ELSEVIER SCIENCE
IRELAND

Mechanisms of Ageing and Development
74 (1994) 121–133

mechanisms of ageing and development

Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse

Rajindar S. Sohal*[a], Hung-Hai Ku[a], Sanjiv Agarwal[a], Michael J. Forster[b], Harbans Lal[b]

[a]*Department of Biological Sciences, Southern Methodist University, Dallas, TX 75275, USA*
[b]*Department of Pharmacology, University of North Texas Health Science Center, Fort Worth, TX 76107, USA*

(Received 25 September 1993; revision received 24 December 1993; accepted 27 January 1994)

Abstract

This study was conducted in order to test the concept that oxidative damage is associated with aging and may be a factor in the modulation of life span in response to variations in caloric intake. Mice fed a diet that was 40% lower in calories (DR) than the ad libitum fed (AL) animals exhibited a 43% extension in average life span and a 61% prolongation in mortality rate doubling time. A comparison of AL and DR mice at 9, 17 and 23 months of age indicated that the protein carbonyl content in the brain, heart and kidney increased with age and was significantly greater in the AL than DR group in each organ at each of the three ages. Mitochondrial state 4 or resting respiratory rate increased with age in the AL, but not the DR group, and was also relatively higher in the former. The rates of mitochondrial superoxide and hydrogen peroxide generation increased with age and were higher in the AL than DR mice in all the three organs at each age. In contrast, there was no clear-cut overall pattern of age-related or dietary-related changes in antioxidant defenses provided by superoxide dismutase, catalase and glutathione peroxidase. Results suggest that mechanisms of aging and life span shortening by enhanced caloric intake are associated with oxidative damage arising from corresponding changes in mitochondrial oxidant production. Protein carbonyl content, and mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation may act as indices of aging.

* Corresponding author.

Abbreviations: AL, ad libitum; DNPH, 2,4-dinitrophenylhydrazine; DR, dietary-restricted; HRP, horseradish peroxidase; MLSP, maximum life span potential; MRDT, mortality rate doubling time; PHPA, *p*-hydroxyphenylacetate; SMPS, submitochondrial particles; SOD, superoxide dismutase.

SSDI 0047-6374(94)01443-P

Key words: Aging; Dietary restriction; Oxygen free radicals; Protein oxidation; Oxidative stress

1. Introduction

A decrease in caloric intake by laboratory strains of mice and rats, in comparison to those fed ad libitum (AL), has been shown to extend their life span as well as postpone or lower the incidence of some age-associated diseases [1–3]. Some, but not all, of the age-related biochemical and physiological changes have also been reported to be delayed by dietary restriction (DR). It is still unclear if the effects of varied caloric intake on life span are primarily due to a slowdown of the basic mechanisms of aging or the minimization of the non-aging-related deleterious effects of overfeeding by AL animals or a combination of the two. Notwithstanding, a number of broadly-stated hypotheses, including one implicating oxidative stress, have been proposed to explain the effects of caloric intake on life span [1–5].

The objective of the present study was to test the relevance and validity of the oxidative stress hypothesis of aging in life span extension by DR [3,5–7]. This hypothesis postulates that oxidative damage to cellular components is a causal factor in aging and in the shortening of life span potential of AL animals or conversely in the prolongation of the life span of DR animals. Oxidative damage is ostensibly due to inherent inadequacy of antioxidant defenses, whereby, cells are constantly under a certain level of oxidative stress, which is defined as the imbalance between oxidant fluxes and antioxidant defenses. It is now well established that the main intracellular site of oxidant generation in the cell is the mitochondrion whereas the primary antioxidant defenses are provided by superoxide dismutase (SOD; which converts $O_2^{\cdot -}$ to H_2O_2) and catalase and glutathione peroxidase (which together eliminate H_2O_2) [8].

Results of previous studies on mammalian systems [9] and insects [10] have indicated that oxidative damage, detectable by the protein carbonyl modifications, is associated with aging and life expectancy. Furthermore, the protein carbonyl content has been shown to be a reliable indicator of molecular reactions of oxygen free radicals [11,12]. The specific aims of the present study were: (i) to determine the effects of age and DR on protein carbonyl content in different organs of the mouse; (ii) to investigate if the rates of mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation are affected by aging and DR; and (iii) to ascertain whether antioxidant defenses vary with age and in response to DR. Results of this study indicate that aging in the mouse is associated with progressive increase in protein oxidative damage and DR reduces such damage probably due to corresponding changes in the rates of mitochondrial oxidant generation.

2. Materials and methods

2.1. Animals

Fifty-nine male C57BL/6NNia mice aged 9 (n = 21), 16 (n = 20), or 23 months (n = 18) of age were used in the current study. The mice were maintained at the

National Institute on Aging/National Center for Toxicological Research Project on Caloric Restriction (NIA/NCTR/PCR) Colonies (Jefferson, AR) until 1 month prior to the target ages, whereupon they were shipped by air to the University of North Texas Health Science Center (UNTHSC) Vivarium. The mice were housed individually at NCTR, beginning at 4 months of age, in 11.5 × 7.5 × 5″ solid bottom polycarbonate cages with wire tops modified into two mouse units by insertion of a stainless steel divider. Half of the mice were on a diet restriction regimen (DR) permitting daily access to 60% of the intake of a companion group of mice given ad libitum access (AL) to the diet (NIH-31, Purina Feeds). The DR mice were fed a special NIH-31 formulation providing a correction for intake of essential nutrients [13]. The mice were maintained on a 12-h light/dark cycle with the light portion beginning at 0600 h and were kept under identical conditions following receipt at the UNTHSC vivarium, with the exception that the DR mice were placed on a night feeding regimen in which they were fed at 2200 h, approximately 2-h prior to the acrophase of the normal circadian feeding cycle of the AL mice. The purpose of this procedure was to provide proper phasing of circadian cycles in the AL and DR groups. Specific pathogen free conditions that had been maintained were verified at both the NCTR/PCR and UNTHSC colonies for all periods of this study.

