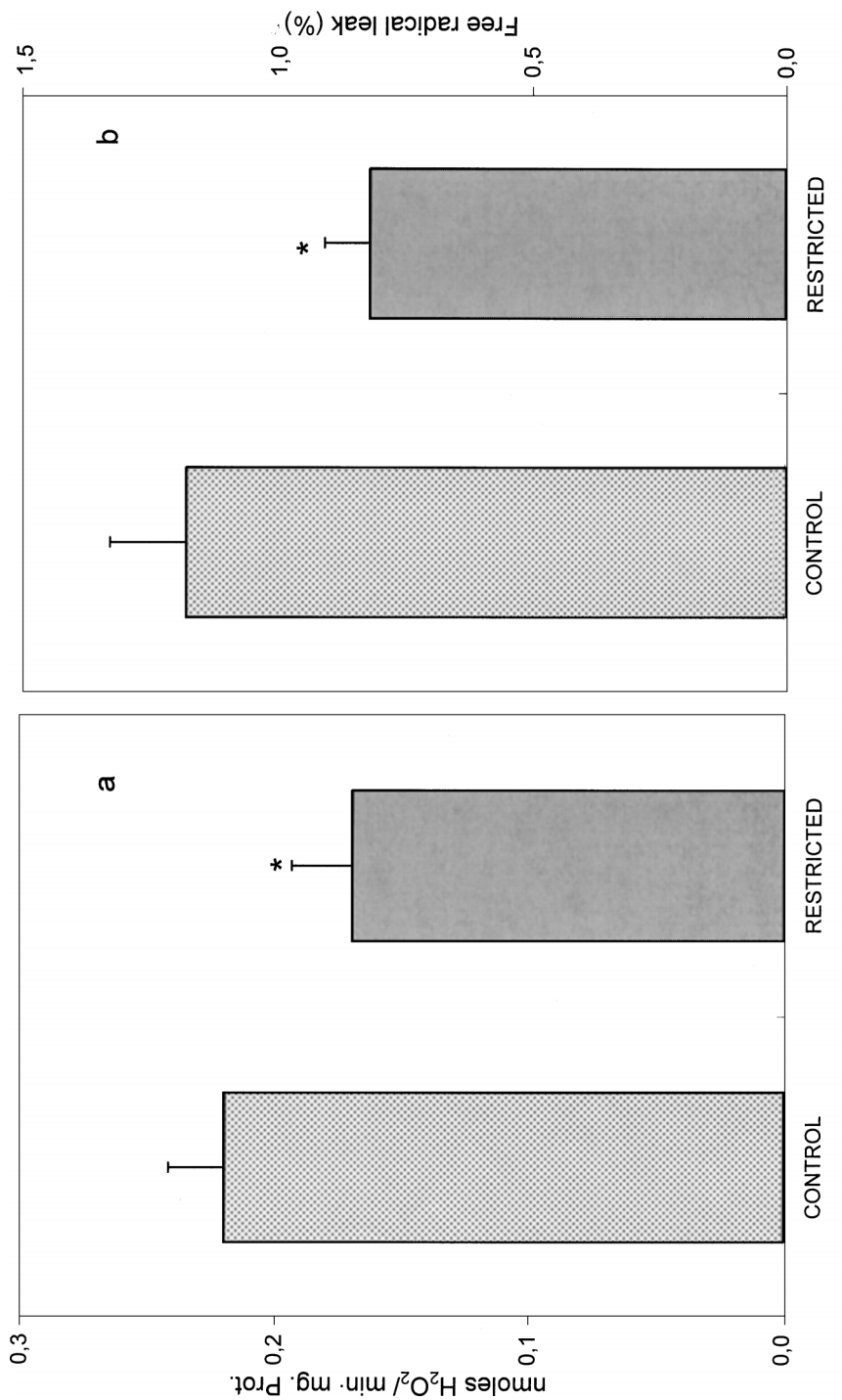

Fig. 1. Effect of short-term caloric restriction on H_2O_2 generation (a) and free radical leak (b) of rat liver mitochondria using pyruvate/malate as substrates. Data are means ± SEM from 6 to 7 animals. Asterisks represent significant differences between control and restricted animals. $^* p < 0.05$.

