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Short-term CR decreases cardiac mitochondrial oxidant production but increases carbonyl content

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Submitted 20 September 2003; accepted in final form 27 October 2003

J. Leeuwenburgh. Short-term CR decreases cardiac mitochondrial oxidant production but increases carbonyl content. Am J Physiol Regul Integr Comp Physiol 286: R254–R259, 2004. First published October 30, 2003; 10.1152/ajpregu.00502.2003.—Lifelong caloric restriction (CR) reduces the rate of mitochondrial oxidant production and the accumulation of oxidized proteins and prevents some of the age-associated decline in 20S proteasome activity. However, few studies have investigated how rapidly the beneficial effects of CR take place. We investigated whether 2 mo of CR in 6-mo-old rats would be of sufficient duration to elicit these beneficial changes. Mitochondrial oxidant production was significantly diminished in the CR rats compared with the ad libitum-fed animals. Short-term CR also caused a significant decrease in mitochondrial superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities, but there were no differences in cytosolic SOD and GPX activities, whereas mitochondrial and cytosolic catalase (CAT) activity was increased with CR. However, protein carbonyl content was significantly elevated in both the mitochondrial and cytosolic fractions from CR rats. Of the three major 20S proteasome activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase), the peptidylglutamyl-peptide hydrolase activity was significantly elevated in the CR animals, possibly because of the fact that there were more oxidized proteins to be degraded. Although fewer oxidants were produced in the CR animals, it is possible that the ability to scavenge oxidants was transiently suppressed because of the reduction in mitochondrial antioxidant enzyme activities, which may explain the observed increases in carbonyl content.

hydrogen peroxide production; superoxide dismutase; glutathione peroxidase; protein oxidation; 20S proteasome activity

CALORIC RESTRICTION (CR) is the only experimental intervention that has consistently been shown to slow the rate of aging and increase mean and maximum life span in a variety of species (51, 56, 57). The exact mechanisms through which CR extends life span have not yet been established, a feat complicated by the fact that CR causes numerous physiological changes. However, there is considerable evidence supporting the role of a reduction in oxidative stress as being causally involved in the anti-aging effects of CR. Lifelong CR attenuates the age-associated increases in mitochondrial superoxide and H2O2 production (6, 8, 50), lipid peroxidation (34), protein oxidation (19, 33), and oxidative damage to DNA (23, 49).

The available data suggest that increased reactive oxygen species (ROS) production and subsequent damage to macromolecules is a primary factor contributing to the aging process (51). Oxidative damage to intracellular proteins may disrupt normal physiological function, since oxidized proteins frequently lose catalytic activity (52) and are also prone to forming large, potentially cytotoxic, aggregates (17, 44). A significant increase in the amount of oxidized protein has been demonstrated to occur during aging and in several age-related diseases, including Alzheimer’s disease, cataract formation, amyotrophic lateral sclerosis, rheumatoid arthritis, and Parkinson’s disease (13, 53, 54).

The extent to which oxidized proteins accumulate is dependent on several factors, including the amount and type of oxidant produced, the ability of the antioxidant defense system to prevent ROS-induced protein damage, and the cell’s capacity to repair or remove oxidized proteins (53). Although the antioxidant defense system appears highly effective, it is not fully capable of preventing all ROS-mediated protein damage (35). Because many oxidative modifications to proteins are irreversible (42), it is essential to have an effective removal mechanism to prevent the accumulation of toxic protein aggregates (44).

The proteasome, a large multicatalytic protease found in the cytosol and nuclei of eukaryotic cells, is responsible for degrading the majority of soluble, intracellular proteins (16). The proteasome exists in two major forms (an ~700-kDa 20S and the much larger ~2,000-kDa 26S). The 20S proteasome contains the catalytic activity, whereas the 26S proteasome is formed when the 20S complexes with two 19S regulatory subunits that confer the ability to hydrolyze ATP and recognize polyubiquitinated proteins (41, 55). The 26S is primarily responsible for degrading ubiquitinated proteins in an ATP-dependent manner (55), although there is compelling evidence that the 20S selectively and rapidly degrades oxidized proteins in an ATP- and ubiquitin-independent fashion (22, 24–27, 37).

