Effects of Aging and Food Restriction on the Antioxidant Enzyme Activity of Rat Livers

Fujiya Gomi¹ and Mitsuyoshi Matsuo²

¹Toyoko Metropolitan Institute of Gerontology and ²Department of Biology, Faculty of Science, Konan University, Hyogo, Japan.

The effects of aging and food restriction on the activities and mRNA levels of antioxidant enzymes in rat livers were examined. Rats were fed ad libitum every day (AL) or ad libitum on every other weekday (FR). At 30 months of age, the catalase and glutathione peroxidase activities were lower, whereas the thiobarbituric acid (TBA) value, an index of lipid peroxidation of the AL rats, was higher than that at younger ages. At 33 months of age, copper/zinc superoxide dismutase (CuZnSOD), catalase, and glutathione peroxidase activities increased, and the TBA value of the FR rats remained unchanged as compared with those at younger ages. Until old age, food restriction gave rather decreasing effects on antioxidant enzyme activities. Furthermore, antioxidant enzyme activities and the TBA values of the FR rats were higher at the end of a fasting period than those at the end of a feeding period.

ACTIVE oxygen species are believed to be usually generated in aerobic cells, and aerobic organisms are provided with antioxidant defense systems which avert damages due to oxidative stress (1,2). The antioxidant defense systems are composed of antioxidant enzymes and biological antioxidants; the former include superoxide dismutase (SOD), catalase, and glutathione peroxidase, and the latter include reduced glutathione, ascorbic acid, and vitamin E. It has been suggested that deteriorative changes in the antioxidant defense system cause accumulation of oxidative damage, which is relevant to the aging process (3).

It is well known that food restriction extends the life spans of rats and mice, and it is thought to retard the aging process in these animals (4). However, the mechanisms by which food restriction extends the life spans of rats and mice are unknown. Although it has been proposed that food restriction retards aging by slowing growth and development (5), by reducing body fat content (6), or by reducing the metabolic rate (7), the results of several studies do not support these hypotheses (8,9). Further, endocrine and neuronal regulation, protein turnover, gene expression, and oxidative damage have been suggested to be involved in the retardation of aging by food restriction (8,10,11).

There are several lines of evidence that food restriction prevents age-related increases in oxidative damage. It has been found that in rats, food restriction suppresses age-dependent increases in the exhalation rates of ethane and pentane, indices of the in vivo lipid peroxidation (12), in the ratio of 8-hydroxy-2′-deoxyguanosine residues to 2′-deoxyguanosine residues, an index of DNA oxidation, in liver DNA (13), and in the liver protein carbonyl group content, an index of liver protein oxidation (14).

The extent of oxidative damage is related to the antioxidant defense capacity. In order to determine the effects of aging and food restriction on liver antioxidant defense systems, we examined the activity and mRNA level of antioxidant enzymes in livers of ad libitum-fed (AL) and food-restricted (FR) rats at various ages.

MATERIALS AND METHODS

Animals
Male Fischer 344 rats at 3 weeks of age were obtained from Charles River Japan (Atsugi, Japan). One hundred and seven rats were housed at a density of 3 or 4 rats per cage and fed on a commercial laboratory chow diet (CRF-1; Oriental Yeast, Tokyo, Japan). After 4 weeks of age, one half of them (AL rats) were fed ad libitum every day, and the other half (FR rats) were fed ad libitum every other weekday—every Monday, Wednesday, and Friday. This latter feeding regime resulted in marked extension of the life spans (12,15). The 50% survival rates of the AL and FR rats were 29 and 37 months, respectively (12). The rats were decapitated under anesthesia at 6, 13, 24, 26, 30, or 33 months of age. Only FR rats were sacrificed at 33 months of age. In the case of FR rats, they were sacrificed under fed condition, i.e., immediately before removal of their diet.

For determination of the effects of intermittent feeding on the activities and mRNA levels of antioxidant enzymes and the TBA value, FR rats were also sacrificed immediately before food supply (after 24 hours of fasting) at 13 and 26 months of age.

