INCREASED EFFECTIVE ACTIVITY OF RAT LIVER CATALASE BY DIETARY RESTRICTION¹

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ABSTRACT

While dietary restriction (DR) increases maximum life span in many animal species, the mechanisms by which this is achieved remain unclear. One possibility is that DR may act in part to reduce free radical levels by retarding age-related declines in rat liver catalase activity. We measured liver cytosolic catalase activity at various times of day in 9-12 month old male (BN X F344)F₁ rats fed ad libitum (AL) or subjected to a 30% DR from 14 weeks of age. Catalase activity (expressed as $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g liver}^{-1}$) in both diet groups reached minimums at 0600 h but activity was 26% higher in DR as compared to AL rats. This traditional expression of catalase activity did not significantly differ between diet groups at other times of day. One must be careful in the interpretation of such data, however, since catalase is rapidly inactivated by its substrate (H_2O_2), thus displaying abnormal enzyme kinetics. In order to avoid this difficulty we evaluated the time period during which the reaction remained linear and multiplied it by its activity to yield the effective catalase activity. Using this method we found a significant increase in catalase activity in DR animals at several H_2O_2 concentrations during the light span. At 1800 h (the beginning of the dark span when the controls initiated peak food intake), fewer and smaller dietary differences were observed and no dietary effects were observed at 2400 h. These data suggest that DR reduces the rate of accumulation of inactive catalase and may contribute to an increased capacity in DR animals to remove free radicals.

INTRODUCTION

Dietary restriction (DR) retards aging and the rate of occurrence of a number of age-related disease processes (1,2). It has been widely speculated that free radicals may be involved in the process of aging and that one of the beneficial effects of DR may be to decrease the generation and/or increase the detoxification of free radicals arising as a result of oxidative energy metabolism (1,3). This notion is appealing because the fundamental requirement for all diet regimens which increase

species-specific maximum life span is reduced energy intake. Although little data has been reported on the possibility that DR may reduce free radical production, the activities of liver enzymes such as catalase (CAT) and superoxide dismutase, which are involved in free radical detoxification, have been reported to be increased by DR (4-7). The age at which these effects of DR are expressed varies (4-7). Further, a fairly extensive literature describes age-related declines in liver and kidney catalase activity in ad libitum (AL) fed rats and mice (8-10). The decomposition of H_2O_2 catalyzed by CAT (reviewed in 11) produces an enzyme-substrate intermediate (Compound I) as a first step followed by a second step where another molecule of H_2O_2 is used to produce oxygen and water. However, Compound I is also subject to addition of a second molecule of H_2O_2 which produces the inactive Compound II (12, 13). There is a direct proportionality between the concentration of H_2O_2 and the rate of Compound II formation which contributes to abnormal kinetics even at subsaturating substrate levels. This abnormal kinetic behavior makes it difficult if not impossible to define activity in international units and thus several alternative methods of evaluation have been developed or are being proposed (14). The method we are suggesting is based on the observed differential rates of Compound II accumulation and/or reactivation to free enzyme which is believed to play a role in H_2O_2 detoxification. In our procedure, we determined the period of time during which CAT remained maximally active (indicating the rate of accumulation of Compound II) in rats fed either AL or subjected to DR. We believe this method provides a new means of quantifying the effective activity of CAT and gives a reasonable estimation as to the effect of DR on CAT.

PROCEDURES

Animals and Housing

A detailed description of husbandry procedures and environmental conditions has been reported elsewhere (15). Briefly, male (Brown Norway x Fischer 344)F₁ rats were bred and raised at the National Center for Toxicological Research in a specific pathogen free environment at 23°C. The rats were maintained on a light-dark cycle with lights on from 0600 to 1800 h. The rats were housed singly in plastic cages with wire metal tops and hardwood chip bedding. Cages were changed weekly and fresh water was always available.

¹Portions of this work were presented at the 1991 meeting of the Federation of American Societies for Experimental Biology (Weindruch, R., Feuers, R.J., Leakey, J. [1991] Caloric restriction (CR) enhances the effective activity of rat liver catalase. FASEB J. 5:A1448).

