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Effects of 60% oxygen inhalation on the survival and antioxidant enzyme activities of young and old rats

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Abstract

Under 60% oxygen, both the 50% and maximum survival times of old rats were markedly shortened, and the maximum survival time of young rats did not change although the 50% survival time was shortened. In addition, the mean body weight of the old rats decreased rapidly, while that of the young rats increased very slowly after the small decrease. In lungs of the young and old rats, the activities of catalase and Mn superoxide dismutase (SOD) were increased, while those of CuZn SOD and glutathione peroxidase remained unchanged. In the liver of the young rats, the activities of Mn SOD and glutathione peroxidase were increased. In the lungs of the old rats, the messenger RNA (mRNA) levels of these antioxidant enzymes were markedly increased. The oxygen-dependent mRNA induction did not correspond to the augmentations of the activities of antioxidant enzymes. The protein levels and activity of CuZn SOD did not changed by 60% oxygen inhalation although the mRNA level was increased to 4.7-fold at 2 weeks of oxygen exposure. Translational efficiency of antioxidant enzymes in old rats might be reduced under oxidative stress. These results indicate that old rats are less tolerant to the oxidative stress of 60% oxygen than young rats because antioxidant enzyme activities are less induced due to low translational efficiency, and suggest that the activities of antioxidant enzymes, not only in the lung but also in the liver, may contribute to the tolerance to oxidative stress.

Keywords: Aging; 60% oxygen inhalation; Survival; Catalase; Glutathione peroxidase; Superoxide dismutase; mRNA; Oxidative stress

1. Introduction

Reactive oxygen species are generated within aerobic cells under conditions of both normal and abnormal metabolism and bring about oxidative stress. To avert damage due to oxidative stress, the cells have antioxidant defense mechanisms composed of both antioxidant enzymes and biological antioxidants (Fridovich, 1978; Sies, 1985). Antioxidant enzymes include SOD, catalase, glutathione peroxidase etc. and biological antioxidants include reduced glutathione, ascorbic acid,

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vitamin E, β-carotene etc. It has been suggested that deteriorative changes in antioxidant defense cause the accumulation of oxidative damage, which is relevant to the aging process (Matsuo, 1993).

The overall antioxidant capacity of rat tissues appears to be maintained without large variation during aging (Matsuo et al., 1992; Matsuo, 1993; Gomi et al., 1995). It is unclear, however, whether the redundancy of antioxidant capacity for emergency use varies with advancing age. Under an atmosphere of more than 96% oxygen, the survival times of old and young rats are similar (65–67 h) (Gomi and Matsuo, 1995) and the activities of catalase and glutathione peroxidase in the lungs of both old and young rats are markedly decreased (Gomi and Matsuo, 1995). These findings indicate that such extremely high concentrations of oxygen induce acute, lethal damage in both old and young rats. It is possible, however, that there may be age-dependent differences in the redundancy of antioxidant capacity in animals under an atmosphere contains a relatively high concentration of oxygen, e.g. 60% oxygen. In oxygen inhalation therapy, a relatively high concentration of oxygen is administered for a long period of time to not only young but also old patients (Dubois et al., 1994). It is of great importance, therefore, to clarify whether or not the redundancy of the antioxidant capacity for emergency use changes with advancing age.

In this study, we examined the survival times and antioxidant enzyme activities of old and young rats under 60% oxygen.

2. Materials and methods

2.1. Animals and oxygen exposure

Female Fischer 344 rats were obtained at 4 weeks of age from Charles River Japan Inc. (Atsugi, Japan) and maintained in the aged animal supply facility of our institute. At 6 (young rats) or 24 months (old rats) of age, rats were placed at a density of less than 3 per cage in polycarbonate cages ($44 \times 29 \times 18$ cm), into which 60% oxygen was supplied at a rate of 1.5 l/min using an oxygen–air mixer (Okazaki Sangyo Ltd., Souka, Japan) from either an oxygen cylinder or an oxygen concentrator (Hai-Sanso TO-90-5L, Teijin Ltd., Osaka, Japan) with an air compressor. For control rats, air was supplied at the same rate. The concentration of oxygen in the cages was monitored with an oxygen analyzer (Model 101-Y, Iijima Products Manufacturing Co. Ltd., Gamagouri, Japan). Rats were fed with a standard animal chow (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum. Cages were changed was two or three times a week, and rats were kept under room air during cage change for about 1 min.