Following a 2-week adaptation period within the UNTHSC vivarium, the mice used in these studies were tested according to a series of behavioral protocols detailed elsewhere [14,15]. Subsequently, the mice were transferred a short distance to the SMU Department of Biological Sciences for sacrifice and biochemical procedures. Body weight history of the mice used in these studies (at approximately 3-week intervals) was provided by the NCTR. The mortality analyses were based upon data provided by NCTR for the 56 designated male C57BL6NNia mice set aside at the outset of the PCR (September, 1986).

2.2. Data analysis

The mortality rate doubling time (MRDT) was determined using Finch's [16] equation: MRDT = ln $2/G$ where G is the slope of the log mortality rate plot (Gompertz plot). Biochemical assays sometimes required pooling of tissue from 2–3 mice. Each set of biochemical data was subjected to a two-way analysis of variance with age and diet as the factors. Planned individual comparisons were performed between the DR and AL groups at each target age, using Student's t-test.

2.3. Biochemical assays

Brain, heart and kidney were removed fro the AL and DR mice of different ages, and tissues were processed for the measurement of carbonyl content, rates of mitochondrial respiration, $O_2^{\cdot -}$ and H_2O_2-generation, and activities of SOD, catalase and glutathione peroxidase.

2.4. Determination of protein carbonyl content

Protein carbonyl content was measured according to Levine et al. [17] using the 2,4-dinitrophenylhydrazine (DNPH) procedure. In each experiment, a 10% (W/V) homogenate of tissue was made in 5 mM phosphate buffer (pH 7.5) containing the protease inhibitors, leupeptin (0.5 μg/ml), aprotenin (0.5 μg/ml) and pepstatin

(0.7 μg/ml) and 0.1% Triton X, using a Teflon and glass homogenizer. The homogenate was centrifuged at 700 *g* and 300-μl aliquots of the resulting supernatant containing 1.6–2.0 mg protein were treated with 300 μl of 10 mM DNPH dissolved in 2 N HCl, or with 2 N HCl alone in the controls. Samples were then incubated for 1 h at room temperature, stirred every 10 min, precipitated with 10% trichloroacetic acid (final conc.), and centrifuged for 3 min at 16 000 *g*. The pellet was washed three times with 1 ml ethanol/ethyl acetate (1:1, v/v) and redissolved in 1 ml 6 M guanidine in 10 mM phosphate buffer-trifluoroacetic acid (pH 2.3). The difference in absorbance between the DNPH-treated and the HCl-treated samples was determined at 366 nm and the results were expressed as nmol carbonyl groups/mg of protein using the extinction coefficient of 22.0 mM^{-1} cm^{-1} for aliphatic hydrazones.

2.5. Isolation of mitochondria and preparation of submitochondrial particles

Mitochondria from the brain were isolated by the method of Ozawa et al. [18]; from the heart by the procedure of Arcos et al. [19]; and from the kidney cortex by the procedure described by Hayden and Steven [20]. Briefly, the brain was homogenized in 10 vols. (w/v) of isolation medium containing 0.3 M mannitol and 0.1 mM EDTA (pH 7.4). The homogenate was centrifuged at 600 *g* for 8 min and the resulting supernatant was recentrifuged at 10 000 *g* for 10 min. The pellet was resuspended in 40 ml of isolation buffer and centrifuged at 5000 *g* for 10 min to pellet the mitochondria. The upper loosely packed white part of the pellet was removed with a cotton-tipped applicator and the remaining brown pellet was resuspended in the isolation buffer.

Pieces of the heart were homogenized in 20 vols. (w/v) of the isolation buffer containing 30 mM nicotinamide, 20 mM EDTA and 0.3 M sucrose (pH 7.4). The homogenate was centrifuged at 700 *g* for 10 min and the pellet discarded; this procedure was repeated once. The supernatant was recentrifuged at 10 000 *g* for 5 min and the resulting pellet was resuspended in 0.25 M sucrose.

The cortices of the kidneys were sliced into small pieces, and homogenized in 20 volumes (w/v) of isolation buffer, consisting of 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 10 mM EGTA, and 0.5 mg/ml bovine serum albumin (pH 7.4). The homogenate was centrifuged at 600 *g* for 10 min, the pellet was discarded and the supernatant was recentrifuged at 8500 *g* for 10 min. The light-colored upper layer of the pellet was carefully removed with a cotton-tipped applicator and the remaining darker part of the pellet was rinsed and resuspended in the isolation buffer.

