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SEQUENTIAL CHANGES IN ACTIVITIES OF SUPEROXIDE DISMUTASE AND CATALASE IN BRAIN REGIONS AND LIVER DURING (−)DEPRENYL INFUSION IN MALE RATS

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Abstract—A continuous s.c. infusion of (−)deprenyl in young male rats at a dose of 2.0 mg/kg/day for 1 week significantly increased total superoxide dismutase (SOD) activities due to increases in both Cu Zn-SOD and Mn-SOD activities in certain brain regions such as the substantia nitra and striatum, but not in the hippocampus or cerebellum, or in the liver. With continuing infusion, enzyme activities of SOD were further increased in the following weeks, reaching a plateau at 3 weeks. In some cerebral cortices the increase became significant at 3 weeks. In contrast to SOD activities, an increase in catalase (CAT) activity became significant only after 2 weeks of infusion, and only in the brain regions where SOD activities were increased earlier. The delay in the increase in CAT activity following deprenyl infusion suggests that this increased CAT activity is an adaptive response to the earlier increase in deprenyl-induced SOD activities rather than a direct effect of deprenyl on CAT activity, although the latter possibility cannot be excluded.

We reported recently that a daily s.c. injection of (−)deprenyl in young rats for 3 weeks (2.0 mg/kg/day) significantly increased activities of superoxide dismutase (SOD‡) and catalase (CAT) in the striatum [1]. Subsequent studies in our laboratory have shown that s.c. infusion of (−)deprenyl in young male rats at the same dose increased activities of both types of SOD (Cu Zn-SOD and Mn-SOD) as well as CAT activities not only in the striatum but also in the substantia nigra (s. nigra) and cerebral cortex [2]. Furthermore, we found that this effect can be seen in animals of different ages and sexes, but that the optimal dose for this effect varies more than 10-fold between young male and female rats, with the optimal dose for the latter being only 0.2 mg/kg/day [3]. In contrast with our finding, the initial work of Knoll [4] did not demonstrate a significant increase in CAT activities in the striatum after 3 weeks of treatment with the drug, although activities of both CAT and glutathione peroxidase tended to be higher in deprenyl-treated rats compared to control rats.

One of many questions stemming from these observations is how quickly these enzyme activities can be increased and when they will reach maximal values, since thus far studies in both laboratories have adhered to the 3 week duration of treatment. In order to clarify these problems, we examined activities of SOD and CAT every week in 4 successive weeks in rats under continuous s.c. infusion of deprenyl.

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‡ Abbreviations: SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; s. nigra, substantia nigra.

MATERIALS AND METHODS

Chemicals

Hydrogen peroxide, xanthine, hydroxylammonium chloride, sulfanilic acid, α-naphthylamine, potassium cyanide, reduced glutathione (GSH) and sodium azide were obtained from Wako Pure Chemicals (Tokyo, Japan). Xanthine oxidase, GSH reductase and SOD were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). NADPH was purchased from the Oriental Yeast Co. (Tokyo, Japan).

Animals

Specific pathogen-free male Fischer-344 rats were purchased at the age of 4 weeks. They were maintained in the clean conventional facility of the institute under standardized conditions. Animals were treated with a continuous s.c. infusion of physiological saline solution (control rats) or (−)deprenyl dissolved in saline by means of osmotic minipumps (Alzet, Alza, Palo Alto, CA, U.S.A.) implanted s.c. in the back for different time intervals so that all animals could be examined at the same age (10–11 weeks). Control animals were given a s.c. infusion of isovolumetric physiological saline solution for 1–4 weeks and four experimental groups were treated with deprenyl infusion s.c. for 1, 2, 3 and 4 weeks, respectively. One control animal treated with a saline solution for the same time period as an experimental group treated with deprenyl infusion was killed at the same time as the experimental animal group. The data for four control animals treated for different time periods (1–4 weeks) with saline infusion were combined into one group and used as control values since the variation of values among rats treated with saline for different periods was very small (see Results) and since we

confirmed previously that a saline infusion does not affect the enzyme activities under study. The dose of deprenyl was always 2 mg/kg/day, which was found to be close to optimal in the young male rats of this strain for increasing SOD and CAT activities in the striatum when given for 3 weeks [1–3].

Animals were killed by decapitation between 1000 and 1200 hr. The brains were immediately removed and several different brain regions [i.e. the hippocampus, striatum, s. nigra and three different (frontal, parietotemporal and occipital) cortical regions of the cerebrum and cerebellum] were obtained by dissection on an ice-cold plate. The s. nigra was homogenized in 0.3 mL of distilled cold water, the striatum in 1 mL and the other brain parts (hippocampus, cortices and cerebellum) in 1.5 or 2 mL, depending on their weights. The resulting homogenates were sonicated for 15 sec in a Sonifier beta-B Branson sonic. The homogenates were next centrifuged for 2 min in an Eppendorf centrifuge. An aliquot of the supernatant was immediately used to determine CAT activity and the rest of the supernatant was stored at −20° until the determination of SOD which was performed within 24 hr of the animals being killed.