2.2. Biochemical analysis

For enzyme assay, rats were sacrificed after either a 1 or 2 weeks of inhalation of 60% oxygen or after 1.5 week inhalation of room air as controls. Tissues were stored in liquid nitrogen until use. For preparation of 10% homogenates (wet weight/volume), frozen tissues were thawed on ice and then homogenized with a Polytron homogenizer (Kinematica GmbH, Luzern, Switzerland).

The activities of SOD, catalase and glutathione peroxidase were assayed according to the methods of Oyanagui (1984), Aebi (1984), Flohé and Günzler (1984), respectively. Thiobarbituric acid (TBA) values were measured according to the method of Uchiyama and Mihara (1978).

Supernatant ($17\,700 \times g$ for 20 min) of lung homogenates (25%) were dissolved in Laemli's buffer and resolved on 5–15% linear gradient polyacrylamide gels. Proteins in the gel were electroblotted onto Immobilon (Millipore). CuZn SOD was detected using anti-human SOD-1 antibody (sc-8637, Santa Cruz Biotechnology, Inc.). The bands for CuZn SOD were visualized using ECL system (Pharmacia Biotech, Uppsala, Sweden).

Total RNA was extracted from rat lungs with a 'QuickPrep total RNA kit' (Pharmacia Biotech). Aliquots of μ0 mg of total RNA were denatured and fractionated on 1% agarose–formaldehyde gels. The RNA was transferred onto nylon membranes, ultraviolet-cross-linked, and hybridized with ^{32}P-labeled probes. The probe for catalase,

glutathione peroxidase and SODs were gifted from Professor S. Goto of Toho University, A. Richardson of the University of Texas, and M. Inoue of Osaka City University, respectively. The washed nylon membrane was exposed to a Kodak X-ray film with an intensifier at −80 °C and the developed film was analyzed using a scanner (The 420oe, PDI Inc., Huntington Station, NY, USA) with the optical analysis software 'QUANTITY ONE' (PDI Inc.). The results are expressed as relative mRNA levels, i.e. the ratio of each antioxidant enzyme mRNA level to that of β-actin mRNA in the lung of control young rats was normalized as 100.

2.3. Statistical analyses

Data were assessed for significance by two-way analysis of variance (ANOVA) and Fisher's PLSD test or Student's *t*-test. Cumulative statistical analysis of rat survival curves was performed by the Kaplan–Meier method; *P*-values of less than 0.05 were considered statistically significant.

3. Results

The effects of long-term oxygen inhalation on the survival times of young and old rats were examined. Fig. 1a shows the survival curve of rats under 60% oxygen. After a shift from an atmosphere of air into that of 60% oxygen at 24 months of age, old rats began to die immediately and all had died within 196 days. Their 50% survival time was 80 days. On the other hand, the 50% and maximum survival times of control old rats were 312 and 414 days, respectively. The maximum survival time of control old rats was longer by 218 days than that of old rats under 60% oxygen. Statistical analysis by the Kaplan–Meier method indicated that the survival curves of old rats under 21 and 60% oxygen were significantly different from each other ($P < 0.0005$, log rank test). Under 60% oxygen after 6 months of age, the 50% and maximum survival times of young rats were 517 and 926 days, respectively. On the other hand, the 50% and maximum survival times of control young rats were 776 and 1000 days, respectively. Although the maximum survival times of control young rats and young rats under 60% oxygen were similar, the 50% survival time of the latter was much shorter than that of the former.

Fig. 1b shows age-dependent variations in the mean body weight of young and old rats under air or 60% oxygen. The body weight of old rats under 60% oxygen began to decrease immediately after a shift into an atmosphere of 60% oxygen and