This rat strain is quite long-lived and responds robustly to DR: male rats fed AL show an average life span of 33 mo and a maximum life span (mean of the longed lived decile) of 40 months whereas rats subjected to DR live an average of 40 mo and, at 46 mo of age, 15% of the cohort remains alive (D. Brand and V. Attwood, National Center for Toxicological Research, personal communication).

Diets, Feeding Strategy, and Food Consumption

All rats were weaned at 3 wks of age and fed the Standard NIH-31 diet (containing 0.315% vitamin mixture and 0.185% mineral mixture) until 14 wks of age. At this time, the rats were randomly assigned to a control group which continued to eat the nonpurified Standard NIH-31 diet *ad libitum* (Group AL) and a group subjected to dietary restriction (Group DR). The DR group received a daily feeding at 1000 h calculated to provide 60% of the AL intake of age-matched AL rats. The restricted rats ate a modified NIH-31 diet which was intended to provide 40% fewer calories (from carbohydrate, fat and protein) but was supplemented with vitamin and mineral fortification mixtures at 1.67 times that in the standard diet. Thus, DR rats were allotted vitamin and mineral intakes which closely matched those of AL controls. However, it was later discovered by the use of a more sensitive means of quantifying food consumption (15) that AL rats wasted some food and therefore the actual consumption level was slightly overestimated. After correcting for the diminished AL intake, it was determined that the DR rats consumed about 70% as many calories when food consumption was monitored for a 1 wk period in AL rats. This was accomplished by a load cell weight measuring device electronically coupled to an automated data acquisition and process control system (15).

Experimental Design

Sixteen AL rats (9-12 mo old) were randomly assigned into four groups of four rats per group and the same was done for 16 DR rats. Beginning at 1200 h, an AL rat was quietly removed from the animal room and killed by decapitation. Blood was collected and the liver was quickly taken and placed on ice. Blood glucose was immediately determined as previously described (16) using the Lifescan™ glucose monitor (Johnson and Johnson, Elkhart, IN). These steps were repeated for the first DR rat, and the sequence continued until four AL and four DR rats had been killed for the 1200 h time point. The entire process was repeated at 1800 h and 0600 h the next morning. The feeding regimen described above was maintained without interruption over the interval when rats were killed.

Tissue Preparation

Fresh liver was rinsed in ice-cold saline, blotted, weighed and homogenized (using a Teflon-glass homogenizer) at 4°C in 3.5 volumes of a buffer designed to stabilize a broad range of enzymes (17). The buffer contained 250 mmol/L sucrose, 24 mmol/L potassium chloride, 10

mmol/L HEPES (acid), 1 mmol/L dithiothreitol, 0.5 mmol/L EDTA, 0.02% (weight/volume) phenylmethylsulfonylfluoride and 10% glycerol at Ph 7.4. The homogenates were centrifuged at 8,000 x g for 20 min at 4°C and the supernatants were further centrifuged at 100,000 x g for 30 min in a Beckman Model LS-75 ultracentrifuge using a 50 Ti rotor. The resultant lipid-free supernatants were quickly aliquoted and stored at -70°C to await analysis as the cytosolic fraction for the CAT assay. Cytosolic protein concentrations were determined by the method of Lowry et al.(18) using bovine serum albumin as the standard.

Catalase Assay

CAT activity was determined spectrophotometrically by measuring the decomposition of H_2O_2 over a broad concentration range (7.8, 15.6, 19.8, 26.0, 40.5 and 62.0 mmol/L) at 240 nm (14). An activity unit was defined in traditional units as the amount of enzyme required to decompose 1 $\mu\text{mol } H_2O_2 \cdot \text{min}^{-1}$. These data were then either expressed as units of enzyme activity per mg of protein or as units of enzyme activity per g wet weight of liver. The rapid inactivation of CAT, which has been thought to be in direct proportion to the concentration of H_2O_2 , is characterized by abnormal kinetics that make it either impossible or at least extremely difficult to define CAT activity in international units. Therefore, many diverse and acceptable definitions of units have been used (14).