Submitochondrial particles (SMPS) were prepared by resuspending the mitochondrial preparation in 4 vols. of 30 mM potassium phosphate buffer (pH 7.0), and sonicated four times, each consisting of 30-s bursts, at 1-min intervals. The sonicated mitochondria were centrifuged at 8250 *g* for 10 min to sediment unfragmented organelles and the supernatant was recentrifuged at 80 000 *g* for 40 min to pellet the SMPS.

2.6. Measurement of mitochondrial state 4 respiration

The rate of resting mitochondrial respiration was measured polarographically with a Clark-type electrode at 37°C using succinate as a substrate. The incubation mixture, to measure state 4 respiration (substrate in excess but no exogenous ADP),

consisted of buffer (154 mM KCl, 5 mM KH_2PO_4, 5 mM K_2HPO_4, 3 mM $MgCl_2$ and 0.1 mM EDTA, pH 7.4), and 40–200 μg of mitochondrial protein. The rate of oxygen consumption was measured after the addition of 7 mM succinate as described previously [21]. The reason for the determination of state 4 respiration was that $O_2^{\cdot -}$ generation by mitochondria occurs under state 4 conditions when the electron carriers are in a reduced state and not under state 3 conditions when they are oxidized [8].

2.7. Measurement of $O_2^{\cdot -}$ generation

The rate of $O_2^{\cdot -}$ generation in the SMPS was measured as SOD-inhibitable reduction of acetylated ferricytochrome *c*, as described by Boveris [22]. Both the system and the reference cuvettes contained 0.2–1.0 mg SMP protein, 0.1 M potassium phosphate buffer (pH 7.4), 7.2 μM acetylated cytochrome *c*, 0.6 μM antimycin A and 7 mM succinate; 200 units of SOD/ml were added to the reference cuvette. The reduction of acetylated cytochrome *c* was monitored at 550 nm. Since both the system and the reference cuvette contained identical ingredients except for SOD in the latter, the reduction of cytochrome *c* was deemed to be specifically due to its interaction with $O_2^{\cdot -}$.

2.8. Measurement of H_2O_2 release by mitochondria

The rate of H_2O_2 released by mitochondria was measured fluorometrically by the method of Hyslop and Sklar [23] by monitoring the oxidation of *p*-hydroxyphenylacetate (PHPA) coupled to the enzymatic reduction of H_2O_2 by horseradish peroxidase. The reaction mixture (3 ml) consisted of ~150 μg of mitochondrial protein, 500 μg PHPA, 4 units of HRP, 7 mM succinate, and buffer composed of 154 mM KCl, 5 mM KH_2PO_4, 5 mM K_2HPO_4, 3 mM $MgCl_2$ and 0.1 mM EDTA (pH 7.4). The rate of H_2O_2 release was determined by following the increase in fluorescence at an excitation of 320 nm and emission of 400 nm. Known concentrations of H_2O_2 were used to establish the standard concentration curve.

2.9. Enzyme assays

SOD activity was measured in the whole body homogenates of the tissues by the 'direct' method of Misra and Fridovich [24] as described previously [25]. Catalase activity was assayed according to Luck [26] as described in detail elsewhere [25]. Glutathione peroxidase activity was measured according to Paglia and Valentine [27] as outlined previously [28].

3. Results

A comparison of oxidative damage, mitochondrial $O_2^{\cdot -}$ and H_2O_2 production and antioxidant defenses was made between the DR and the AL mice at 9, 17 and 23 months of age.

3.1. Effect of DR on body weight

Age-dependent changes in the body weight of the three groups of mice, kept under the AL and the DR regime, and killed at 9, 17 and 23 months of age are presented

in Fig. 1. Whereas, the average body weight of the mice under the DR regime remains relatively stable from about 3 months to 23 months of age, those under the AL regime show an age-related increase of about 100%, reaching a peak around 17 months of age, followed by a gradual decline of about 15% at 23 months of age.

A two-way analysis of variance on the 23-month-old target group resulted in a significant Age × Diet interaction ($F[23,368] = 18.3$, $P < 0.001$) in support of this observation.

3.2. Mortality characteristics

The survivorship curves of the mice under the DR and the AL regime are presented in Fig. 2A. The average life span of the DR mice (30.6 ± 1.2 months) was 43% longer than that of the AL mice (21.5 ± 1.0 months). As compared to the AL group, the MRDT, calculated from the slope of the Gompertz plots (Fig. 2B), was 61% longer in the DR group (3.68 and 5.93 months, respectively).

3.3. Protein carbonyl content

The protein carbonyl content in all the three organs, i.e. brain, heart and kidney increased progressively and statistically significantly with age in both the AL and the DR mice (Fig. 3) (all $F_s > 74$, $P_s < 0.001$). In each organ and at each age, the amount of protein carbonyls was greater in the AL than the DR group ($F_s > 63$, $P_s < 0.001$). Furthermore, the differences between the two groups tended to increase with age of the mice. The greatest difference in the carbonyl content be-