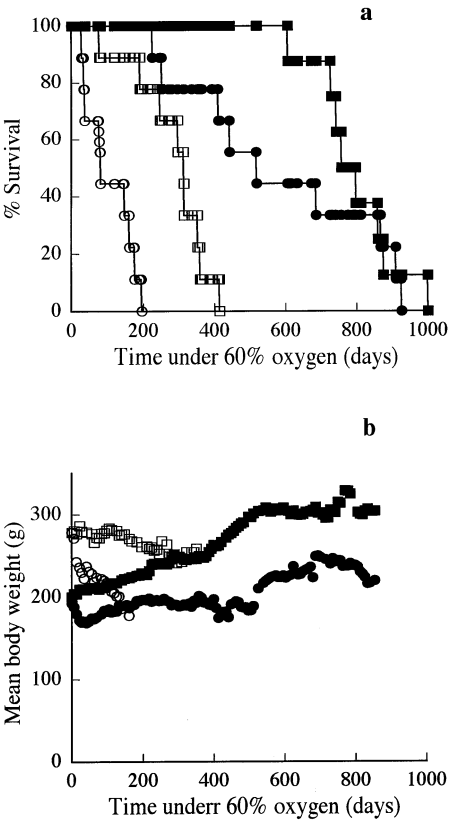

Fig. 1. Survival and body weight of rats under air or 60% oxygen. The survival curves (a) and mean body weight variation (b) of young and old rats under air or 60% oxygen are shown. Young and old rats were exposed to 60% oxygen from 6 months of age (closed circles) and 24 months of age (open circles), respectively, and control young and old rats to air at the same rate as that of the oxygen flow from 6 months of age (closed squares) and 24 months of age (open squares), respectively. Cumulative statistical analysis by the Kaplan–Meier method showed that the survival curves of old rats under 21 and 60% oxygen were significantly different from each other ($P < 0.0005$, log rank test).

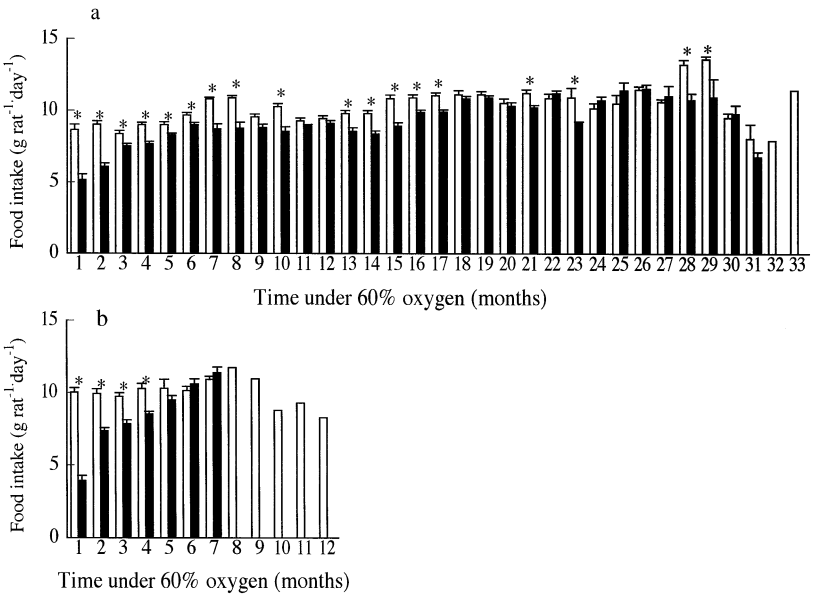

Fig. 2. Food intake of rats under air or 60% oxygen. Variations in the monthly food intakes of young (a) and old rats (b) under 60% oxygen (closed columns) or air (open columns) are shown. Young and old rats were kept under oxygen or air from 6 months of age and 24 months of age, respectively, as described in the legend of Fig. 1. Asterisks indicate that statistically significant differences between the daily food intakes of rats under 60% oxygen and the corresponding controls as determined by Student's t-test ($P < 0.05$).

continued to decrease until death. The body weight of control old rats decreased slowly with advancing age. The body weight of young rats under 60% oxygen decreased for 30 days after the shift and then increased slowly as compared with control young rats.

Fig. 2 shows the daily food intakes of young and old rats calculated every month under air or 60% oxygen. The food intake of rats under 60% oxygen tended to be lower than that of control rats. The food intake of old rats under 60% oxygen decreased by 60% for 1 month after the shift and was lower than that of control old rats for the initial 3 months after the shift. The food intake of young rats under 60% oxygen decreased by 40% for 1 month after the shift and tended to be lower for 16 months than that of control young rats.

The effects of 60% oxygen inhalation on the antioxidant capacity of the lungs and liver were examined. Fig. 3 shows the antioxidant enzyme activities and TBA values of lungs in young and old rats under 60% oxygen for 1 or 2 weeks. The catalase activities of both young and old rats increased after the 1 or 2 weeks of inhalation (Fig. 3a), and the Mn SOD activities increased only after 2 weeks of inhalation (Fig. 3d). Neither the glutathione peroxidase nor the CuZn SOD activity changed after inhalation (Fig. 3b and c). The TBA values, which are the indices of in vivo lipid peroxidation, also did not change, although the value after the 2 weeks of inhalation was lower in young than in old rats.

Fig. 4 shows the antioxidant enzyme mRNA levels in lungs of young and old rats under 60% oxygen for 1 or 2 weeks. Interestingly, all the antioxidant enzyme mRNA levels of old rats were increased after 2 weeks of inhalation; the Mn SOD mRNA level was increased even after 1 week of inhalation. The glutathione peroxidase mRNA level of young rats was higher under air than that of old rats but was decreased significantly after 1 or 2 weeks of inhalation. The CuZn SOD mRNA level of young rats was higher under air or after 1 week of inhalation than that of old rats, and that of old rats was increased after the 2 weeks of inhalation.