The accumulation of oxidized proteins with age may be the result of, among other things, a decrease in proteasome activity. Indeed, in both cell culture and tissues, proteasome activity has been shown to decline with age (3, 11, 15, 43, 47, 52). Lifelong CR has been shown to reduce the rate of mitochondrial oxidant production (23, 50) and the accumulation of oxidized proteins (19), along with preventing some of the ecoeducation.
age-associated decline observed in proteasome activity (43). Much less is known about the effects of short-term CR on these same parameters. Furthermore, the magnitude of the effects of CR appear to be dependent on both time and degree of restriction (58). Therefore, the purpose of this study was to determine whether 2 mo of CR in young rats was of sufficient duration to cause alterations in H2O2 production, antioxidant enzyme activity, protein oxidation, and 20S proteasome activity in the heart.

**EXPERIMENTAL PROCEDURES**

*Animals.* Ad libitum-fed (AL; n = 9) and CR (n = 9) male Fischer 344 (F344) rats were obtained from the National Institute of Aging colony (Harlan Sprague Dawley, Indianapolis, IN). CR was initiated at 14 wk of age (10% restriction), increased to 25% restriction at 15 wk, and maintained at 40% restriction from 16 wk of age until the termination of the experiment. CR animals were fed the NIH31 rat diet. All animals had unrestricted access to water. The animals were individually housed in a temperature (20 ± 2°C)- and light (12:12-h light-dark cycle)-controlled environment. At 344 (F344) rats were obtained from the National Institute of Aging colony (Harlan Sprague Dawley, Indianapolis, IN). CR was initiated at 14 wk of age (10% restriction), increased to 25% restriction at 15 wk, and maintained at 40% restriction from 16 wk of age until the termination of the experiment. CR animals were fed the NIH31 rat diet. All animals had unrestricted access to water. The animals were individually housed in a temperature (20 ± 2°C)- and light (12:12-h light-dark cycle)-controlled environment. At 14 wk of age, after 2 mo of 40% CR and was killed with an intraperitoneal injection of pentobarbital sodium (10 mg/100 g body wt), and the heart was removed rapidly. All experimental procedures received approval from the University of Florida’s Institute on Animal Care and Use Committee.

*Preparation of mitochondrial and cytosolic extracts.* Mitochondrial and cytosolic protein fractions were isolated using differential centrifugation. Briefly, both ventricles were finely minced and homogenized on ice in 1:10 (wt/vol) ice-cold isolation buffer (in mM: 200 mannitol, 70 sucrose, 1 EDTA, and 10 Tris HCl, pH 7.4) using a Potter-Elvehjem glass homogenizer. The homogenates were centrifuged at 700 g for 10 min, and the resulting supernatants were centrifuged at 8,000 g for 10 min. The 8,000-g supernatant (representing the crude cytosolic fraction) was immediately frozen at −80°C for later analysis. The pellet was resuspended in a small volume of isolation buffer and centrifuged at 8,000 g for 10 min. All centrifugation steps were carried out at 4°C. The final mitochondrial pellet [consisting of subsarcomemal mitochondria (SSM)] was suspended in 750 μl of isolation buffer and used immediately for measurement of mitochondrial H2O2 production.

*Oxidant production.* H2O2 production was measured in intact mitochondria over a period of 15 min at 37°C following the method of Barja (7). Briefly, incubation buffer (145 mM KCl, 30 mM HEPES, 5 mM KH2PO4, 3 mM MgCl2, 0.1 mM EGTA, and 0.1% fatty-acid free BSA, pH 7.4) was added to test tubes followed by the addition of mitochondria (0.25 mg protein/ml), horseradish peroxidase (5.7 U/ml), homovanillic acid (0.1 mM), and substrate (2.5 mM pyruvate/malate) so that the total volume was equal to 1.5 ml. The tubes were then incubated in a shaking water bath at 37°C for 15 min, and the reaction was stopped by placing the tubes in ice and adding 0.5 ml cold stop solution (0.1 M glycine, 25 mM EDTA-NaOH, pH 12.0). Fluorescence was determined at 312 nm excitation and 420 nm emission using a SPECTRAMAX Gemini XS dual-scanning microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Arbitrary fluorescence units were converted to known amounts of H2O2 using a glucose-glucose oxidase standard curve. All measurements were performed in duplicate, and results were expressed as nanomole H2O2 produced per minute per milligram protein.

*Antioxidant enzyme activity.* Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed according to Oyanagui (36) with slight modifications. One unit of SOD activity is defined as the concentration of enzyme that inhibits nitrate formation from hydroxylamine in the presence of xanthine oxidase by 50%. Cu/Zn-SOD activity was determined in the cytosol while Mn-SOD activity was measured in the mitochondrial fraction. Selenium-dependent glutathione peroxidase (GPX; EC 1.11.1.9) activity was assayed at 37°C according to Flohe and Gunzler (21), with H2O2 as the substrate. GPX activity is expressed as nanomole NADPH per minute per milligram protein. Catalase activity (CAT; EC 1.11.1.6) was measured at 25°C using the method developed by Aebi (2). CAT activity is expressed as units per milligram protein.

