Short-Term Caloric Restriction and Sites of Oxygen Radical Generation in Kidney and Skeletal Muscle Mitochondria

RICARDO GREDILLA,[a] SHARON PHANEUF,[b] COLIN SELMAN,[b]
SUMA KENDAIAH,[b] CHRISTIAAN LEEUWENBURGH,[b] AND GUSTAVO BARJA[a]

[a]*Department of Animal Biology–II (Animal Physiology), Faculty of Biology, Complutense University, 28040 Madrid, Spain*

[b]*Biochemistry of Aging Laboratory, College of Health and Human Performance, University of Florida, Gainesville, Florida 32611, USA*

ABSTRACT: Mitochondrial free radical generation is believed to be one of the principal factors determining aging rate, and complexes I and III have been described as the main sources of reactive oxygen species (ROS) within mitochondria in heart, brain, and liver. Moreover, complex I ROS generation of heart and liver mitochondria seems especially linked to aging rate both in comparative studies between animals with different longevities and in caloric restriction models. Caloric restriction (CR) is a well-documented manipulation that extends mean and maximum longevity. One of the factors that appears to be involved in such life span extension is the reduction in mitochondrial free radical generation at complex I. We have performed two parallel investigations, one studying the effect of short-term CR on oxygen radical generation in kidney and skeletal muscle (gastrocnemius) mitochondria and a second one regarding location of mitochondrial ROS-generating sites in these same tissues. In the former study, no effect of short-term caloric restriction was observed in mitochondrial free radical generation in either kidney or skeletal muscle. The latter study ruled out complex II as a principal source of free radicals in kidney and in skeletal muscle mitochondria, and, similar to previous investigations in heart and liver organelles, the main free radical generators were located at complexes I and III within the electron transport system.

KEYWORDS: reactive oxygen species; mitochondria; caloric restriction

INTRODUCTION

Mitochondria are the most important sources of reactive oxygen species (ROS) in healthy tissues because the main generator, the electron transport chain, is located at the inner mitochondrial membrane. They are also important targets of oxidative damage and are considered one of the main determinants of aging.[1–5] Mitochondrial

Address for correspondence: Dr. Gustavo Barja, Departmento de Biología Animal–II, Facultad de Biología, Universidad Complutense, Madrid 28040, Spain. Voice: +34-91-394-49-19; fax: +34-91-394-490-35.
gbarja@bio.ucm.es

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free radical generation and the ensuing accumulation of oxidative damage can be responsible for the age-related decline in maximum functional capacities of tissues.

Caloric restriction (CR) is the only known nongenetic manipulation that extends maximum longevity in a wide variety of animals. In the last years, new insights about the underlying mechanism involved in the CR life-extension effect have been reported. Thus, CR decreases oxidative damage and its deleterious effects during aging, at least in part by decreasing mitochondrial oxygen radical production.[6–9] The magnitude of such reduction seems to depend on implementation time and the specific tissue, probably due to sensitivity differences to CR regimen.[10–13]

Although there is general agreement pointing to mitochondrial free radical generation as one of the principal causes of aging, controversy still exists concerning the sources of those reactive species within mitochondria.

Pioneer studies on the location of mitochondrial ROS-generating sites specially emphasized complex III as the major contributor to mitochondrial ROS production,[14] although contribution of complex I was suggested by others based on studies in submitochondrial particles.[15] During many decades, the view that complex III was the main or even the only source of ROS within mitochondria prevailed. However, recent investigations unequivocally show that complex I generates ROS in intact functional mitochondria;[16–21] see Barja[4] for review). Furthermore, complex I seems to play a decisive role in aging as deduced from caloric restriction investigations and comparative studies between birds and mammals. We have reported recently that the decrease in mitochondrial free radical production observed in animals subjected to CR takes place exclusively at complex I both in heart and liver mitochondria.[7–9] Regarding comparative studies, the lower mitochondrial ROS generation observed in long-lived birds versus short-lived rodents also was reported to occur mainly at complex I.[16–18] The importance of complex I ROS generation is also supported by its implication in the development of age-related pathologies such as Parkinson disease[22,23] However, most of the investigations described above were performed in tissues like heart (mainly) and liver, and almost no information exists in skeletal muscle (a postmitotic tissue) and kidney (the other mainly responsible for age related death in rodents).