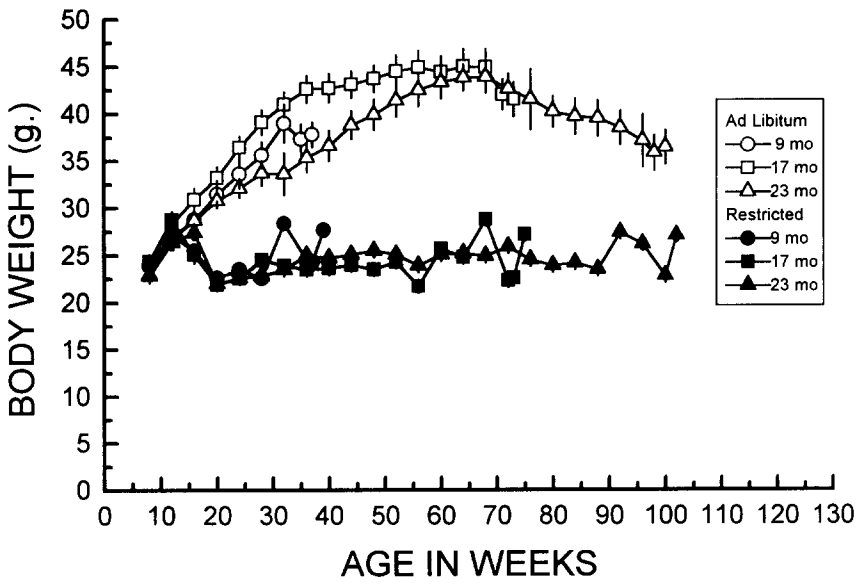

Fig. 1. Effects of age and varied food intake on the mean body weight (± S.E.) of three different groups of mice, which were killed at 9, 17 and 23 months of age and used for the biochemical assays. Mice were fed either ad libitum (AL) or 60% (DR) of the amount eaten by the AL group. Weights were recorded at 3-week intervals. *Indicates $P < 0.05$ when compared with age-matched AL group.

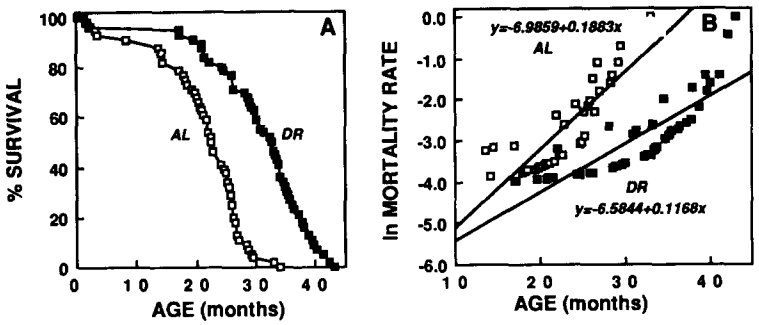

Fig. 2. (A) Survivorship curves of the ad libitum fed (AL) and dietary restricted (DR) mice. (B) Mortality rates of the ad libitum fed and dietary restricted (DR) mice, graphed on a semilogarithmic scale (Gompertz plots).

tween the AL and the DR group was found in the kidney followed by the heart and the brain.

For the heart and kidney, the apparent age-dependent differences between AL and DR groups resulted in significant age × diet interactions ($F_s > 11.1$, $P_s < 0.001$).

3.4. Mitochondrial oxygen consumption

The rates of $O_2^{\cdot -}$ and H_2O_2 production by mitochondria are known to be highest when the components of the electron transport chain are in the most reduced state, i.e. state 4 (excess substrate but no exogenous ADP) [22]. The rates of mitochondrial state 4 respiration, using succinate as a substrate, are presented in Fig. 4. In both the AL and the DR group, the state 4 respiration increased with age. The rate of O_2 consumption by mitochondria from all organs of AL animals increased dramatically with age, whereas no increase at any age was evident for brain and kidney of DR mice. An increase did occur for heart mitochondria of DR mice, although not until after 17 months. In contrast, an age-related increase in O_2 consumption by heart mitochondria of AL mice occurred earlier, after 9 months of age. For brain and kidney, the different effects of age in AL and DR mice resulted in significant

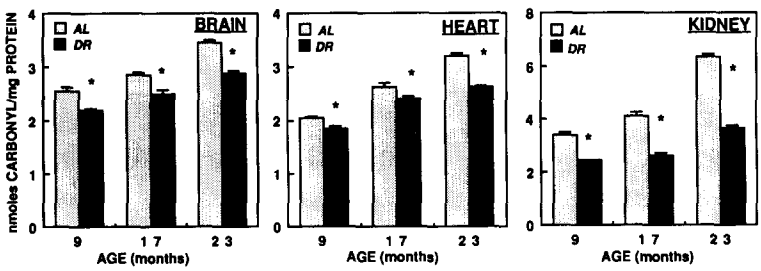

Fig. 3. Comparison of protein carbonyl content in the homogenates of brain, heart and kidney between the ad libitum fed (AL) and dietary restricted mice at 9, 17 and 23 months of age. Carbonyl content was measured by the DNPH method of Levine et al. [17]. Results are average ± S.E.M. of 3–10 determinations.