For the purpose of these studies the time required for the observable inactivation of CAT at a particular concentration of H_2O_2 was defined as the time to reach a nonlinear loss of absorbance versus time. This time was determined for each reaction and multiplied by $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ to yield the effective CAT activity. Thus, effective CAT activity depicts the catalytic capacity of the enzyme over the period of its maximal activity. We believe that describing the effective CAT activity by this method takes into account the period of time required for inactivation of the enzyme by H_2O_2 (i.e., resistance to substrate inhibition). Additionally, this type approach has been validated as an useful mathematical model for expression of CAT activity (19).

Statistics

Data analysis was done using Statistical Analysis Systems (SAS) programs (Cary, NC). Food consumption was evaluated by cosinor regression (20) while all other data were analyzed by ANOVA or Student's t-test (two-tailed) as indicated. Results are reported as mean $\pm$ SEM. A probability level of <0.05 was considered as statistically significant.

RESULTS

Body Weight and Food Consumption.

The average body weight at 1100 h of the 16 AL animals used in this study was 467.9 ± 41.6 grams and the weight of the 16 DR rats was 295.2 ± 13.7 grams which equates to a 36% reduction. Food consumption in AL animals

Figure 1: Circadian rhythm of food consumption (g/h) in 9-12 mo old ad libitum fed (BN X F344)F₁ male rats. Values are expressed as mean $\pm$ SEM. The shaded bar along the time scale denotes the period of dark. Relative to a standard light-dark cycle, lights on would occur at 0600 h and lights off at 1800 h. Cosinor regression analysis indicated the presence of circadian differences ($p < 0.01$) and the acrophase (the time of peak food consumption) was calculated to be 2142 h.

was characterized by a strong circadian component as determined by cosinor regression analysis (Fig. 1; $P < 0.01$). Acrophase (i.e., the calculated time of maximum food intake) was at 2142 h with the majority of the food consumed during the dark. However, a significant amount of food was also consumed during the light span. The food intake of the AL rats averaged 16.5 ± 0.3 g/d. The rats subjected to DR were fed during the mid-light span and ate all food (11.5 g) within a 2 h period.

Blood Glucose and Cytosol Protein Content

Blood glucose levels for rats from the AL and DR groups showed similar patterns across the 24 h time span (Table 1; $P < 0.02$, ANOVA). Maximum observed levels of blood glucose did not differ between diet groups and occurred at 1200 h. Blood glucose dropped thereafter and was significantly lower by 2400 h in the DR group than it was at 1200 h. At 0600 h blood glucose levels from both AL and DR groups reached minimums and rats from the DR group showed significantly lower blood glucose levels when compared to rats from the AL group. The intergroup differences at 2400 h approached statistical significance ($P = 0.07$).

Table 1. Blood glucose and liver cytosolic protein from AL and DR rats at four times of day¹

Time h	Blood glucose		Cytosolic protein	
	AL mg/100ml	DR	AL mg/g wet weight	DR
0600	61.8 $\pm$ 1.9 ^{a,*}	54.3 $\pm$ 1.2 ^{a,*}	77.8 $\pm$ 4.4 ^{a,*}	103.8 $\pm$ 8.7 ^{a,*}
1200	79.5 $\pm$ 3.8 ^b	79.0 $\pm$ 2.8 ^b	73.4 $\pm$ 7.6 ^{a,*}	102.2 $\pm$ 8.7 ^{a,*}
1800	70.3 $\pm$ 3.5 ^{a,b}	65.0 $\pm$ 6.0 ^{a,b}	83.9 $\pm$ 8.9 ^{a,b}	97.8 $\pm$ 7.4 ^a
2400	68.0 $\pm$ 1.7 ^{a,b}	59.8 $\pm$ 3.2 ^a	94.0 $\pm$ 4.7 ^b	99.9 $\pm$ 9.6 ^a

¹Each value is the mean $\pm$ SEM for four rats fed ad libitum (AL) or subjected to dietary restriction (DR). Means with different superscript letters designate statistically significant differences ($P < 0.05$) within a diet group across time of day as analyzed by ANOVA.