Biochemical Analyses
The livers from the sacrificed rats were isolated and cut into small pieces. The pieces were immediately frozen in liquid nitrogen, in which they remained frozen for less than 2.5 years prior to use.

For the preparation of liver 10% homogenates (wet weight/volume), the frozen liver pieces were thawed on ice and then homogenized with a Polytron homogenizer (Kinematica GmbH, Luzerne, Switzerland). The homogenates were divided into aliquots and stored in liquid nitrogen for less than 2 weeks until use, at which time they were thawed and subjected to antioxidant enzyme activity determination. SOD activity was determined by the nitrite method...
(16); catalase activity was determined on the basis of the rate of hydrogen peroxide decomposition (17); glutathione peroxidase activity was determined on the basis of the rate of oxidation of NADPH coupled to a glutathione-glutathione reductase system (18). TBA value was determined according to the method of Uchiyama and Mihara (19).

For the determination of mRNA levels, frozen liver pieces were powdered at -196°C. Total RNA was extracted from the powdered liver pieces with a QuickPrep Total RNA Kit (Pharmacia Biotech, Uppsala, Sweden). Ten μg of total RNA was denatured and electrophoretically fractionated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane, ultraviolet ray-crosslinked, and hybridized to each of 32P-labeled probe. Human CuZnSOD cDNA (460 base pairs, bp) probe and human manganese superoxide dismutase (MnSOD) cDNA (1300 bp) probe, rat catalase cDNA (1596 bp) probe, and mouse glutathione peroxidase cDNA (500 bp) probe were gifts from Profs. M. Inoue of Osaka City University, S. Goto of Toho University, and A. Richardson of University of Texas, respectively. Chick β-actin cDNA (770 bp) probe was purchased from Oncor Inc. (Gaithersburg, MD). The nylon membrane was washed 3 times with 2X SSC/0.2% SDS for 10 min at room temperature and twice with 0.2X SSC/0.2% SDS for 30 min at 65°C. After washing, the nylon membrane was exposed to a sheet of Kodak XAR film with an intensifier at -80°C for 3 hours to 3 days. The film was then developed and analyzed with a scanner (420 oe, PDI Inc., Huntington Station, NY) and the optical analyzing software Quantity One (PDI Inc.). For determination of the mRNA levels of a given antioxidant enzyme, all samples from young AL, young FR, young fasted FR rats and several samples from old AL rats were run on a gel, and all samples of livers from old AL, old FR, old fasted FR rats and several samples of young AL rats were run on another gel. The results are expressed as relative mRNA levels, the ratio of mRNA levels for each antioxidant enzyme/the β-actin mRNA level, that is corrected relative to the mRNA level of young AL rats (the relative mRNA level of young AL rats must be 1.0).

**Statistical analysis.**—The effects of aging and food restriction on body weight, food intake, liver antioxidant enzyme activities, and liver antioxidant enzyme mRNA levels were tested for significance by a two-way (age vs feeding regime) analysis of variance (ANOVA) and Fisher PLSD test.

**RESULTS**

Male rats were grown under two different feeding regimes, i.e., ad libitum feeding and food restriction. Figure 1A shows age-dependent changes in the mean body weights of AL and FR rats. The body weights of rats, determined monthly, were analyzed with two-way ANOVA. For these monthly body weights, the effects of age and feeding regime and their interaction were found to be significant (p < .0001, < .0001, and < .0001). The mean body weight of the AL rat was significantly less at 25–30 months of age than at 15–21 and 23–24 months of age and significantly less at 26–30 months of age than at 22 months of age (p < .05). The mean body weight of the AL rats increased until about 13 months of age, remained at about 450 g until 24 months, and then decreased rapidly. The mean body weight of FR rats increased much more slowly until 12 months than that of AL rats and remained about 200 g until 33 months of age. Figure 1B shows age-dependent changes in the mean food intakes per week of the AL and FR rats. For these food intakes, the effects of age and feeding regime, and their interaction were found to be significant (p < .0001, < .0001, and = .0015, respectively). The Fisher
PLSD test revealed that for the AL rats, these food intakes were significantly less at 24, 27, or 30 months of age than at 18 months of age (p = .0003, .0051, and < .00001, respectively). The mean food intake of the AL rats was about 115 g per week from 15 until 27 months of age and then decreased rapidly. The mean food intake of the FR rats was about 60 g per week until 33 months of age. At 15 months of age, the mean food intake per week and mean body weight of the FR rats were about 55% and 44% of those of the AL rats, respectively.