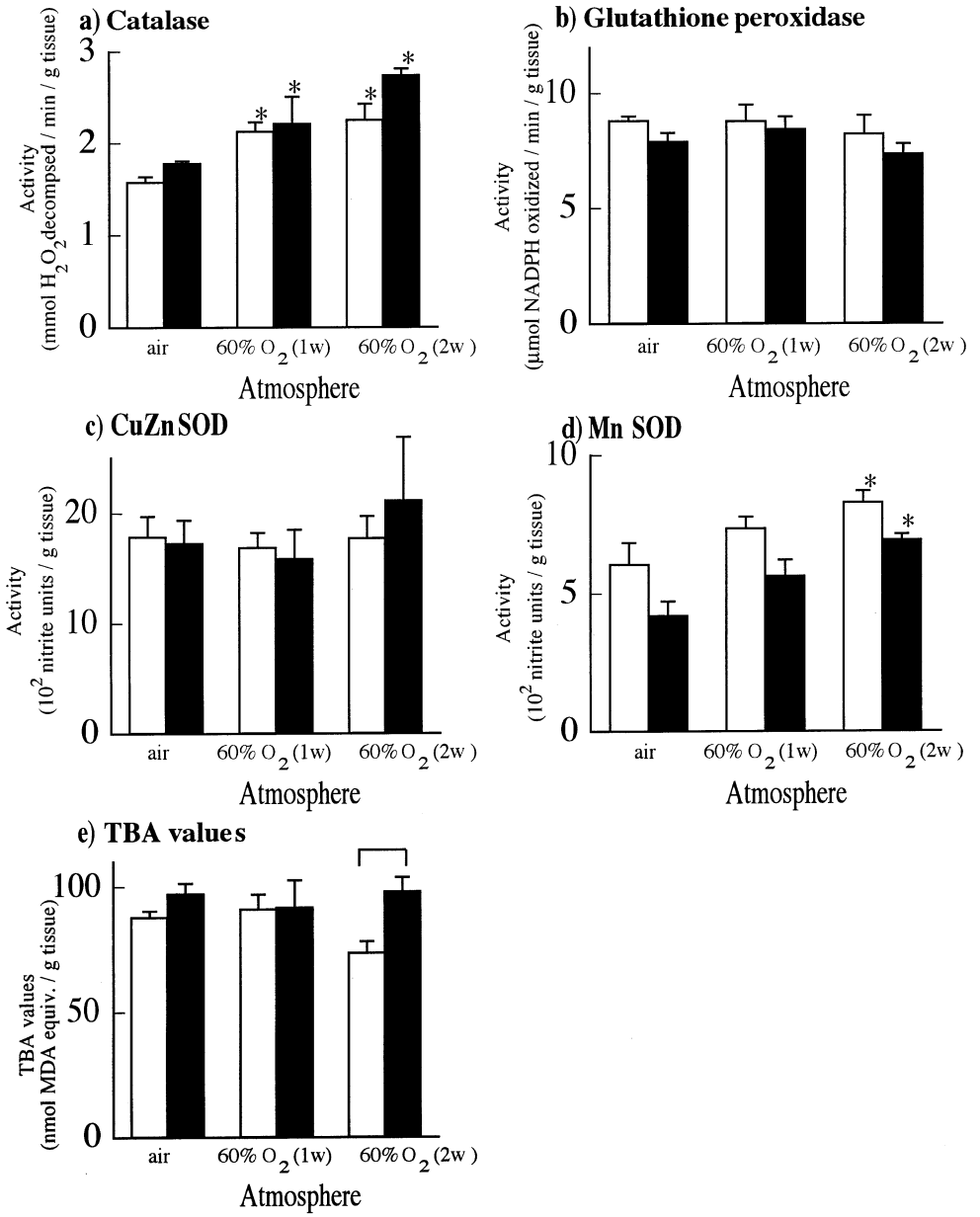

Fig. 3. Antioxidant enzyme activities and thiobarbituric acid values in the lungs of young and old rats under air or 60% oxygen for 1 or 2 weeks. The activities of catalase (a), glutathione peroxidase (b), CuZn SOD (c), Mn SOD (d), and the TBA values (e) of young (open columns) and old rats (closed columns) under air or 60% oxygen for 1 or 2 weeks are shown. Young and old rats were kept under oxygen or air from 6 months of age and 24 months of age, respectively, as described in the legend of Fig. 1. Asterisks indicate statistically significant differences between the enzyme activities of rats under 60% oxygen and the corresponding controls. Pairs of columns linked with a line indicate statistically significant differences between the two TBA values. Statistical significance was determined by ANOVA and Fisher's PLSD ($P < 0.05$).