Differences between diet groups at a specific time of day were evaluated by the two tailed t-test with * indicating significant differences between diet groups.

Cytosolic protein content (mg/g liver) did not change across the 24 h span in the rats from the DR group. In contrast, the AL-fed rats showed maximum levels at 1800 and 2400 h with the value at 2400 h being significantly greater than those observed at 0600 and 1200 h (Table 1; $P < 0.01$, ANOVA). The recovery of cytosolic protein was higher in rats in the DR group as compared to AL rats at 0600 and 1200 h ($P < 0.01$, t-test).

CAT Activity in Traditional Units

Determination of cytosolic CAT activity in $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ using 26 mmol/L H_2O_2 in the reaction mixture showed no effects of diet at any time of day (Table 2). However, at 0600 h, DR animals showed a $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g liver}^{-1}$ mean value which was 26% higher than those found in AL rats ($P < 0.01$). For both modes of data expression, there was a statistically significant difference across the 24 h span, with maximums observed at 1200 and 1800 h and minimums at 0600 h ($P < 0.01$, ANOVA) for AL and DR rats. The use of other subsaturating or saturating substrate concentrations in the reaction mixtures yielded similar results (data not shown).

Table 2: Catalase Activity from AL and DR Rat Liver cytosols at Four Times of Day¹

Time h	Catalase Activity		Catalase Activity	
	AL $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$	DR	AL $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g liver}^{-1}$	DR
0600	1730 $\pm$ 90 ^a	1610 $\pm$ 140 ^a	133 $\pm$ 1 ^{a,*}	167 $\pm$ 17 ^{a,*}
1200	2790 $\pm$ 320 ^b	2590 $\pm$ 200 ^b	211 $\pm$ 45 ^b	266 $\pm$ 43 ^b
1800	2600 $\pm$ 50 ^b	2590 $\pm$ 270 ^b	219 $\pm$ 27 ^b	260 $\pm$ 49 ^b
2400	2160 $\pm$ 90 ^a	1830 $\pm$ 130 ^a	202 $\pm$ 11 ^b	185 $\pm$ 28 ^a

¹Each value is the mean $\pm$ SEM for four rats fed ad libitum (AL) or subjected to dietary restriction (DR). Reactions were performed in the presence of 26 mmol/L H_2O_2 . Means with different superscript letters designate statistically significant differences ($P < 0.05$) within a diet group across time of day as analyzed by ANOVA. Differences between diet groups at a specific time of day were evaluated by the two tailed t-test with * indicating significant differences between diet groups.

Effective CAT Activity

Effective CAT activities were also determined using a range of H_2O_2 concentrations at each of the four times of day. At 0600 h the effective enzyme levels were significantly higher in DR cytosols at several intermediate H_2O_2 concentrations (Fig. 2A). Values from DR animals were 42, 58 and 52% higher at H_2O_2 concentrations of 15.6, 19.8, and 26.0 mmol/L, respectively than those observed in cytosols from AL rats. The effective CAT activity was not influenced by diet at H_2O_2 concentrations of 7.8, 15.6, 26.0 or 62 mmol/L for rats killed at 1200 h (Fig. 2B). However, at this same time of day, values were 62 and 64% higher in cytosols from DR rats at H_2O_2 concentrations of 19.8 and 40.5 mmol/L, respectively. Maximum levels were observed at H_2O_2 concentrations of 26.0 and 40.5 mmol/L in DR cytosols and 26.0 mmol/L in AL cytosols. Evaluation of cytosols from animals killed at 1800 h (beginning of the dark span) showed that dietary differences were smaller with significantly increased effective CAT activities detected only for

Figure 2: Effective activity of liver catalase in AL (●) and DR (○) rats at different times of day. Means with different letter superscripts within a diet group and H_2O_2 concentration indicate statistically significant differences across time of day ($P < 0.05$, ANOVA). An * identifies means which were significantly different between diet groups at a specific time of day and concentration of H_2O_2 ($P < 0.05$, student t-test).