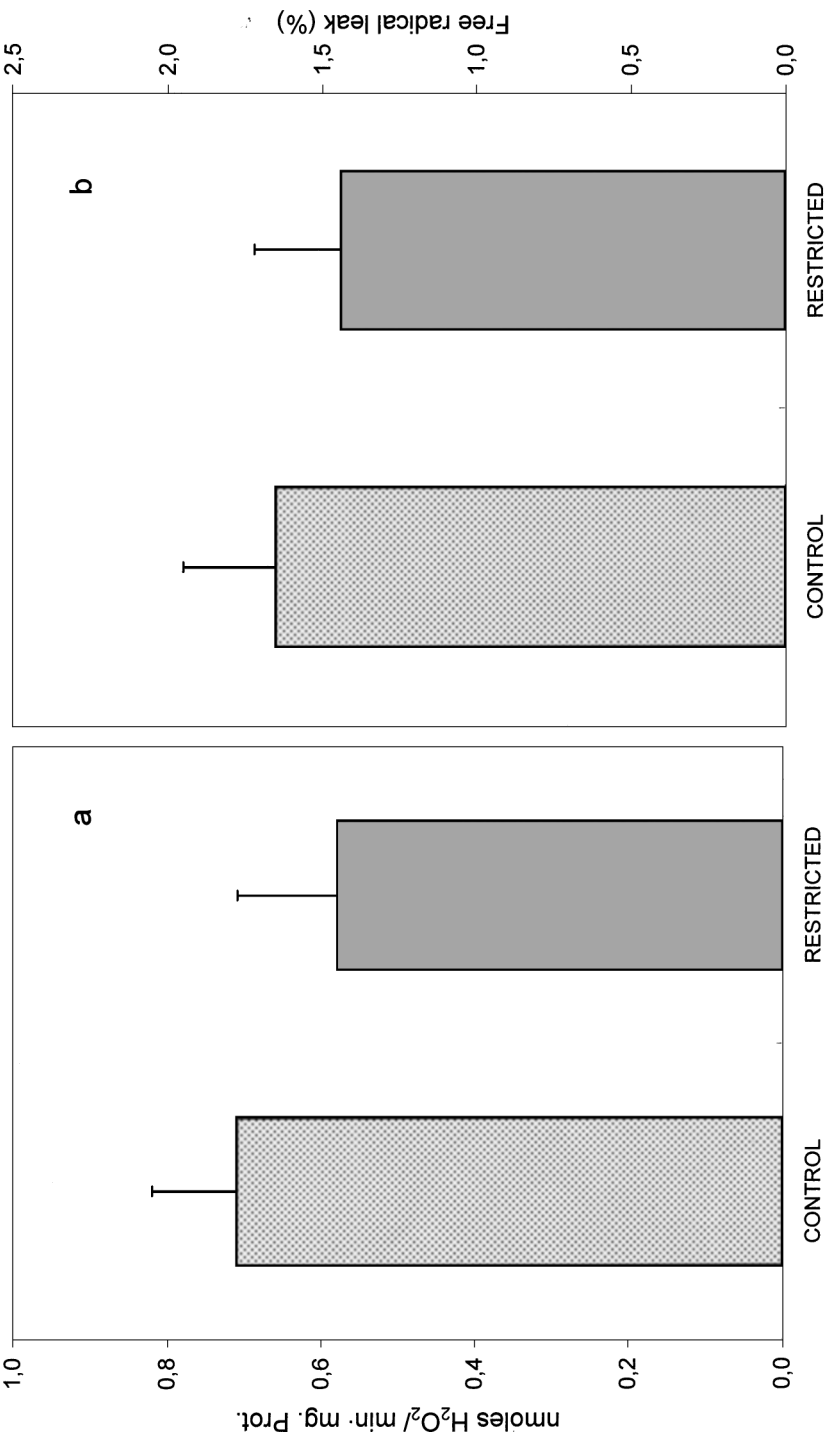

Fig. 2. Effect of short-term caloric restriction on H_2O_2 generation (a) and free radical leak (b) of rat liver mitochondria using succinate as substrate. Data are means ± SEM from 6 to 7 animals.