In the current investigation, we studied the effect of short-term caloric restriction on mitochondrial free radical generation in kidney and skeletal muscle as well as the location of free radical generators sites.

MATERIALS AND METHODS

Animals and Experimental Design

Caloric Restriction Study

Both *ad libitum* and caloric restricted male Fischer 344 rats were obtained from the National Institute of Aging colony (Indianapolis, IN, USA) at 4 months of age. Caloric restriction was started at 3.5 months of age (10% restriction), increased to 25% restriction at 3.75 months of age, and maintained from 4 months at 40% restriction until the termination of the experiment at 6 months of age. From 4 months onward, all animals were housed individually under a 12-hour-light/12-hour-dark

photoperiod and an ambient temperature of 18–20°C. After 8 weeks of acclimation, two animals per day (one *ad libitum* and one caloric restricted) were killed after anesthesia with haloperidol.

Location Study

Male Fischer 344 rats were obtained from Iffa Credo (Lyon, France) several weeks before being killed. Rats were fed *ad libitum* and caged individually under a 12-hour-light/12-hour-dark photoperiod and an ambient temperature of 18–20°C. Animals were killed by decapitation at 6 months of age.

Mitochondrial Isolation

Mitochondria were subsequently isolated from kidney and gastrocnemius muscle by differential centrifugation. Gastrocnemious muscles were immediately dissected and minced after decapitation. Homogenization was performed in isolation buffer A (225 mM mannitol, 75 mM sucrose, 1 mM EDTA, 0.2% BSA, pH 7.4) in a 1:10 dilution (wt/vol). The homogenate was centrifuged at 700*g* for 10 minutes, and the resulting supernatant was centrifuged twice at 8,000 *g* for 10 minutes. The mitochondrial pellet was resuspended in isolation buffer A without BSA. Kidneys were rapidly removed and homogenized in isolation buffer B (210 mM Mannitol, 70 mM sucrose, 5 mM Hepes, 1 mM EDTA, pH 7.35). The procedure performed to obtain the mitochondrial pellet was the same used in skeletal muscle. The mitochondrial pellet was resuspended in isolation buffer B without EDTA. All the isolation procedures were performed at 4°C. Mitochondrial protein was measured by the Biuret method.

Mitochondrial H_2O_2 Generation and Oxygen Consumption

The rate of mitochondrial H_2O_2 production was assayed 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.[32] The assays were performed in incubation buffer (145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% BSA, pH 7.4), and the reaction conditions were 0.25 mg/mL of mitochondrial protein, 6 U/mL of horseradish peroxidase, and 0.1 mM homovanillic acid (total volume 1.5 mL). The reactions were started by the addition of 2.5 mM pyruvate/2.5 mM malate or 5 mM succinate as substrates. Some of the assays with succinate were performed in the presence of 2 μM rotenone to avoid the backward flow of electrons to complex I. In some assays, 2 μM antimycin A and 11 μM thenoyltrifluoroacetone (TTFA) were added to succinate + rotenone-supplemented mitochondria, and 2 μM rotenone was added to pyruvate/malate-supplemented mitochondria to determinate the maximum rates of H_2O_2 production of complexes III, II, and I, respectively. After 15 min of incubation at 37°C, the reaction was stopped transferring the samples to a cold bath and adding 0.5 mL of 0.1 M glycine containing 25 mM EDTA-NaOH (pH 12.0). Known amounts of H_2O_2 produced by glucose oxidase with glucose as substrate were used as standards. In the CR study, fluorescence was determined using a fluorescent microplate reader (GeminiXS; Molecular Devices, Sunnyvale, CA), whereas in the location study the fluo-

rescence of the total reaction volume (2 mL) was measured using a LS50B Perkin Elmer fluorometer (Buckinghamshire, UK).

Mitochondrial oxygen consumption was measured in a Clark-type O_2 electrode (Oxygraph, Hansatech, Norfolk, UK) in the absence (State 4) and in the presence (State 3) of ADP (500 μM) in the same conditions used for H_2O_2 measurements. All the assays were performed within 2 hours after the isolation of mitochondria.