Obviously, the variations in the antioxidant enzyme activities of lungs in rats under 60% oxygen did not reflect those in the antioxidant enzyme mRNA levels. The mRNA level of catalase in young rats remained unchanged after oxygen inhalation, although the activity increased. The level of glutathione peroxidase mRNA was reduced in young rats after inhalation and was increased by 90% in old rats after 2 weeks of inhalation, although there were no age- or oxygen-dependent differences in the activity. The level of CuZn SOD mRNA was higher in young than in old rats and was increased about 4.7-fold in old rats after 2 weeks of inhalation, although there were no age- or oxygen-dependent differences in the activity. The level of Mn SOD mRNA was increased more than 4.5-fold in old rats after inhalation, while the activity rose by 65% in old rats only after 2 weeks of inhalation.

To determine the reason of this inconsistency between mRNA levels and enzyme activities, we determined the protein levels under oxidative

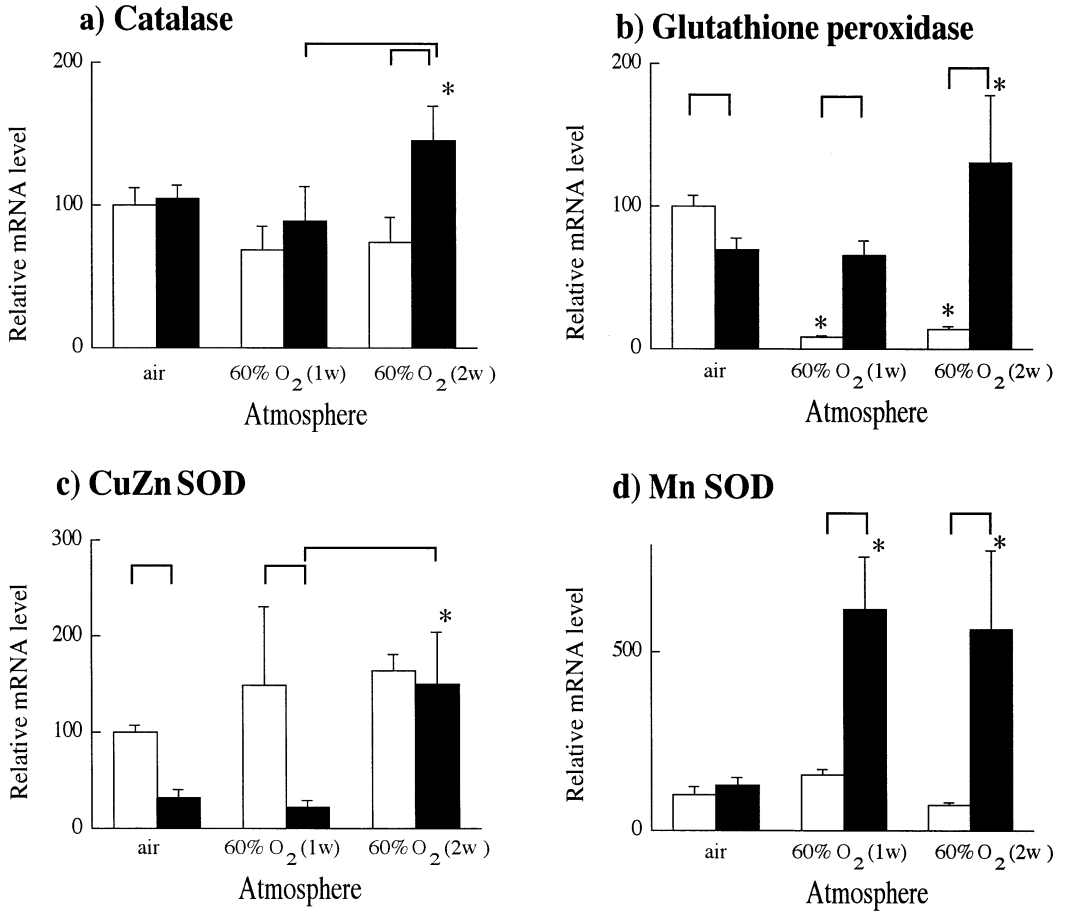

Fig. 4. Antioxidant enzyme mRNA levels in the lungs of young and old rats under air or 60% oxygen for 1 or 2 weeks. The relative mRNA levels of catalase (a), glutathione peroxidase (b), CuZn SOD activity (c), and Mn SOD (d) of young (open columns) and old rats (closed columns) under air or 60% oxygen for 1 or 2 weeks are shown. Young and old rats were kept under oxygen or air from 6 months of age and 24 months of age, respectively, as described in the legend of Fig. 1. Asterisks indicate statistically significant differences between the antioxidant enzyme mRNA levels of rats under 60% oxygen and the corresponding controls. Pairs of columns linked with a line indicate that the difference between the two antioxidant enzyme mRNA levels was statistically significant. Statistical significance was determined by ANOVA and Fisher's PLSD ($P < 0.05$).

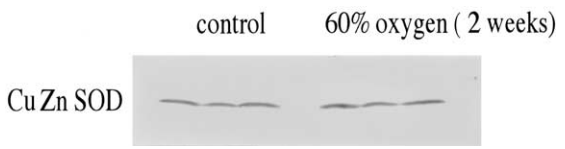

Fig. 5. Western blot analysis of CuZn SOD of 24 months old rat lung exposed to 60% oxygen and control.

stress. Most inconsistent old rat lung SOD protein levels were determined by Western blot analysis. Oxygen exposure for 2 weeks increased mRNA levels 4.7-fold but activities did not changed in old rat lung (Figs. 3 and 4). The protein levels were not changed by oxygen exposure (Fig. 5). This result indicates that the reason of inconsistency between the mRNA level and the enzyme activity must exist in translational level.

Fig. 6 shows the antioxidant enzyme activities in the livers of young and old rats under 60% oxygen for 1 or 2 weeks. The catalase activities of young and old rats did not change after oxygen inhalation, although that activity of young rats was higher than that of old rats (Fig. 6a). The glutathione peroxidase activity of young rats was increased considerably after inhalation, but that of old rats was decreased (Fig. 6b). The CuZn SOD activity of young rats was increased after 1 week of inhalation and that of old rats was decreased after 2 weeks of inhalation (Fig. 6c). The Mn SOD activity of young rats was increased after inhalation and was higher than that of old rats (Fig. 6d). The TBA value increased in old rats only after 2 weeks of inhalation (Fig. 6e).