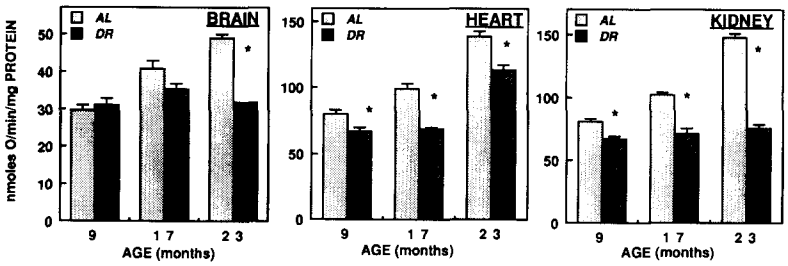

Fig. 4. Effect of age on mitochondrial state 4 or resting respiration in the brain, heart and kidney of mice fed ad libitum (AL) or on a calorically restricted diet (DR). State 4 respiration was measured polarographically by a Clark-type electrode in the presence of 7 mM succinate and no exogenous ADP. Results are average ± S.E.M. of 3–6 determinations.

Age × Diet interaction ($F_s > 14$, $P < 0.001$). A significant overall effect of diet on mitochondrial state 4 O_2 consumption was also evident for all organs ($F_s > 27$, $P < 0.001$), even though the 9-month-old AL and DR groups tended to be more closely matched for O_2 consumption when compared with the older mice.

3.5. *Mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation*

The rate of $O_2^{\cdot -}$ generation by submitochondrial particles increased with age in both the AL and the DR group in all the three organs (Fig. 5) ($F_s > 52$, $P_s < 0.001$). At all ages and in each organ, the rate of $O_2^{\cdot -}$ production was significantly higher in the AL than the DR group ($F_s > 16$, $P_s < 0.001$). The relative differences between the AL and DR groups were highest in the brain.

The rates of H_2O_2 release by mitochondria followed a pattern similar to that observed for $O_2^{\cdot -}$ generation. H_2O_2 release tended to increase with age in all the three organs in both the AL and the DR group (Fig. 6) ($F_s > 50$, $P_s < 0.001$). At all ages and in each organ studied, the rate of H_2O_2 release was higher in the AL than the DR group ($F_s > 26$, $P_s < 0.001$). Again, the highest relative differences

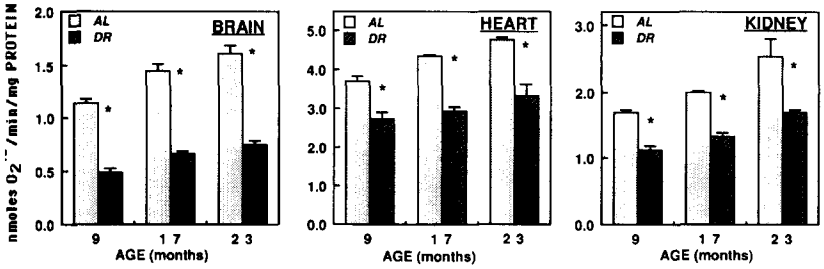

Fig. 5. Rates of mitochondrial $O_2^{\cdot -}$ generation from brain, heart and kidney of ad libitum fed (AL) and dietary restricted (DR) mice at 9, 17 and 23 months of age. Rate of $O_2^{\cdot -}$ production was measured in submitochondrial particles (SMPS) as SOD-inhibitable reduction of acetylated cytochrome *c*. Both the system and the reference cuvette contained 0.2–1.0 mg submitochondrial protein, 0.6 μM antimycin A, 7 mM succinate, and 7.2 μM acetylated cytochrome *c*; 200 units of SOD/ml were added to the reference cuvette. Values are average of 4–8 determinations ± S.E.M.

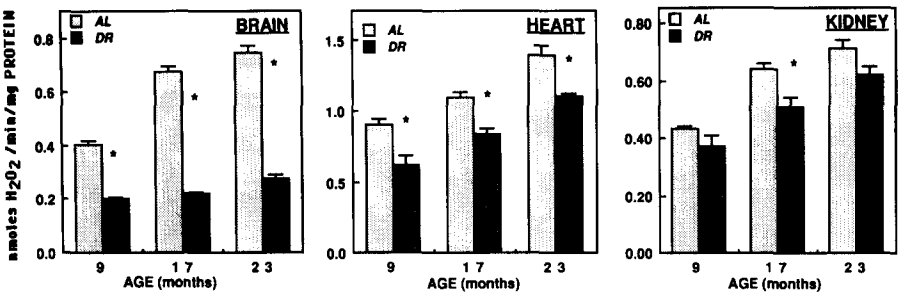

Fig. 6. Rates of H_2O_2 release by mitochondria from the brain, heart and kidney of mice fed ad libitum or on caloric restricted diet (DR) at 9, 17 and 23 months of age. Rate of H_2O_2 release was measured in isolated mitochondria as an increase in fluorescence due to the reduction of H_2O_2 by horseradish peroxidase. The reaction mixture included 20–200 μg protein, 500 μg PHPA, 4 units of horseradish peroxidase and 7 mM succinate. Results are average ± S.E.M. of 4–6 measurements.

between the AL and DR group were observed in the brain, where the rate of H_2O_2 release was more than twofold higher in the AL than the DR group. These large differences for brain were partly due to a relatively smaller effect of age in DR mice, when compared with AL mice (Age × Diet interaction; $F(2,29) = 27.9$, $P < 0.001$).

3.6. Comparison of antioxidant defenses

SOD activity. SOD activity did not exhibit a clear-cut trend either in relation to aging or in response to varied caloric intake. As shown in Fig. 7, SOD activity was higher in the DR than the AL group at some ages in some organs, but was lower or similar at other ages.