Statistical Analyses

Comparisons were statistically analyzed with Student's *t* tests. The minimum level of statistical significance was set at $P < .05$ for all the analyses.

RESULTS

Caloric Restriction: Mitochondrial ROS Generation and Oxygen Consumption

Well-coupled mitochondria were isolated from kidney and skeletal muscle as indicated by the respiratory control ratio (RCR). In pyruvate/malate-supplemented skeletal muscle mitochondria, RCR values were 10.94 ± 1.65 and 11.46 ± 1.35 for *ad libitum* and CR group, respectively; in kidney, RCR values were 14.74 ± 1.07 in *ad libitum* and 14.03 ± 1.59 in the restricted group. Caloric restriction did not change oxygen consumption in either state 4 or state 3 in kidney or skeletal muscle mitochondria (data not shown). Mitochondrial ROS generation was measured in skeletal muscle mitochondria respiring with complex I–linked substrates (pyruvate/malate). However, because we did not detect ROS production in kidney mitochondria using pyruvate/malate as substrates, we supplemented those particular mitochondrial suspensions with a complex II–linked substrate (succinate). No changes in H_2O_2 production were found between the two dietary groups either in skeletal muscle or in kidney mitochondria (data not shown).

Location of ROS-Generating Sites within Mitochondria

Detectable amounts of H_2O_2 were released by kidney and skeletal muscle mitochondria in basal conditions when pyruvate/malate or succinate were added as substrates (FIGS. 1 and 2). Measurement of mitochondrial oxygen consumption allowed us to test that the mitochondrial suspensions were well coupled (TABLE 1).

When rotenone was added to pyruvate/malate supplemented mitochondria, ROS generation was significantly increased in both tissues ($P < .001$; FIG. 1A, B). Further

TABLE 1. Location study: oxygen consumption of pyruvate/malate-supplemented mitochondria in kidney and skeletal muscle (gastrocnemius)

	State 4	State 3	RCR
Skeletal muscle	15.63 ± 1.60	131.68 ± 3.86	8.68 ± 0.85
Kidney	18.19 ± 3.34	119.36 ± 22.73	6.53 ± 0.35

[a]Results are means ± SEM from four different animals and were expressed in nmoles O_2/min·mg protein.

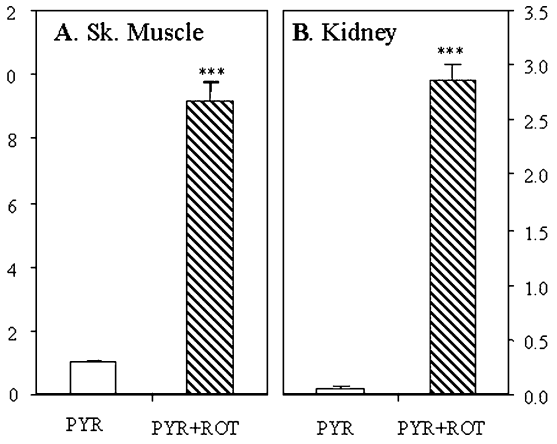

FIGURE 1. Location study. Effect of rotenone (ROT) on the basal rates of H_2O_2 production (nmoles H_2O_2/min · mg prot) with pyruvate/malate (PYR) as substrate in skeletal muscle (**A**) and kidney (**B**) mitochondria. Results are means ± SEM from four different animals. Asterisks represent statistical significance comparing H_2O_2 production with and without rotenone (ROT) in the same tissue and substrate. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