4. Discussion

The inhalation of pure oxygen is well known to be lethal to adult rats and mice. In the present study, we showed that the inhalation of normobaric 60% oxygen, a concentration that is not particularly high, is also hazardous for adult rats (Fig. 1). We also found that 60% oxygen is much more toxic toward old rats than young rats: the 50% survival times of old and young rats were reduced to one quarter and two thirds, respectively, under 60% oxygen as compared with those under air (Fig. 1). Interestingly, a small population of young rats showed tolerance to the oxidative stress of 60% oxygen, the less than 20% survival time of young rats was similar under either air or 60% oxygen (Fig. 1). It has been reported that neonatal rats are tolerant to nearly 100% oxygen (Clark and Lambertsen, 1971). This population of young rats might retain an antioxidant capacity similar to that of neonatal rats.

Another piece of evidence for the low tolerance of old rats to the oxidative stress is that their mean body weight continued to rapidly decrease under 60% oxygen (Fig. 2). The mean body weight of young rats increased slowly under 60% oxygen after the decrease for 30 days after a shift from an atmosphere of air into that of 60% oxygen. However, the mean body weight of young rats under 60% oxygen was lower at all time points than that of age-matched controls. It has been reported that the body weight of young male Wistar rats increases slowly under 60% oxygen after a decrease for 3 days (van Klaveren et al., 1997), and that the body weight of male Sprague–Dawley rats decreased by about 13% under more than 95% oxygen for 48 h (Khatsenko et al., 1997).

The daily food intake of young and old rats calculated every month decreased by 40–60% at the beginning of the oxygen inhalation and then gradually increased to become similar to that of the age-matched controls after 5–9 months (Fig. 1b). As described above, however, the mean body weight of old rats under 60% oxygen decreased rapidly, and the mean body weight of young rats under 60% oxygen did not reach that of the age-matched controls although it increased (Fig. 2). Thus, the variation in the food intake was not directly related to that in the body weight. It is well known that food restriction, which corresponds to 50–60% of food intake under ad libitum feeding, results in life-span extension (Weindruch and Walford, 1988; Nolen, 1972). It appears, however, that the decrease in the food intake of rats under 60% oxygen is not beneficial for life-span extension.

