

# Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart<sup>1</sup>

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## SPECIFIC AIMS

In this investigation the effect of caloric restriction (CR), the only experimental manipulation that decreases aging rate, on mitochondrial reactive oxygen species (ROS) generation and oxidative damage to mitochondrial (mtDNA) and nuclear (nDNA) DNA was studied in the heart of Wistar rats. The site in the respiratory chain where the change in ROS production occurs and the mechanism causing it were also studied.

## PRINCIPAL FINDINGS

### 1. Effect of caloric restriction on H<sub>2</sub>O<sub>2</sub> production rates of rat heart mitochondria

Short-term 40% caloric restriction (6 wk of restriction) did not change the basal or maximum rates of H<sub>2</sub>O<sub>2</sub> production or oxygen consumption of rat heart mitochondria with any substrate.

When 40% caloric restriction was applied on the long-term (1 year of restriction), succinate-supported H<sub>2</sub>O<sub>2</sub> production continued to show lack of differences between ad libitum and restricted animals (Fig. 1B). However, the pyruvate/malate-supplemented rates of mitochondrial H<sub>2</sub>O<sub>2</sub> generation were significantly lower (45%) in old restricted animals than in old controls (Fig. 1A). This did not happen in the case of maximum complex I H<sub>2</sub>O<sub>2</sub> generation (with pyruvate/malate+rotenone), which was similar in ad libitum-fed and restricted animals. Mitochondrial oxygen consumption in states 4 and 3 were not modified by long-term restriction with any substrate. The fraction of electrons out of sequence that reduce O<sub>2</sub> to oxygen free radicals at the respiratory chain (the percent free radical leak) instead of reaching cytochrome oxidase to reduce O<sub>2</sub> to water was also calculated. Long-term caloric restriction significantly decreased the free radical leak with pyruvate/malate (by 43%) and did not change it with succinate.

No significant differences in rates of H<sub>2</sub>O<sub>2</sub> genera-

tion were found between young adult and old control animals (Fig. 1A, B).

### 2. Effect of caloric restriction on oxidative damage to mitochondrial and nuclear DNA

Neither short- nor long-term caloric restriction modified steady-state levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in nDNA. In agreement with the lack of changes in mitochondrial oxygen radical generation, 8-oxodG levels in the mtDNA were not affected by short-term caloric restriction (Fig. 2A). Similarly, the lack of differences in mtDNA 8-oxodG between young adult and old animals fed ad libitum (Fig. 2B) agreed with their absence of differences in mitochondrial H<sub>2</sub>O<sub>2</sub> generation (Fig. 1). However, the mtDNA 8-oxodG levels of long-term caloric restricted old animals were significantly lower (by 30%) than those of old controls fed ad libitum (Fig. 2B). Again, this was consistent with the decrease in pyruvate/malate-supplemented H<sub>2</sub>O<sub>2</sub> generation observed after long-term caloric restriction (Fig. 1A).

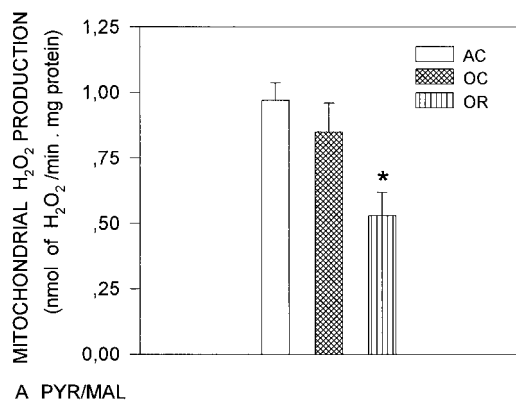
## CONCLUSIONS

In this investigation we show that CR decreases H<sub>2</sub>O<sub>2</sub> generation and oxidative damage to mtDNA in rat heart mitochondria. Furthermore, the decrease in mitochondrial ROS production occurs at complex I and is not due to a diminution in mitochondrial oxygen consumption, but rather to a lower degree of reduction of the complex I generator that decreases its percent-age free radical leak.