Despite the lack of clear trends, analyses of SOD activity in brain and heart (but not kidney) yielded significant main effects of Age, and a Diet × Age interaction ($F_s > 7.0$, $P < 0.01$).

Catalase activity. Catalase activity exhibited an age-related increase in the brain in both the DR and the AL groups ($F(2,39) = 65.8$, $P < 0.001$) (Fig. 7). However, more complex patterns across ages were observed for heart and kidney. In the heart of AL mice, there was a 25% decline in catalase activity between 9 and 17 months of age, but a 10% increase during the same period for the DR groups. No age-related changes were observed for catalase in the kidney of the AL group, whereas there was an 18% decrease between 9 and 17 months of age in the DR mice. The different aging patterns for heart and kidney in AL and DR mice each resulted in significant Age × Diet interaction ($F_s > 22$, $P_s < 0.001$). Overall, the DR group exhibited a higher level of catalase activity than the AL group in each organ ($F_s > 30$, $P_s < 0.001$). The only exception was the 17% greater catalase activity of AL mice at 9 months of age in the heart.

Glutathione peroxidase. There was no consistent pattern of glutathione peroxidase activity across all organs (Fig. 7). Activity of this enzyme in the brain showed an overall increase between 9 and 23 months of age, but with a different pattern for DR and AL mice. The DR mice showed an increase at an earlier age than AL mice (by

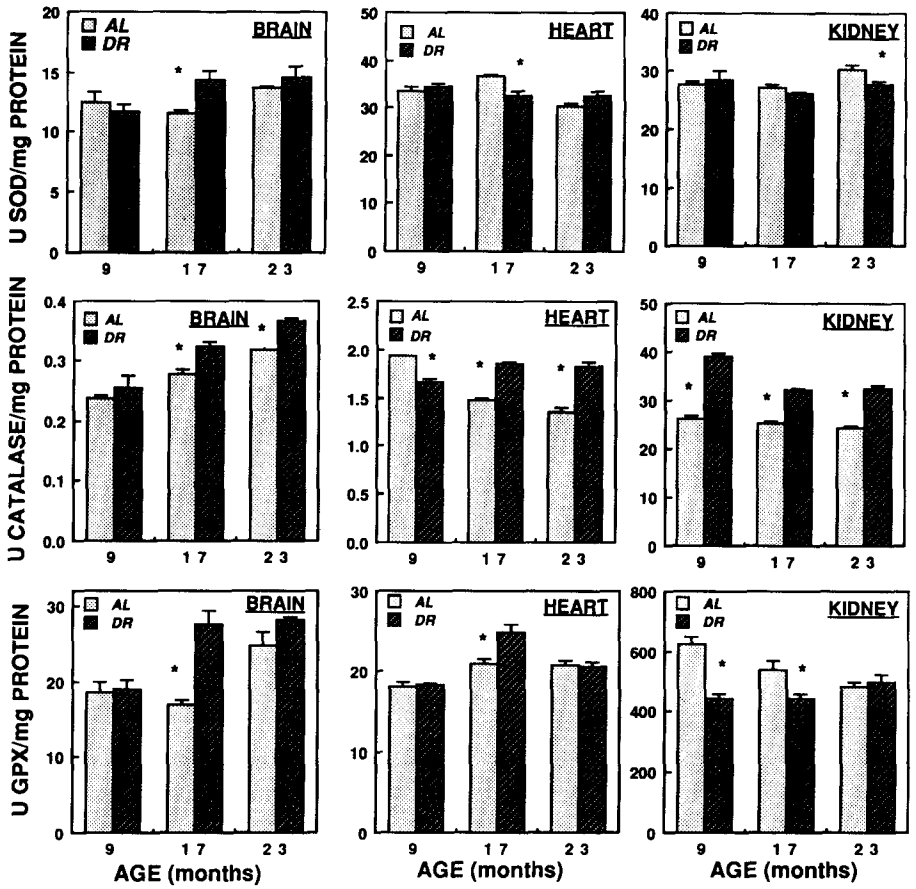

Fig. 7. Activities of superoxide dismutase (top), catalase (middle) and glutathione peroxidase (bottom) in the homogenates of the brain, heart and kidney from ad libitum fed (AL) and dietary restricted (DR) mice at 9, 17 and 23 months of age. Results are average ± S.E.M. of 3–11 determinations.

17 as opposed to 23 months), but no further increase thereafter. On the other hand, enzyme activity tended to decrease with age in the kidney of the AL group, but not in the DR group. In the heart, enzyme activity was relatively higher in the DR group at 17 months of age, but no differences between the groups existed at 9 or 23 months of age. In the case of each organ, the trends described resulted in a significant Age × Diet interaction ($F_s > 11$, $P_s < 0.005$).

4. Discussion

Results of this study suggest that relatively high caloric intake, as achieved by the AL mice, results in a potentially deleterious alteration in mitochondrial state 4 or resting respiration rate as well as the autoxidizability of the components of the mitochondrial electron transport chain producing $O_2^{\cdot -}$ and H_2O_2. Results also indicate

that both aging and caloric intake are associated with increased protein oxidative damage, which seems to result from enhanced production of oxidants rather than a uniform attenuation in antioxidant defenses.