The main adaptive response of rats to oxidative stress is considered to be the induction of antioxidant enzymes. This induction may be critical for

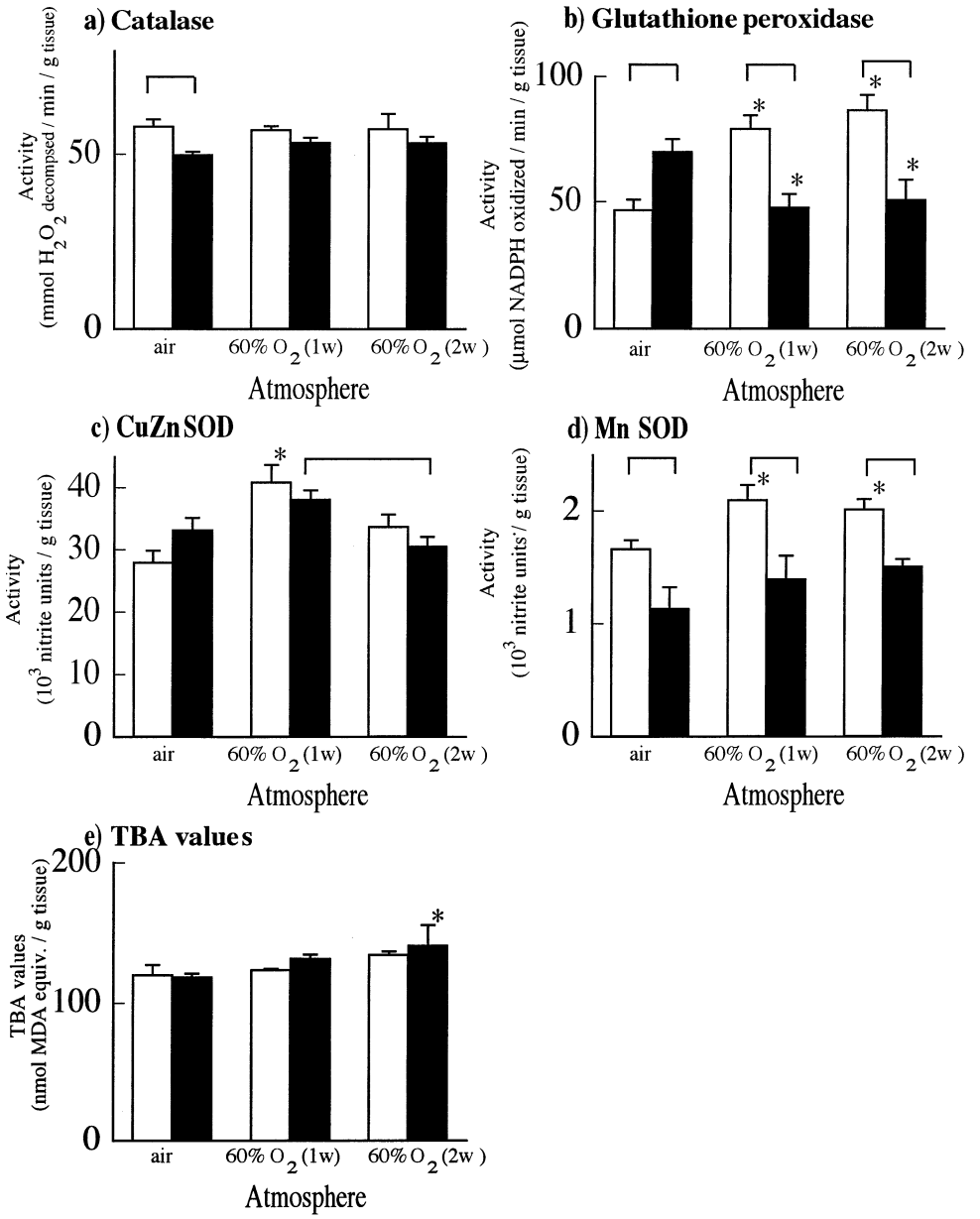

Fig. 6. Antioxidant enzyme activities and thiobarbituric acid values in the livers of young and old rats under air or 60% oxygen for 1 or 2 weeks. The activities of catalase (a), glutathione peroxidase (b), CuZn SOD (c), Mn SOD (d), and the TBA values (e) of young (open columns) and old rats (closed columns) under air or 60% oxygen for 1 or 2 weeks are shown. Young and old rats were kept under the oxygen or air from 6 months of age and 24 months of age, respectively, as described in the legend of Fig. 1. Asterisks indicate that the difference between the enzyme activities or TBA values of rats under 60% oxygen and the corresponding controls were statistically significant. Pairs of columns linked with a line indicate that the difference between the two enzyme activities was statistically significant. Statistical significance was determined by ANOVA and Fisher's PLSD ($P < 0.05$).