The effects of aging and food restriction on the liver antioxidant enzyme activities and TBA values of rats were determined. In the case of the liver CuZnSOD activity, the effects of age and feeding regime were found to be significant (p = .0008 and < .0001, respectively). Figure 2A shows that at 6–24 months of age, the CuZnSOD activity of the FR rats was lower than that of the AL rats, and that the CuZnSOD activity of the FR rats was higher at 33 months of age than at 13–30 months of age. In the case of the MnSOD activity, the effects of age and feeding regime were found to be significant (p < .0001 and .0097, respectively), but no linear increase or decrease with advancing age was detected (Figure 2B). At 24 months of age, the MnSOD activity of the FR rats was significantly higher than that of the AL rats (Figure 2B). In the case of catalase activity, the effects of age and feeding regime, and the interaction of age and feeding regime were found to be significant (p < .0001, = .0278, and .0029, respectively). Figure 2C shows that at 6 and 13 months of age, the catalase activity of the FR rats was lower than that of the AL rats. The activities of the AL and the FR rats decreased with advancing age until 30 months of age. The catalase activity of the FR rats was significantly higher at 33 months of age than at 30 months of age. For glutathione peroxidase activity, the interaction of age and feeding regime was found to be significant (p = .0056). Figure 2D shows that the glutathione peroxidase activity of the AL rats was lower at 30 months of age than at 6, 24, and 26 months of age. The glutathione peroxidase activity of the FR rats was higher at 30 and 33 months of age than at 6–26 months and the glutathione peroxidase activity at 6 months of age was lower than that of the AL rats. Interestingly, the CuZnSOD, catalase, and glutathione peroxidase activities of the FR rats were higher or tended to be higher at 33 months of age than at 30 months of age (Figures 2A, C, and D). In the case of the TBA value, the effect of age and the interaction of age and feeding regime were found to be significant (p < .0001 and = .0221, respectively). Figure 2E shows that the TBA

![Graphs showing the effects of aging and food restriction on liver antioxidant enzymes and TBA values.](https://academic.oup.com/biomedgerontology/article-abstract/53A/3/B161/540461/5622638)
value of the AL rats increased with advancing age, and that of the FR rats remained unchanged from 13 months of age until 33 months of age. At 30 months of age, the TBA value of the FR rats was significantly lower than that of the AL rats.

Figure 3 shows the effect of intermittent feeding on the antioxidant enzyme activities and TBA values in the FR rats at 13 and 26 months of age. The liver antioxidant enzyme activities and TBA values were determined of the AL rats, the FR rats sacrificed at the end of a nonfasting day (fed FR rats; same as FR rats in Figure 2), and the FR rats sacrificed at the end of a fasting day (fasted FR rats). Except for MnSOD activity, the two-way ANOVA revealed a significant effect of feeding condition on CuZnSOD activity ($p = .0042$), and each of catalase and glutathione peroxidase activities and the TBA values ($p < .0001$). CuZnSOD activity of the young fasted FR rats (Figure 3A), catalase activities of the young and old fasted FR rats (Figure 3C), and glutathione peroxidase activities of the young and old fasted FR rats (Figure 3D) were significantly higher than those of the corresponding fed FR rats. The CuZnSOD activity of the young fed FR rats was significantly lower than that of the young AL rats (Figure 3A). The MnSOD activity of old AL, old fed FR, old fasted FR rats was lower than those of the corresponding young rats (Figure 3B). For catalase activity, the effect of age and interaction of age and feeding condition were found to be significant ($p < .0001$ and $< .0001$, respectively). The catalase activity of the young fasted FR rats was significantly higher than that of the old fasted FR rats, and that of the young AL rats was significantly higher than that of the old AL rats (Figure 3C). The catalase activity of the young fed FR rats was lower than that of young AL rats, and that of the old fasted rats was higher than that of the old AL rats (Figure 3C). The TBA value of the young fasted FR rats was significantly higher than that of the young AL rats (Figure 3D). The TBA value of both the young and the old fasted FR rats was significantly higher than those of the corresponding fed FR rats (Figure 3E). The TBA value of the old fasted FR rats was significantly higher than that of the old AL rats (Figure 3E).