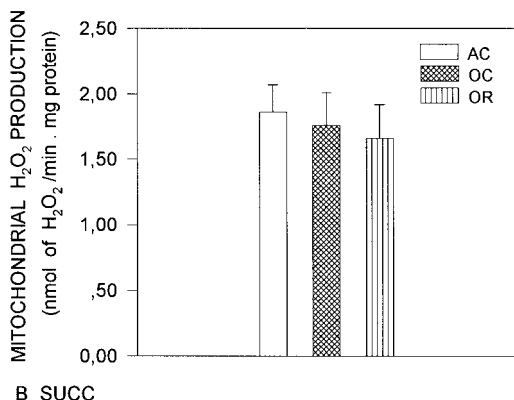
According to the mitochondrial free radical theory of aging, mitochondrial ROS production is a cause of

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A PYR/MAL



B SUCC

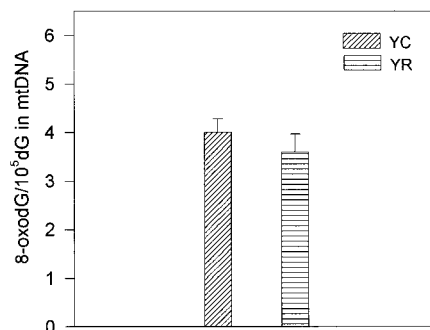
**Figure 1.** Effect of aging and long-term (1 year) caloric restriction on the basal rates of H<sub>2</sub>O<sub>2</sub> production of rat heart mitochondria respiring with pyruvate/malate (pyr/mal) (A) or succinate (succ) (B) as substrates. Succinate assays were performed in the presence of rotenone. AC = adult controls (7 months of age); OC = old controls (24 months of age); OR = old restricted (24 months of age). Results are means  $\pm$  SE from seven different animals in each group except for succinate in OC ( $n=6$ ). \*Significant difference between OC and OR ( $P<0.02$ ).

aging. Since aging is progressive, occurring at a similar rate at all ages, causes of aging should not increase in old age. What should increase with age is the final consequence of those causes, like the accumulation of mtDNA mutations with age consistently described in many previous investigations. Mitochondrial H<sub>2</sub>O<sub>2</sub> production is higher in short- than in long-lived species, which is probably a cause of the higher rate of accumulation of mtDNA mutations observed in the former kind of animals. However, in each species a rather constant rate of ROS production (high or low) is expected at different ages. Consistent with those theoretical expectations as well as with other investigations, no differences in mitochondrial H<sub>2</sub>O<sub>2</sub> generation between young adult and old rats were found in the present study. In agreement with this, no age-related differences in oxidative damage to mtDNA were observed.

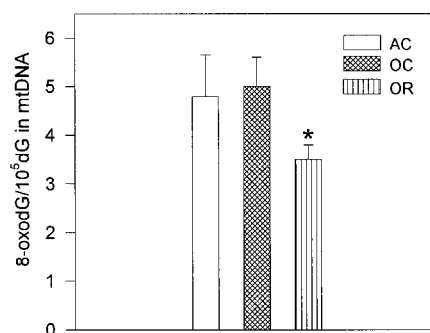
One previous study showed, as we did, that the rate of mitochondrial H<sub>2</sub>O<sub>2</sub> production is lowered by CR. Our study further clarifies that such a decrease does not

simply avoid an increase in ROS generation with age, which would only avoid putative increases in the aging rate at old ages. Instead, the CR-induced decrease in H<sub>2</sub>O<sub>2</sub> generation is more important because it lowers the rate of mitochondrial H<sub>2</sub>O<sub>2</sub> generation below that normally present in control animals of any age. This is consistent with the idea that it can be a cause of the slow down of the aging rate induced by CR at all ages. In agreement with the decrease observed in mitochondrial H<sub>2</sub>O<sub>2</sub> generation, 8-oxodG also decreased in mtDNA and did not change in nDNA. This agrees with the localization of mtDNA (not of nDNA) very near the mitochondrial free radical source, although putative CR-induced changes in mitochondrial 8-oxodG repair could also be involved.