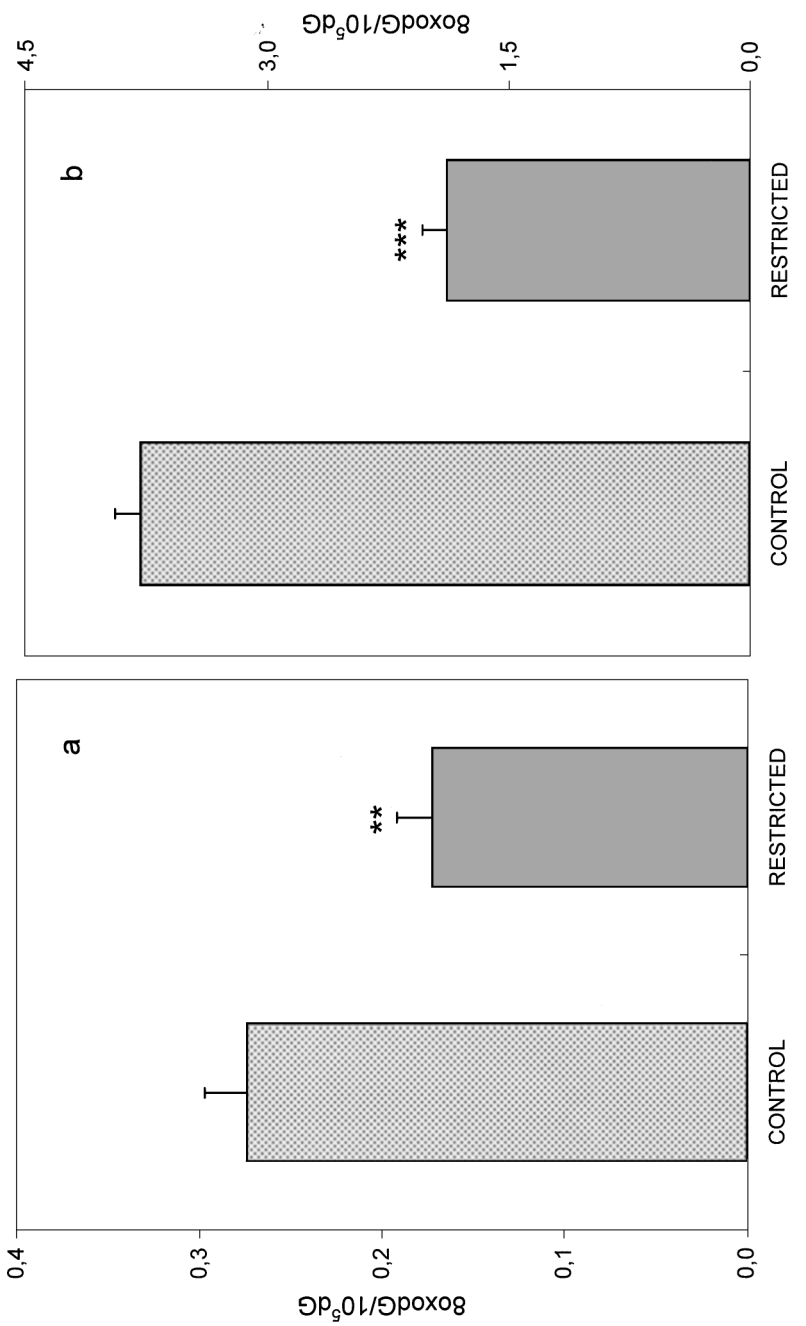

Fig. 3. Effect of short-term caloric restriction on oxidative damage to liver nuclear (a) and mitochondrial (b) DNA measured as 8-oxodG. Data are means ± SEM from 7 animals. Asterisks represent significant differences between control and restricted animals. ** $p < 0.01$; *** $p < 0.001$.