**RESULTS**

*Body weight and heart weights.* The body weights (g) and heart weights (g) of the CR animals were significantly lower than those of the AL animals (387.8 ± 5.0 vs. 271.2 ± 5.1 and 1.04 ± 0.02 vs. 0.77 ± 0.02, respectively, P < 0.0001 for both). However, the heart-to-body weight ratio (g/kg) was significantly lower in the CR groups (0.02 vs. 0.77).
significantly higher in the CR animals (2.67 ± 0.04 vs. 2.83 ± 0.03, \(P < 0.01\)).

**Oxidant production and antioxidant enzyme activity.** We determined \(\text{H}_2\text{O}_2\) production in the isolated mitochondria immediately after the isolation procedure. Mitochondria from CR rats produced significantly less \(\text{H}_2\text{O}_2\) compared with AL rats (Table 1). \(\text{H}_2\text{O}_2\) generated by the mitochondria can be scavenged by mitochondrial antioxidant enzymes or released in the cytosol (6). The potential for oxidative stress is therefore dependent on both the amount of \(\text{H}_2\text{O}_2\) produced and its removal by antioxidant enzymes (6, 32, 33). Therefore, we also measured the activity of the major antioxidant enzymes, SOD, GPX, and CAT, in the mitochondria and cytosol (Table 1). There were no differences in cytosolic SOD and GPX activities between the AL and CR animals (Table 1). In striking contrast, short-term CR caused a significant reduction in mitochondrial SOD and GPX activities (Table 1). Moreover, the reduction in SOD (39%) and GPX (19%) is approximately equal to a 2:1 ratio, which may reflect the production of two superoxide anion molecules for every \(\text{H}_2\text{O}_2\) molecule produced. These data suggest that the amount of \(\text{H}_2\text{O}_2\) produced in the mitochondria may be responsible for the reduction in antioxidant enzyme activities. In both mitochondria and cytosol, however, CAT activity was significantly increased in the CR animals (Table 1).

**Protein oxidation.** The measurement of protein carbonyls has been used extensively as a marker of protein oxidation, and there is strong evidence that carbonyl content increases with age in a variety of tissues (4, 11, 52). Moreover, CR can reduce the age-associated accumulation of protein carbonyls (29, 50). We determined carbonyl levels in the AL and CR animals using both a highly sensitive ELISA (Fig. 1, A and B) and Western blot analysis (Fig. 2) and found that protein carbonyls were significantly increased in the CR animals.

**20S proteasome activity.** The 20S proteasome has three main proteolytic activities (chymotrypsin-like, trypsin-like, and PGPH) that cleave peptides on the carboxyl side of specific amino acid residues (40). The chymotrypsin-like activity cleaves after large hydrophobic residues such as phenylalanine and tyrosine; the trypsin-like after basic residues such as arginine, lysine, and histidine; and the PGPH after acidic residues like aspartate and glutamate. We measured these three activities in heart cytosol from AL and CR animals and found that, in all cases, the mean activity tended to be higher in the CR animals (Fig. 3), although the only statistically significant increase was in the PGPH activity (\(P < 0.05\), Fig. 3C).

**DISCUSSION**

We found that mitochondrial \(\text{H}_2\text{O}_2\) production was significantly decreased after 2 mo of CR, but, despite this decline, there was an increase in mitochondrial and cytosolic protein carbonyl content, as determined using two different analytic methods. The decrease in oxidant production is in agreement with previous reports showing that long-term CR (8 mo or more) decreases mitochondrial oxidant production in rat heart.

![Fig. 1. Protein carbonyl content in heart cytosol (A) and mitochondria (B) from 6-mo-old ad libitum (AL)-fed and calorie-restricted (CR) male Fischer 344 (F344) rats measured using an ELISA (12) with slight modification (45). For cytosolic carbonyls (A), \(n = 9\) for AL and \(n = 7\) for CR; **\(P < 0.01\). For mitochondrial carbonyls (B), \(n = 8\) for AL and \(n = 5\) for CR; **\(P < 0.01\).](http://ajpregu.physiology.org/)