The resting or state 4 mitochondrial respiration was found to increase with age in all the three organs of the AL mice. DR was found to prevent this age-related alteration in the brain and the kidney and delay or lessen its magnitude in the heart. Since the age-related increase in state 4 respiration occurred consistently in the AL but not the DR group, this alteration would seem to be related to caloric intake rather than aging and could thus be considered to be a feature of mitochondrial adaptation to the nutrient fluxes.

The rate of mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation is highest under state 4 conditions (excess substrate but no exogenous ADP) in the coupled mitochondria, when the components of the electron transport chain are in a reduced state [8]. Autoxidation of ubisemiquinone is believed to be the main source of $O_2^{\cdot -}$ generation. In contrast, under state 3 conditions when the rate of O_2 consumption by mitochondria is relatively high and the electron carriers are in an oxidized state, there is no detectable H_2O_2 release by mitochondria. Thus the most probable explanation for the observed positive correlation between the rate of state 4 respiration and the rate of $O_2^{\cdot -}$ and H_2O_2 release by mitochondria is that the increase in the state 4 respiration is a reflection of the enhanced diversion of oxygen to the formation of $O_2^{\cdot -}$ and H_2O_2 and not of uncoupling. This interpretation is supported by the fact that experimental uncoupling of mitochondria, which enhances the rate of oxygen utilization leads to a decrease in the rate of $O_2^{\cdot -}$ and H_2O_2 generation [8] as also shown by us previously [29].

Since the rates of mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation were found to increase with age in all the three organs in both the AL and the DR mice, albeit to a lesser extent in the latter, this alteration would seem to be a marker of an age-related change which is modulated by the amount of caloric intake. Further evidence supporting the link between mitochondrial generation of $O_2^{\cdot -}$ and H_2O_2 and aging is provided by previous studies on both insects and mammals [30–32]. Rates of mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation were found to not only increase with age, but were also inversely correlated with the life expectancy of flies [30–32]. Similarly, mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation in liver [33], kidney and heart [21] were shown to be inversely related to the maximum life span potential of different mammalian species [33]. Stadtman et al. [9] have demonstrated the accretion of protein carbonyl content in three different model systems of aging. The findings of the present study, indicating that protein carbonyls increase with age in all the three organs of the AL group and this effect is retarded, but not eliminated, by dietary restriction, suggests that protein oxidative damage is linked to the rate of aging and is not simply a by-product of over-feeding in the AL group. A similar observation was made in a very short-term study by Youngman et al. in the rat liver [34]. Dietary restriction was initiated at 3 weeks of age and ended at 15 weeks of age. Altogether, such findings support the concept that oxidative damage may be a contributory factor in aging [35] and is affected by caloric intake.

The mechanism underlying the age- and dietary-related accumulation of protein oxidative damage is more likely to involve corresponding elevations in mitochon-

drial oxidant generation rather than decreases in the levels of antioxidant defenses. This view is supported by the observation that unlike the mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation, antioxidant defenses, in general, do not decline with age in the AL or the DR group. Although catalase activity tends to be higher in the DR than the AL mice, the absence of a decline in its activity as a function of age indicates that variations in catalase levels cannot explain the age-related increase in protein oxidative damage. Notwithstanding, further studies are needed to resolve the relative contributions of prooxidants and antioxidants in lessening the oxidative damage associated with dietary restriction.

In summary, results indicate that protein oxidative damage is associated with the aging process and that dietary restriction, which prolongs life span, lessens this damage. The underlying mechanism may involve alterations in the inner mitochondrial membrane affecting both the state 4 respiration and $O_2^{\cdot -}$ and H_2O_2 generation. Because the levels of oxidative damage as well as mitochondrial $O_2^{\cdot -}$ and H_2O_2 generation increase with age and are diminished by the life prolonging regime of dietary restriction, they may be acting as biomarkers of aging.

5. Acknowledgments

This research was supported by the grants R01AG7657 and R01AG7695 from the National Institutes of Health — National Institute on Aging. We gratefully acknowledge the assistance by the National Center for Toxicological Research, Jefferson, AR in the maintenance of the animals and providing the data on mortality and body weights of the mice.

6. References

1 C.M. McCay, M.F. Crowell and L.A. Maynard, The effect of retarded growth upon the length of lifespan and upon ultimate body size. *J. Nutr.*, *10* (1935) 63–79.
2 R. Weindruch, R.L. Walford, S. Fligiel and D. Guthrie, The retardation of aging in mice by dietary restriction: Longevity, cancer, immunity and lifetime energy intake. *J. Nutr.*, 116 (1986) 641–654.
3 B.P. Yu, Update on food restriction and aging. In M. Rothstein (ed.), *Review of Biological Research in Aging*, Vol. IV, Raven Press, New York, 1987, pp. 495–505.
4 E.J. Masoro and R.J.M. McCarter, Aging as a consequence of fuel utilization. *Aging Clin. Exp. Res.*, *3* (1991) 117–128.
5 A. Richardson, Changes in the expression of genes involved in protecting cells against stress and free radicals. *Aging Clin. Exp. Res.*, *3* (1991) 403–405.
6 D. Harman, Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.*, *11* (1956) 298–300.
7 R.S. Sohal and R.G. Allen, Relationship between oxygen metabolism, aging and development. *Adv. Free Rad. Biol. Med.*, *2* (1986) 117–160.
8 B. Chance, H. Sies and A. Boveris, Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.*, *59* (1979) 527–603.
9 E.R. Stadtman, Protein oxidation and aging. *Science*, *257* (1992) 1220–1224.
10 R.S. Sohal, S. Agarwal, A. Dubey and W.C. Orr, Protein oxidative damage is associated with life expectancy of houseflies. *Proc. Natl. Acad. Sci. USA*, *90* (1993) 7255–7259.
11 E.R. Stadtman and C.N. Oliver, Metal-catalyzed oxidation of proteins. *J. Biol. Chem.*, *266* (1991) 2005–2008.