Figure 4 shows the liver antioxidant mRNA levels of the AL and FR rats at 13 and 26 months of age. The mRNA levels were determined simultaneously with the antioxidant enzyme activities. Two-way ANOVA of CuZnSOD mRNA levels showed that the effect of feeding condition and the interaction of age and feeding condition were significant ($p = .0002$ and .0386, respectively). In the case of MnSOD mRNA levels, the effects of feeding condition and age, and their interaction were found to be significant ($p = .0026$, .0109, and .0465, respectively). For catalase mRNA level, the effect of age was found to be significant ($p = .0013$). In the case of glutathione peroxidase mRNA levels, the effects
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Figure 4. The effects of intermittent feeding on the liver antioxidant enzyme mRNA levels of young and old FR rats. The liver CuZnSOD (A), MnSOD (B), catalase (C), and glutathione peroxidase (D) mRNA levels of rats at 13 (open columns) and 26 (closed columns) months of age are shown. Each column represents a mean value for 5–7 rats with the standard error of the mean. A pair of asterisks or daggers indicates a statistically significant (*p < .05) difference between the two values. Each FR rat value accompanied by the letter F is significantly different from the corresponding AL rat value, and each young rat value accompanied by the letter G is significantly different from the corresponding old rat value (*p < .05).

**DISCUSSION**

In the present study the liver catalase and glutathione peroxidase activities of the AL rats at 30 months of age were lower, whereas the TBA value was higher than those at 6–26 months of age (Figure 2). It has been reported that in the livers of male Fischer 344 rats, catalase activity decreases with advancing age while glutathione peroxidase activity remains unchanged. Many conflicting findings of age-related change in SOD activity have been published (3); e.g., there are reports that the SOD activity of rat brains decreased, increased, or remained unchanged with advancing aging.

In the livers of the FR rats at 33 months of age in the present study, CuZnSOD, catalase, and glutathione peroxidase activities were increased, whereas the TBA values remained unchanged (Figure 2). Previously, we found that the exhalation rates of ethane and pentane, indices of the in vivo lipid peroxidation, of the AL rats increase at 28–30 months of age, and that these age-dependent increases in the rates are suppressed by food restriction (12). These findings suggest that in the FR rats, the increase in the antioxidant enzyme activities may suppress lipid peroxidation in old age.

The activities of CuZnSOD at 6–24 months of age, cata-
lase at 6 and 13 months of age, and glutathione peroxidase at 6 months of age were lower in the livers of the FR rats than in those of the AL rats (Figure 2A). It was previously reported, however, that in livers of male Fischer 344 rats, CuZnSOD (20,21) and catalase (20,22) activities were higher under food restriction than under ad libitum feeding, and that in livers of female Wistar rats, CuZnSOD, catalase, and glutathione peroxidase activities were higher or tended to be higher under food restriction than under ad libitum feeding (23). These findings differ from ours.

The liver CuZnSOD, catalase, and glutathione peroxidase activities of the fasted FR rats in the present study were higher than the fed FR rats (Figure 3). These liver antioxidant enzyme activities were increased and decreased in response to the intermittent feeding in the FR rats.

Other than intermittent feeding, feeding daily 60% of the mean food intake of ad libitum fed rats was often used for food restriction of rats. In those cases, when diet was provided to the food restricted rats, they consumed all of their food within 4 to 6 hours (24). This means that there is a short term cycle of feeding (4–6 hours) and fasting (18–20 hours) within 24 hours different from the 48-hour cycle in the present study (24 hours fed and 24 hours fasted). Rao et al. (24) reported sacrificing animals after 12 hours fasting to minimize diurnal variation between the ad libitum-fed group and dietary restricted group. So it was possible for them to collect specimens of livers of dietary restricted rats resembling our fasted FR rat liver specimens. Fasted state must be more beneficial for higher activities of liver antioxidant enzymes of the FR rats. It might be at least one of the reasons of differences between others and our results of liver antioxidant enzyme activities of food-restricted rats until old age.