the survival of rats under oxidative stress. It has been reported that neonatal rats were tolerant to nearly 100% oxygen (Clark and Lambertsen, 1971), and that adult rats pretreated with 85% oxygen gained tolerance to exposure to nearly 100% oxygen (Yam et al., 1978). This tolerance seems to result from the augmentation of antioxidant enzyme activity in the lungs (Clark and Lambertsen, 1971; Yam et al., 1978; Crapo and Tierney, 1974). Further, when rats had been injected intravenously with liposomes containing catalase and SOD, their survival time under nearly 100% oxygen was extended (Turrens et al., 1984). Transgenic mice overexpressing human CuZn SOD showed increased tolerance to hyperoxia (White et al., 1991).

Under 60% oxygen, the activities of catalase and Mn SOD in lungs of young and old rats were enhanced (Fig. 3a and d). However, this augmentation of antioxidant enzyme activity appeared not to contribute to the complete tolerance of old rats to 60% oxygen, for 50% survival was reduced under 60% oxygen (Fig. 1). For young rats, the activities of glutathione peroxidase and Mn SOD in the liver, as well as those of catalase and Mn SOD in the lungs, were induced under 60% oxygen (Fig. 6). To gain tolerance to the oxidative stress of 60% oxygen, rats may need the augmentation of antioxidant enzyme activities not only in the lungs but also in the livers and probably other tissues.

After 2 weeks of inhalation of 60% oxygen, the mRNA levels of catalase, glutathione peroxidase, CuZn SOD and Mn SOD in the lungs of old rats were increased 1.4-, 1.9-, 4.7-, and 4.5-fold, respectively, while those of catalase and SODs in the lungs of young rats remained unchanged and that of glutathione peroxidase was reduced (Fig. 4). In the lungs of old rats, the oxygen-dependent mRNA induction did not correspond to the augmentations of the activities of antioxidant enzymes (Fig. 3). The protein levels of CuZn SOD in old rat lung did not change by 60% oxygen inhalation (Fig. 5). These observations suggested that the translational efficiency of antioxidant enzymes in the lungs of old rats is decreased. For under 60% oxygen, the increase in CuZn SOD activity must be needed in lung, its mRNA level was increased. But the activity was not increased because of the translational level problem. No increase in CuZn SOD activity should again increased mRNA level to 4.7-fold.

It has been reported that the translational efficiency of Mn SOD was reduced in rat lungs under high concentrations of oxygen. Under more than 95% oxygen, the level of Mn SOD mRNA in rat lungs was increased 3-fold, while its activity was reduced by 50% (Clerch and Massoro, 1993). Under 85% oxygen, the level of Mn SOD mRNA in rat lungs was increased 6-fold for 3 days and 3.4-fold for 5 days, while no significant increase in the activity was detected for 5 days (Ho et al., 1996). Rat lung peroxidredoxin I and II show the changes in translational efficiency during early development (Kim et al., 2001). The mechanism of translational change is not clear.

The inhalation of 60% oxygen affected the antioxidant enzyme activities in the liver, which was not directly exposed to the high concentration of oxygen (Fig. 6). In the livers of young rats, the activities of glutathione peroxidase, CuZn SOD and Mn SOD increased after 1 and 2 weeks of oxygen inhalation. In the livers of old rats, the activity of glutathione peroxidase decreased after oxygen inhalation and that of CuZn SOD decreased after 2 weeks of inhalation. These results showed that the inhalation of 60% oxygen affects antioxidant defense in various tissues, as well as the lungs.

During 2 weeks after 60% oxygen exposure, antioxidant enzyme activities in lung and liver were increased more in young rats than in old rats. Increased activities should work in reducing hazardous ROS, hydrogen peroxide or superoxide, which are produced under 60% oxygen. After 2 weeks of inhalation, the TBA value was higher in the lungs of old than young rats (Fig. 3e), and the value was increased in the liver of old rats (Fig. 6e). These findings suggested that under high concentrations of oxygen, oxidative stress was greater in the tissues of old than young rats. So, under 60% oxygen, young rats showed more tolerance because of increased antioxidant enzyme activities and reduced oxidative stress as shown in their survival (Fig. 1).

In summary, old rats are less tolerant to the oxidative stress of 60% oxygen than young rats because the induction of antioxidant enzyme activities were less in old rats maybe due to low translational efficiency in old rats. The activity of antioxidant enzymes not only in the lung but also in the liver may contribute to the tolerance to oxidative stress.

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