12 E.R. Stadtman, P.E. Starke-Reed, C.N. Oliver, J.M. Carney and R.A. Flyod, Protein modifications in aging. In I. Emerit and B. Chance, (eds.), *Free Radicals and Aging*, Birkhauser, Basel, 1992, pp. 64–72.
13 W.M. Witt, C.D. Brand, V.G. Atwood and O.A. Soave, A nationally supported study on caloric restriction of rodents. *Lab Animal 18* (1989) 37–43.
14 M.J. Forster and H. Lal, Neurobehavioral biomarkers of aging: Influence of genotype and dietary restriction. *Biomed. Environ. Sci., 4* (1991) 144–165.
15 M.J. Forster and H. Lal, Within-subject behavioral analysis of recent memory in aging mice. *Behav. Pharmacol., 3* (1992) 337–349.
16 C.E. Finch, *Longevity, Senescence and the Genome*, University of Chicago Press, Chicago, 1990.
17 R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shaltiel and E. Stadtman, Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol., 186* (1990) 464–478.
18 K. Ozawa, K. Seta, H. Takeda, K. Ando, H. Handa and C. Araki, On the isolation of mitochondria with high respiratory contrast from rat brain. *J. Biol. Chem., 59* (1966) 501–510.
19 J.C. Arcos, R.S. Sohal, S.C. Sun, M.F. Argus and G.E. Burch, Changes in ultrastructure and respiratory control in mitochondria of rat heart hypertrophied by exercise. *Exp. Mol. Pathol., 8* (1968) 49–65.
20 P.J. Hayden and J.L. Stevens, Cysteine conjugate toxicity, metabolism and binding to macromolecules in isolated rat kidney mitochondria. *Mol. Pharmacol., 37* (1990) 468–476.
21 H.-H. Ku and R.S. Sohal, Comparison of mitochondrial prooxidant generation and antioxidant defenses between rat and pigeon: Possible basis of variation in longevity and metabolic potential. *Mech. Ageing Dev., 72* (1993) 67–76.
22 A. Boveris, Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. *Methods Enzymol., 105* (1984) 429–435.
23 P.A. Hyslop and L.A. Sklar, A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: Its use in the simultaneous fluorimetric assay of cellular activation processes. *Anal. Biochem., 141* (1984) 280–286.
24 H.P. Misra and I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem., 247* (1972) 3170–3175.
25 R.S. Sohal, L. Arnold and W.C. Orr, Effect of age on superoxide dismutase, catalase, glutathione reductase, inorganic peroxides, TBA-reactive material, GSH/GSSG, NADPH/$NADP^+$ and NADH/NAD^+ in *Drosophila melanogaster*. *Mech. Ageing Dev., 56* (1990) 223–235.
26 H. Luck, Catalase. In H. Bergmeyer, (ed.), *Methods of Enzymatic Analysis*, Academic Press, New York, 1965, pp. 885–894.
27 D.E. Paglia and W.N. Valentine, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med., 70* (1967) 158–169.
28 R.S. Sohal, L.A. Arnold and B.H. Sohal, Age-related changes in antioxidant enzymes and prooxidant generation in tissues of the rat with special reference to parameters in two insect species. *Free Rad. Biol. Med., 10* (1990) 495–500.
29 R.S. Sohal and B.H. Sohal, Hydrogen peroxide release by mitochondria increases during aging. *Mech. Ageing Dev., 57* (1991) 187–202.
30 K.J. Farmer and R.S. Sohal, Relationship between superoxide anion radical generation and aging in the housefly, *Musca domestica*. *Free Rad. Biol. Med., 7* (1989) 23–29.
31 R.S. Sohal, B.H. Sohal and U.T. Brunk, Relationship between antioxidant defenses and longevity in different mammalian species. *Mech. Ageing Dev., 53* (1990) 217–227.
32 R.S. Sohal, Hydrogen peroxide production by mitochondria may be a biomarker of aging. *Mech. Ageing Dev., 60* (1991) 189–198.
33 R.S. Sohal, I. Svensson, B.H. Sohal and U.T. Brunk, Superoxide anion radical production in different animal species. *Mech. Ageing Dev., 49* (1989) 129–135.
34 L.D. Youngman, J.-Y.K. Park and B.N. Ames, Protein oxidation associated with aging is reduced by dietary restriction of protein or calories. *Proc. Natl. Acad. Sci. USA, 89* (1992) 9112–9116.
35 W.A. Pryor, The free radical theory of aging revisited: A critique and a suggested disease specific theory. In H.R. Warner, R.N. Butler, R.L. Sprott and E.L. Schneider (eds.), *Modern Biological Theories of Aging*, Raven Press, New York, 1987, pp. 89–112.