![Fig. 2. Protein carbonyl content in heart cytosol from 6-mo-old AL and CR male F344 rats using the OxyBlot Protein Oxidation Detection Kit (Intergen); \(n = 9\) for AL and \(n = 7\) for CR; **\(P < 0.01\). A representative Western blot for cytosolic carbonyls using the OxyBlot kit is also shown.](http://ajpregu.physiology.org/)
found that 6 wk of 40% restriction did not significantly alter the rate of H₂O₂ production in heart mitochondria compared with AL-fed animals. However, it was reported that oxidative production was decreased by 15% after 6 wk of CR, a percent reduction that is remarkably similar to what we have found. Furthermore, the rates of H₂O₂ production (using pyruvate/malate) obtained in their study and ours were very similar (1.1 ± 0.1 vs. 0.9 ± 0.1 for Gredilla et al. compared with 1.06 ± 0.04 vs. 0.91 ± 0.05 in our study), and it is likely that the lack of statistical significance obtained by Gredilla et al. (23) was the result of the higher SEs and smaller number of animals used (n = 7). Additionally, Lee et al. (31) found that ROS production induced by tert-butyl hydroperoxide was significantly lower in cardiac mitochondria obtained from 6-mo-old CR F344 rats compared with 6-mo-old AL-fed rats. It is also worthwhile to point out that two functionally different populations of cardiac mitochondria exist (38). SSM are located beneath the plasma membrane, whereas intermitochondrial (IFM) are found in parallel rows between the myofilaments, and differences in the oxidative capacity of these two populations have been reported (20). Interpreting CR-induced changes in oxidant production is complicated by the fact that most mitochondrial isolation procedures yield either SSM alone or a mixed population of SSM and IFM. In that regard, it is of interest that our results are compatible with those of Lee et al. (31), considering that the isolation procedure used in both cases yielded SSM and additionally that the age (6 mo) and strain of rats (F344) used were identical. Gredilla et al. (23) used an isolation procedure that yielded a mixed population of SSM and IFM and also used a different age (14 wk) and strain of rat (Wistar). Oxidant production may vary depending on whether it is measured in SSM alone or a mixture of SSM and IFM; furthermore, age and strain differences may also have an influence. Future studies in our laboratory will focus on isolating SSM and IFM separately and measuring oxidant production in both populations to further characterize the differences between them.

Although we found a reduction in oxidant production, protein carbonyls in the heart were increased with short-term CR. This finding was unexpected, since previous studies have shown that longer durations of CR reduce oxidative DNA damage, protein damage, and lipid peroxidation (19, 23, 39, 48). However, there are at least two reports that lipid peroxidation is increased in animals subjected to short-term CR (18, 28). Of particular interest is the study by Davis et al. (18), which compared lipid peroxidation potential in hepatic microsomes from young (3.5 mo) and old (27 mo) mice that had their caloric intake restricted by either 25% or 50% of AL intake. Hepatic microsomes from young mice on the 25% restricted diet exhibited higher peroxidation potential than the other groups. Unfortunately, neither study offered substantial reasons as to why lipid peroxidation may be elevated with short-term restriction. However, in combination with our data, it does appear that there may be some transient, negative effects of short-term CR in young animals, and the underlying mechanisms should be more thoroughly investigated. The use of gene arrays or proteomics may be especially useful in determining which genes or proteins are differentially expressed with short-term CR.

It should be noted that protein carbonyls can be generated via both oxidative and nonoxidative mechanisms (1), and there has been some criticism of the method. However, compared with other methods for measuring protein oxidation, the measurement of carbonyls using DNPH is convenient, fast, and inexpensive and therefore remains widely used. Furthermore, we used two different methods for detecting carbonyl groups, Western blotting and a highly sensitive ELISA (12, 45), to confirm our findings.

In an attempt to explain the increase in protein carbonyls, we further investigated whether antioxidant enzyme activity changed in response to the reduction in H₂O₂ production. We showed that both mitochondrial SOD and GPX were significantly lower in the CR animals but that the cytosolic isoenzymes remained unaffected. Additionally, we found that both mitochondrial and cytosolic CAT activities were increased with CR. Considering that mitochondrial catalase is not believed to play a major role in H₂O₂ removal in the heart (5), it is possible that the decline in Mn-SOD and GPX activities contributed to the increase in mitochondrial carbonyl content. Indeed, Bota et al. (9) found that young (3- to 6-mo-old) Sod2⁺/⁺ mice, which exhibit a 50% decrease in Mn-SOD activity, have an 80% increase in the total levels of protein carbonyls compared with young Sod2⁻/⁻ mice. In our study, we found an ~40% decrease in Mn-SOD activity, which may account for the increase in mitochondrial protein carbonyls. Although we did not measure it, Lon protease activity may also have been reduced in the CR animals. Lon is an ATP-stimulated protease found in mammalian mitochondria that likely plays an important role in degrading oxidized mitochondrial proteins (9).
However, given that cytosolic SOD and GPX activity were not altered by CR and catalase activity increased, it is unclear why the carbonyl content in the cytosol was also elevated. Because the 20S proteasome is the major cytosolic protease responsible for degrading oxidized proteins, we measured the three major proteolytic activities of this proteasome to determine whether a decline in 20S proteasome activity could be responsible for the increased cytosolic carbonyl content.