assays performed using 15.6 mmol/L H_2O_2 a 35% increase Fig. 2C). The maximum values at this time point were observed at 26.0 mmol/L in DR cytosols and at H_2O_2 concentrations of 26.0 mmol/L or greater in AL cytosols. The trend toward fewer significant effects of DR during the dark span continued and was even more pronounced for rats killed at 2400 h when no significant differences occurred at any substrate concentration (Fig. 2D) between AL and DR animals. Maximum activities at this time point were at 26.0 mmol/L H_2O_2 for cytosols from both diet groups. When comparing the panels of Figure 2 it is apparent that at low or intermediate substrate concentrations, maximum values were obtained during the mid-light span in both diet groups. Circadian components were not observed when assays were performed using the highest H_2O_2 concentrations.

DISCUSSION

The detoxification of H_2O_2 by CAT proceeds through two types of reactions (11,21). The enzyme combines with one molecule of H_2O_2 as an obligatory step to form an intermediate known as Compound I. In the absence of an appropriate hydrogen donor other than H_2O_2 , catalytic activity is expressed when another molecule of H_2O_2 is utilized to yield free enzyme plus $2H_2O_2$ and O_2 . Alterna-

tively, Compound I may bind another molecule of H_2O_2 to produce the inactive Compound II (yielding abnormal kinetics) which can be converted back to Compound I and H_2O through an enzymatically catalyzed reduction utilizing NADPH as substrate. A second NADPH dependent reduction of Compound I results in release of free enzyme and H_2O (11). Catalase can also catalyze a second type of reaction. In the presence of Compound I, other appropriate hydrogen donors (e.g., lower to aliphatic alcohols) stimulate peroxidatic activity which yields free enzyme H_2O and an aldehyde as products.

Because CAT displays complex kinetics, the quantification of its activity is complicated. By calculating activity as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ and multiplying this by the time span of proportional conversion of substrate to product at maximal rates, the effective amount of enzyme activity could be determined at a given concentration of H_2O_2 . Our data suggest that this approach can distinguish the effects of diet on H_2O_2 detoxification which were not readily apparent when a traditional approach was used. The increased effective activity due to DR suggests that the accumulation of the inactive form of CAT (Compound II) occurred at a slower rate in cytosols from restricted rats. There are many potential nonmutually exclusive explanations for this action of DR including: 1) an increased rate of

catalysis of a second molecule of H_2O_2 in the presence of Compound I; 2) an altered enzyme structure having a higher affinity for H_2O_2 or an increased rate of dissociation of the enzyme-substrate complex; 3) there may be an increased concentration of catalase (a possibility supported by a report from Richardson's group (7) that DR increases levels of rat liver catalase mRNA); and 4) higher levels of NADPH to facilitate the NADPH-dependent reduction of Compound II (13). The data reported herein do not distinguish between these possibilities but do suggest that DR may produce higher levels of enzyme activity and slow the accumulation of Compound II at certain times of day.

There is literature which describes liver CAT activity using traditional analysis in rodents subjected to DR although no prior studies provide circadian information and all appear to have employed mid-morning killing. Koizumi et al. (4) first reported that DR (50% of AL) initiated at 3 wks of age in female mice from a long-lived strain resulted in 40% increases in activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ in 600 g supernatants using 26 mmol/L H_2O_2) at 12 mo of age. The difference was even more pronounced at 24 mo of age. Male Fischer 344 rats subjected to DR (60% of ad lib intake from 6 wks of age) showed sporadic differences in cytosolic CAT activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$) during the first year of life (5). At 24 mo of age, activity declined in both diet groups but was 130% higher in the DR group. As was the case in our hands, the variation among animals for CAT activity was often large and values resembled (but were less than) those reported herein. The higher CAT activity we report may relate to strain differences with the shorter-lived Fischer 344 rat (22) giving lower values than the long-lived (BN X F344) F₁ hybrid. Richardson's group (7) also studied male Fischer 344 rats subjected to the same DR regimen as just described and evaluated liver cytosolic CAT activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$) using a relatively low H_2O_2 concentration (15 mmol/L). They could not detect an effect of DR at 4 or 12 mo of age; however, by 21 mo an age-related decline in CAT activity in the AL group occurred which was retarded by DR. This same group reported earlier (6) that at 18 mo of age both cytosolic CAT activity (10.3 mmol/L H_2O_2) and CAT mRNA levels were increased by DR relative to control values. Our observed values are in reasonable agreement with those of the latter two reports.