Neither the location of the site in the respiratory chain responsible for the decrease in ROS production or the mechanism allowing it have been previously investigated. In the only previous caloric restriction study available dealing with mitochondrial ROS generation (Sohal *et al.*, 1994a) that location was not possible because only one substrate (succinate) was used, and it was added to mitochondria in the absence of rotenone. In such situation, electrons flow from succinate to complex II and complex III, but also backward to complex I, making impossible to discriminate which of these complexes is responsible for the caloric restriction effect. In the present study, we used both succinate and pyruvate/malate as substrates. With succinate (plus rotenone), the electrons flow through the ROS generators of complexes II and III, whereas with pyruvate/malate they flow through complexes I and III. Since caloric restriction decreased H_2O_2 production with pyruvate/malate but did not reach statistical significance with succinate (plus rotenone), CR must decrease ROS generation at a complex I site, although lack of significance does not unequivocally demonstrate that complexes II or III are not involved.

Concerning the mechanism of action of caloric restriction, whereas some favor a hypometabolic hypothesis (Greenberg and Boozer, 2000; Orr and Sohal, 2000), others have shown that total body 24 h metabolic activity does not change in caloric-restricted rats (McCarter *et al.*, 1985). Our results are consistent with the second of these interpretations because mitochondrial oxygen consumption was not modified by caloric restriction either in States 4 or in 3. What decreased was the percentage of total electron flow leading to ROS generation at complex I instead of reaching cytochrome oxidase to reduce oxygen to water, i.e., the free radical leak was decreased in caloric restriction. This means that restricted mitochondria can release less ROS per unit electron flow in the respiratory chain. One mechanism allowing this was clarified in the present investigation. The rate of ROS production decreased in the restricted mitochondria with pyruvate/malate, a situation in which complex I is only partially reduced. Addition of rotenone to pyruvate/malate-supplemented mitochondria causes 100% reduction of complex I. Interestingly, the difference in ROS production between restricted and *ad libitum*-fed animals disappeared after addition of rotenone to pyruvate/malate-supplemented mitochondria. This means that restricted mitochondria have lower ROS production and free radical leak because the degree of reduction of their complex I generator is lower than in control mitochondria.

In the present study oxidative damage to mitochondrial and nuclear DNA were also measured. Both kinds of DNA showed lower levels of oxidative damage measured as 8-oxodG in caloric-restricted than in *ad libitum*-fed animals. A single previous study (Chung *et al.*, 1992) has also found a decrease in 8-oxodG in rat liver mitochondrial DNA, although this was observed after long-term caloric restriction. Taking into account the strong proximity of mitochondrial DNA to the inner mitochondrial membrane, it is most probable that the decrease in mitochondrial ROS production induced by caloric restriction is responsible for the decrease in 8-oxodG in mitochondrial DNA, which can be involved in the antiaging effect of this experimental manipulation. A decrease in mitochondrial DNA mutations has been also described in rat liver after caloric restriction (Kang *et al.*, 1998). In any case, this is the first report simultaneously describing decreases in ROS production and in oxidative DNA damage in the mitochondria after caloric restriction. Concerning nuclear DNA, other authors have detected, like us, the decrease in 8-oxodG induced by caloric restriction in rat liver (Chung *et al.*, 1992; Kaneko *et al.*, 1997), although their experiments concern exclusively long-term restriction. In another report, no significant differences in 8-oxodG were observed between control and restricted animals in mouse liver (Sohal *et al.*, 1994b). Decreased levels of 5-hydroxymethyluracil have been also reported after caloric restriction in rat liver genomic DNA (Djuric *et al.*, 1992). Since caloric-restricted mitochondria secrete less H_2O_2 to the outside and H_2O_2 is a relatively stable molecule, which can diffuse throughout the cell, it is possible that the decrease in 8-oxodG in nuclear DNA is also due, at least in part, to the decrease in mitochondrial H_2O_2 production. However, the possibility can not be ruled out that increases in repair of 8-oxodG in the nuclear DNA of the restricted animals are involved.

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