Of the three major 20S proteasome activities, the PGPH activity was significantly elevated in the CR animals. Despite this, these animals had higher levels of protein carbonyls compared with the AL-fed group. Therefore, the increase in carbonyls cannot be explained as being the result of a reduction in proteasome activity. Conversely, the PGPH activity may have increased because of the fact that there were more oxidized proteins present to be degraded. Furthermore, protein carbonyls produced via direct or indirect oxidation of amino acid side chains may be considered intermediate products of carbonyls produced via direct or indirect oxidation of amino acids (46). It is possible that protein carbonyls appeared lower in the AL animals because oxidized proteins in these rats had already undergone more extensive cross-linking and would therefore have fewer free carbonyl groups to be detected by the assay. From this viewpoint, the increase in PGPH activity can also be better explained, since minimal oxidative damage appears to increase proteasome activity, whereas more extensive oxidation products inhibit proteasome activity (46, 53).

Additionally, more oxidants may have been able to cross the mitochondrial membranes and enter the cytosol without being scavenged because of the strong reduction in mitochondrial SOD and GPX activities. Furthermore, because we only measured mitochondrial oxidant production, we cannot rule out the possibility that oxidants produced from other sources (peroxisomes, NADPH oxidase, etc.) could have contributed to the observed increase in protein carbonyls.

Although 20S proteasome activity is measured under a wide variety of conditions, most studies show that there is a decline in PGPH activity with age (11, 14, 15, 43), and Shibatani and Ward (43) found that CR was able to partially prevent this decline (26-mo-old CR animals had PGPH activity equivalent to that of 7-mo-old AL-fed animals). Furthermore, the expression of several genes involved in protein turnover, including the 20S proteasome subunit, was found to decline by at least twofold in gastrocnemius muscle from old (30-mo) mice compared with adult (5-mo) mice (30). CR was able to completely or partially reverse many of these changes, offering support for the idea that one mechanism by which CR prolongs life is through increased protein turnover and therefore reduced accumulation of damaged proteins. Our finding that in young animals 2 mo of CR resulted in significant increases in PGPH activity indicates that one of the anti-aging effects of CR may indeed be the result of increased proteasome activity and that this effect is manifested in a relatively short period of time. Shringarpure and Davies (44) propose that cellular aging results from both increased mitochondrial oxidant production and decreased proteasome activity. The fact that as little as 2 mo of CR, a known life-prolonging intervention, resulted in a significant decline in mitochondrial H$_2$O$_2$ production and an increase in PGPH proteasome activity offers further support for this hypothesis.

In summary, 2 mo of CR significantly reduced mitochondrial H$_2$O$_2$ production and mitochondrial SOD and GPX activities. Surprisingly, mitochondrial and cytosolic protein carbonyl content were significantly increased along with PGPH proteasome activity. The following two possible explanations for the increase in protein carbonyls can be offered: 1) protein oxidation may be temporarily elevated in the heart after short-term CR or 2) more extensive oxidation and cross-linking of proteins has already occurred in AL-fed animals, making it appear as if oxidative protein damage is increased with short-term CR. It should be noted that we examined only one tissue, and other organs may or may not respond in the same manner. We chose the heart, since there is convincing evidence that this postmitotic tissue accumulates oxidative damage with age and that CR can prevent at least some of this damage (33, 49, 50). Additionally, because we used only one time point for our measurements, we cannot fully determine how quickly after the onset of CR these changes are occurring. To the best of our knowledge, this is the first reported study to demonstrate a reduction in key mitochondrial antioxidant enzymes and an increase in protein carbonyls with short-term CR. Therefore, future studies are required to more fully characterize the response to short-term CR in different tissues and to determine the time course at which CR produces its various effects.

ACKNOWLEDGMENTS

We thank Dr. Colin Selman for critical reading and editing of this manuscript.

GRANTS

This research was supported by National Institute on Aging Grants R01-AG-17994 and AG-21042 to C. Leeuwenburgh.

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