Thus, to date no consistently detectable effects of DR on CAT activity have been found in Fischer 344 rats until they approach mid-life. At 12 months of age rats from the long-lived (BN x F344)F₁ hybrid are relatively younger than are 12 mo old Fischer 344 rats (i.e., the 10th decile survival for ad lib fed Fischer 344 rats is 28 mo [22] vs. 40 mo for the (BN x F344)F₁ hybrid [Brand and Attwood, personal communication]). Also, the level of restriction employed in the present study (30%) was slightly less than that (40%) used in the studies on Fischer 344 rats. Therefore, using a circadian approach in conjunction with evaluation of effective activity we were able to distinguish a significant effect of DR which supports the possibility that free radical detoxification by CAT

is positively influenced by DR relatively early in life. Our finding of an increased CAT activity in young, restricted animals from a long-lived strain agrees with the finding of Koizumi *et al* (4) in mice.

Most enzyme activities show significant circadian variations and many of these rhythms are strongly influenced by feeding activity in rodents (23). Additionally, maintenance of enzyme activity and regulation of these rhythms often deteriorate with aging (24). We observed that while food consumption in AL (BN x F344)F₁ rats peaked during the dark span, considerable feeding also occurred during the light span. This light and dark feeding behavior of AL rats probably contributed to their relatively low amplitudes for glucose rhythms and may also explain the peak for blood glucose which occurred during the mid-light in AL and DR rats (the time DR rats were fed). It is probable that the feeding pattern of the AL rats was influenced by their sharing a room with the morning-fed DR rats. Future DR studies would benefit by "phase shifting" the feeding schedule of DR rats to approximate the period of peak food intake of the AL group (25).

Cytosolic protein content from AL rats was out of phase with glucose levels and was significantly lower than the values for cytosolic protein seen in DR rats (which did not change across the time of day). This impact of DR on the amount and rhythmicity of cytosolic protein directly affects the outcome of data expressed as U/mg protein and may mask dietary influences on enzyme activity by underestimating actual enzyme levels. Use of U/g tissue indicated that DR increased traditional CAT activity to levels higher than in controls at the beginning of the light span. However, significant dietary differences in this value were not detected at other circadian stages. The tendency for maximum values to occur during the mid-light span suggests a clear association of CAT activity with feeding in the DR group. The possibility exists that feeding results in an increased activity of energy metabolism which leads to free radical generation and an increased CAT activity.

Significant circadian effects were also observed for the effective CAT activity. The dietary differences were most overt during the light span. During the dark span, AL values increased while DR values decreased. Because the AL rats eat the majority of their food during the dark whereas the DR rats eat in mid-morning, an association between peak effective CAT activity and food intake may exist. This also would accord with the aforementioned possibility that free radicals are formed as a result of oxidative energy metabolism.

Utilization of a new approach to quantify CAT activity has provided additional insight into a mechanism by which DR may act to increase the rates of free radical detoxification. In accord with previous reports, DR appears to increase the level of enzyme activity as a result of increased enzyme concentration or, as recently suggested, by translational or posttranslational effects (26). Importantly, DR also appears to produce another distinct outcome which provides an advantage for free radical detoxification: CAT appears to be less susceptible

to oxidation and inactivation by H_2O_2 . This intriguing possibility warrants additional study. The thorough characterization of the influences of DR on free radical metabolism should provide insights into the role of free radicals on aging and age-associated diseases.

ABBREVIATIONS

AL, ad libitum; CAT, Catalase; DR, Dietary Restriction

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