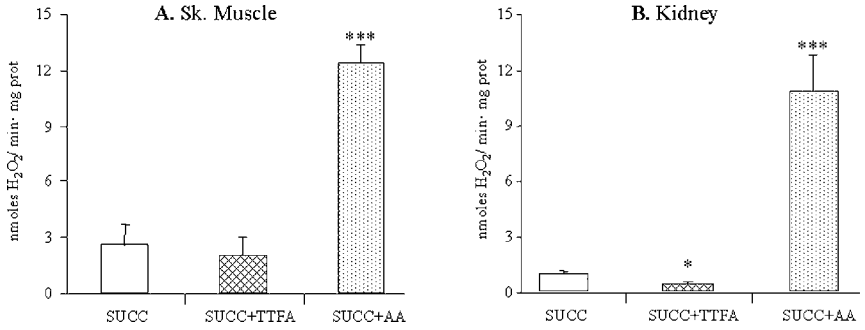

FIGURE 2. Location study. Effect of thenoyltrifluoroacetone (TTFA) and antimycin A (AA) on the basal rates of H_2O_2 production with succinate (SUCC) as substrate in skeletal muscle (**A**) and kidney (**B**) mitochondria. Rotenone was added with succinate in all the assays. Results are means ± SEM from four different animals. Asterisks represent statistical significance comparing H_2O_2 production with and without AA or TTFA in the same tissue and substrate. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

indications about the role of complex I in ROS generation were obtained after supplementing mitochondria with succinate. Similar to what has been observed previously,[4] H_2O_2 production was strongly decreased when rotenone was added to succinate-supplemented kidney or skeletal muscle mitochondria (data not shown). ROS generation was not increased after the addition of TTFA to succinate plus srotenone-supplemented mitochondria in either kidney or skeletal muscle (FIG. 2A, B); it was not changed in skeletal muscle, and it was even decreased in kidney after TTFA addition. Finally, Antimycin A strongly increased H_2O_2 generation when added to succinate plus rotenone-supplemented mitochondria (FIG. 2A, B) in both tissues.

DISCUSSION

Long-term caloric restriction has been shown to extend maximum life span, at least in part, by decreasing mitochondrial free radical generation.[6,8,9,24] It is noteworthy that whenever CR was implemented in long-term experiments by different investigators, a decrease in the rate of ROS generation has been always observed (see Barja[5] for review). However, when short-term CR has been studied, different results have been reported in free radical generation rate depending on the tissue. Thus, significant decreases in mitochondrial ROS production has been described to occur in liver,[7] whereas the trends to decrease were observed in heart after 6 weeks[8] and 4 months[12] of CR did not reach statistical significance. In this investigation, short-term caloric restriction (2 months) was not enough to decrease ROS generation either in kidney or skeletal muscle mitochondria.

Because we did not detect any H_2O_2 production in kidney mitochondria in the CR study, when pyruvate/malate was used as substrate, we supplemented mitochondria with succinate. Mitochondrial ROS generation with succinate in the absence of rotenone has been reported to be higher than with pyruvate/malate as substrate.[9,21,25] The lack of detectable amounts of mitochondrial H_2O_2 production in kidney could be caused by a sensitivity problem because the assays of the CR study were performed in a fluorescent microplate reader and only a fraction of the total reaction volume (300 μL out of 2,000) could be used in the measurements. This decreases sensitivity by sevenfold. Indeed, free radical generation in pyruvate/malate-supplemented kidney mitochondria was detected when we investigated the location of ROS-generating sites within mitochondria. In this location study, we read the fluorescence of the whole reaction volume (2 mL) in a fluorometer cuvette. This suggests that the whole volume of reaction should be used to avoid sensitivity losses, especially when rates of free radical generation are low. When using succinate in the absence of rotenone, electrons flow from complex II to complex III but also to complex I through reverse electron flow. In such conditions, possible sensitivity problems were avoided, and we could study the effects of the CR regimen on ROS generation at complexes I and III.

The only previous study investigating dietary restriction and ROS generation in kidney mitochondria used succinate as substrate without rotenone addition in mice.[6] Although in that study decreases in mitochondrial ROS generation were observed in CR animals, the implementation times were longer than those used in the current investigation. It has been reported that the implementation time has a strong effect

in dietary restriction studies.[10,11] The lack of effect on H_2O_2 generation observed here after only 8 weeks can be because of such time dependence.