The site in the respiratory chain where ROS generation is decreased in CR and the mechanism allowing it have never been investigated. Heart mitochondria produce ROS at complexes I and III. In the only previous CR study available, localization was not possible because only one substrate (succinate) was used, and it was added to mitochondria in the absence of rotenone. In such a situation, electrons flow from succinate not

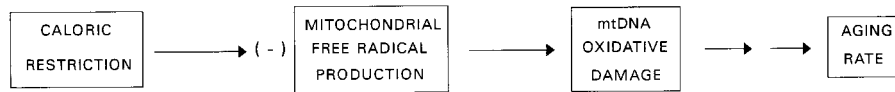


A SHORT-TERM RESTRICTION (6 WEEKS)



B LONG-TERM RESTRICTION (1 YEAR)

**Figure 2.** Effect of short-term (6 wk; A) and long-term (1 year; B) caloric restriction on steady-state levels of oxidative damage (8-oxodG/10<sup>5</sup>dG) in rat heart mitochondrial DNA (mtDNA). YC = young controls (14 wk of age); YR = young restricted (14 wk of age); AC = adult controls (7 months of age); OC = old controls (24 months of age); OR = old restricted (24 months of age). Results are means  $\pm$  SE. The number of animals per group was 6 in YR and OC and 7 in the rest of the groups. \*Significant difference between OC and OR ( $P<0.02$ ).



only to complex III, but also backward to complex I, making it impossible to discern which of the two complexes (complex I or complex III) is responsible for the CR effect. In our study, we used both succinate and pyruvate/malate as substrates. With succinate (+rotenone), the electrons flow only through the complex III generator whereas with pyruvate/malate they also flow through complex I. Since CR decreases  $H_2O_2$  production with pyruvate/malate but not with succinate (+rotenone), the ROS generator site responsible for the CR-induced decrease must be situated at complex I. Similarly, previous investigations showed that  $H_2O_2$  generation of heart mitochondria with succinate as substrate is lower in long-lived than in short-lived species, but this difference disappeared when the assays were performed with succinate + rotenone. This meant that the difference in the rate of ROS production between the two kinds of animals occurred, as between control and CR rats, at complex I.

Although some investigators have favored a hypometabolic mechanism concerning the mechanism of action of CR, others have shown that total body 24 h metabolic activity does not change in CR rats. Our results are consistent with the second of these interpretations because mitochondrial oxygen consumption was not modified by CR. What decreased in CR was the percentage release of ROS per total electron flow in the respiratory chain (the free radical leak). At least one

mechanism allowing this was elucidated in the present report. With pyruvate/malate, complex I is only partially reduced. Addition of rotenone to pyruvate/malate-supplemented mitochondria causes a 100% reduction of complex I. 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 CR mitochondria have a lower rate of ROS production and free radical leak because the degree of reduction of their complex I generator is lower than in control mitochondria.

The experiments described in this investigation support the idea that the decrease in complex I ROS generation of heart mitochondria can be involved in the life extension effect of CR for two other reasons. First, the decrease in ROS production seems to be time dependent (like the life extension effect of CR), because it occurs after 1 year (starting at 12 months of age) but not after 6 wk of restriction. Second, the quantitative variations in the different parameters studied indicate that a strong proportionality of dose-response occurs in the different subsequent steps. Thus, a 40% CR (which decreases aging rate by 30–50%) led to a 45% decrease in the rate of mitochondrial  $H_2O_2$  generation and to a 30% decrease in oxidative damage to mtDNA. **FJ**