In the present study, the liver MnSOD, and glutathione peroxidase mRNA levels of the young fasted FR rats and the CuZnSOD and glutathione peroxidase mRNA levels of the old fasted FR rats were higher than those of corresponding fed FR rats (Figure 4). These mRNA level enhancements of glutathione peroxidase in young fasted FR rats and glutathione peroxidase in old fasted FR rats corresponded to the antioxidant enzyme activities enhancements of the fasted FR rats (Figures 3 and 4). However, no correlation was detected between the activity and the mRNA level of the following antioxidant enzymes in the fasted FR rats and those of the fed FR rats: in the young fasted FR rats, the CuZnSOD activity increased, while the mRNA remained unchanged (Figures 3A and 4A); in the old fasted FR rats, the CuZnSOD mRNA level increased, while the activity remained unchanged (Figures 3A and 4A); in the young fasted FR rats, the MnSOD mRNA level increased, while the activity unchanged (Figures 3B and 4B); in the young and old fasted FR rats, the catalase activity increased, while the mRNA level remained unchanged (Figures 3C and 4C). This fact that no correlation was detected between the liver activity and the liver mRNA level of the above antioxidant enzymes might be explained in terms of a time lag between the mRNA level and activity increases.

Interestingly, the liver TBA value of the FR rats was also increased in response to fasting (Figure 3D). Lipid peroxidation appears to have been enhanced in the livers of the FR rats during fasting. In the old AL rats, most of the liver antioxidant enzyme activities decreased and the TBA value increased after fasting, while in the old FR rats, the activities increased and the value remained unchanged after feeding (Figure 2). Thus, the decreases in the activities of antioxidant enzymes appear to have caused an increase in the TBA value. In the fasted FR rats, however, the antioxidant enzyme activities, except the MnSOD activity, and the TBA value increased at 33 months of age (Figure 3). Presumably, the metabolism of rats changes during fasting. If 24-hour fasting causes a rapid increase in lipid peroxidation, then antioxidant enzyme activities may be induced during fasting to reduce the level of oxidative stress.

This increase in the TBA value after fasting is consistent with the finding that the liver and skeletal muscle TBA values of rats were higher during fasting than during refeeding (25). The mechanism by which lipid peroxidation in rat livers is enhanced during fasting has not yet been elucidated. As discussed below, the ketone body increases during fasting. Ketone body or its derivatives might be involved in the formation of TBA-reacting material.

During starvation, liver glycogen is consumed, free fatty acids are released into the blood vessels from adipose tissue, and ketone body is formed by β-oxidation of the fatty acids in liver mitochondria (26). Ketone body is an important energy source for hearts, muscles, and many peripheral tissues. Such energy metabolism during fasting would be relevant to oxidative stress. Acceleration of glycogen consumption and fatty acid oxidation results in removal of excess energy from the body, which might result in mild oxidative stress and thus yield a hormesis-like effect. This might result in an increase in life span in rats.

**Acknowledgments**

We thank Dr. Yoko Uchida of Tokyo Metropolitan Institute of Gerontology (TMIG) for technical advice on Northern analysis, and Dr. Takao Kaneko and Mr. Shoichi Tabara of TMIG for maintaining the animals. We also thank Prof. Masayasu Inoue of Osaka City University for superoxide dismutase probes, Prof. Sataro Goto of Tobo University for a catalase probe, and Prof. Arlan Richardson of the University of Texas for a glutathione peroxidase probe. This work was supported in part by a research fund for the TMIG project Aging and Gene Expression.

Address correspondence to Dr. Fujiiya Gomi, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashi-ku, Tokyo, 173-0015, Japan. E-mail: gomi@tmig.or.jp

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Received January 3, 1997
Accepted October 21, 1997

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