Concerning skeletal muscle, a previous investigation reported a decrease in superoxide production after long-term CR in mice submitochondrial particles.[24] However, a recent study in functional isolated mitochondria showed that caloric restriction only tended (nonsignificantly) to lower H_2O_2 generation in skeletal muscle of the restricted rats.[26] The current study reports no significant changes in mitochondrial ROS generation in skeletal muscle after 2 months of restriction. However, the mitochondrial rate of H_2O_2 generation observed in restricted animals was 11% lower than in *ad libitum* fed rats. The magnitude of such reduction interestingly is very similar to that found in rat heart mitochondria after 6 weeks or 4 months (15% and 13%, respectively) of CR.[12] It is possible that tissues differ in their sensitivity to CR implementation times; decreases on mitochondrial free radical generation after short-term CR would be easily observed in organs such as the liver (more affected by digestive and metabolic changes), whereas in heart and skeletal muscle such decrease would not reach statistical significance. Thus, longer CR time periods would be needed to observed statistical significant decreases in those muscular tissues.

Regarding the sites where mitochondrial ROS generation took place, results were similar to previous studies in other tissues. Various respiratory chain inhibitors were used to localize the free radical generator sites. The rate of free radical production in the respiratory chain increases as a function of the degree of reduction of the autoxidizable electron carriers.[27] When the respiratory chain is blocked with an inhibitor, the degree of reduction of the electron carriers situated on the substrate side strongly increases, whereas those on the oxygen side become oxidized. Thus, an increase in ROS production after the addition of an inhibitor indicates that the free radical generator site is located on the substrate side in relation to the inhibitor. On the other hand, a decrease in ROS production after the addition of an inhibitor means that the free radical generator is situated on the oxygen side.

In this investigation, we report that complexes I and III are the main sites of free radical generation. Complex I generates radicals because the addition of rotenone largely increased ROS generation in pyruvate/malate-supplemented mitochondria and strongly decreased it in succinate plus rotenone-supplemented mitochondria. Moreover, when antimycin A was added to mitochondria oxidizing succinate (plus rotenone) the mitochondrial rate of H_2O_2 generation also was increased, thus indicating complex III ROS generation. TTFA did not increase the rate of free radical generation in succinate plus rotenone-supplemented mitochondria, ruling out complex II as a principal source of ROS in rat kidney and skeletal muscle mitochondria.

One of the characteristics of aging is its universality.[28,29] The mechanisms causing aging are probably similar, especially in closely related species of the same phylogenetic group. Several lines of evidence support the mitochondrial free radical theory of aging. This theory postulates that aging rate is determined, at least in part, by the rate of mitochondrial free radical production.[4,5] Therefore, it makes sense that the main mitochondrial ROS generator sites are the same in different tissues within a species and in different species of the same phylogenetic group. It has been described that the main source of ROS related to aging in heart and brain is located at complex I in a wide group of mammalian species.[8,30] Recent studies indicate that complex I is a main site of free radical generation in liver, heart, and brain mitochondria.[8,9,16,17,19–21] However, previous controversial results had been reported when

kidney was studied,[25] questioning the role of complex I on ROS production of kidney mitochondria. In such investigation, the addition of rotenone to NADH-linked supplemented kidney mitochondria did not increase H_2O_2 generation; this led to ruling out complex I as a main source of free radicals. However, when we added rotenone to succinate-supplemented kidney mitochondria a strong decrease in the H_2O_2 generation was observed, supporting the role of complex I in ROS generation in those conditions. This conclusion agrees with other investigations reporting that the rate of H_2O_2 production of succinate-supplemented mitochondria seems to come mainly from complex I (not from complex III) because of reverse electron flow from complex II to complex I.[9,21,31] This explains why succinate-supported H_2O_2 production is largely blocked after addition of rotenone. Our results support the role of complex I in free radical generation in kidney mitochondria, not only when the organelles oxidize complex II–linked substrates, but also when supplementing them with complex I–linked substrates. Because similar results were obtained in skeletal muscle mitochondria, the present investigation supports the idea that complex I is a main ROS generator in many different tissues, suggesting that complex I plays an important role in ROS generation and, possibly, in the determination of the rate of aging.

Together with previous information,[4,5,33] these results are consistent with an important role of complex I ROS generation in the determination of aging rate in at least heart, brain, kidney, and skeletal muscle.

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