The livers were quickly removed and perfused with cold saline. A small portion of the largest lobe (about 1 g) was homogenized with 9 vol. of 0.25 M sucrose. One half of the homogenate was frozen and stored overnight, and then thawed, centrifuged at 25,000 *g* and supernatant dialysed against 500 vol. of 50 mM potassium phosphate (pH 7.8) and 1 mM EDTA, in order to determine SOD activities.

The other half of the homogenate was centrifuged at 1000 *g* for 10 min and the supernatant fraction was centrifuged at 10,000 *g* for 20 min. The resulting supernatant was used for determination of the activities of CAT, on the day that the animals were killed.

Enzyme assays

SOD. The activity of SOD was assayed by the method of Elstner and Heupel [5] which is based on the inhibition of nitrite formation from hydroxylammonium in the presence of O_2^- generators. Nitrite formation from hydroxylammonium chloride was determined under the following conditions. The incubation mixture (2 mL total volume) consisted of phosphate buffer, pH 7.8 (65 mM, 1 mL), xanthine oxidase (40 μg protein, 0.3 mL), xanthine (1.5 μmol, 0.1 mL) and hydroxylammonium chloride (1 μmol, 0.1 mL). The reaction was started by the addition of xanthine oxidase and was conducted at 25° in a water bath for 20 min. The determination of nitrite as a product of hydroxylammonium chloride was assessed in a 0.5-mL sample of the above reaction mixture with sulfanilic acid (0.01 mM) and α-naphthylamine (0.001 mM) (total volume, 1.5 mL). The optical density of the mixture was determined at 530 nm. Addition of SOD (or 0.015 mL of supernatant) to the incubation mixture yielded an inhibition of nitrite formation. A curve of activity units vs percentage of inhibition was recorded with known amounts of purified SOD from Sigma, which contained 3600 U/mg protein as assayed by the method of McCord and Fridovich [6]. Approximately one-fifth of the cavity unit yielded a 50% inhibition of hydroxylammonium chloride oxidation. The amount of SOD activity units of the samples was calculated using this curve.

Total SOD activity was also measured in some samples in terms of its ability to prevent the reduction of cytochrome *c* by an enzymatic source of O_2^- [6]. A highly significant linear relationship between values obtained by the two methods used ($r = 0.997$) was demonstrated. Therefore, the Elstner and Heupel method [5] was used continuously throughout the experiments since a far greater number of samples can be assayed simultaneously by this method than by the standard method using cytochrome *c* [6].

Differentiation of the two different types of SOD (Cu Zn-SOD and Mn-SOD) was performed by the addition of potassium cyanide (5×10^{-4} M) to the incubation medium. Cu Zu-SOD activities were defined as activities inhibited by potassium cyanide. The difference between total and KCN-inhibited enzyme activities was defined as Mn-SOD [7].

CAT. CAT activity was assayed by the method described by Beers and Sizer [8] in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. The incubation mixture contained 0.05 M potassium phosphate, pH 7.0, 0.02 M hydrogen peroxide and a sample (0.05 mL) of the supernatant fluid, in a final volume of 3 mL. The decrease in absorbance was recorded at 240 nm for 2 min. The rate of decrease in absorbance per min was calculated from the initial linear portion of the curve (45 sec). The value of 0.0394 $cm^2/\mu mol$ proposed by Nelson and Kiesow [9] was used as the extinction coefficient of H_2O_2. One unit of CAT was defined as the amount of enzyme which decomposed one μmole of H_2O_2 per minute at 25° and pH 7.0 under the specified conditions. Protein concentration was determined by the method of Lowry *et al.* [10].

Statistical analysis. All values were expressed as means ± SD. Values were analysed by means of ANOVA. When the difference was significant with respect to drug treatment duration, any two sets of values of different rat groups were compared by using Scheffe's test. P values lower than 0.05 were judged to be significant.

RESULTS

Figure 1 summarizes sequential changes in the activities of CAT, total SOD and two different forms of SOD in the s. nigra and striatum in control rats and rats treated with deprenyl for different time periods. In these tissues, total SOD activities significantly increased by 1 week after the start of deprenyl infusion in comparison to respective control values; this was due to increases in both forms of SOD. These activities further increased with 2 weeks of treatment and reached a plateau at 3 or 4 weeks. In contrast to SOD activities, CAT activities after 1 week of treatment remained at levels almost identical to respective values in the control group. However, activities at 2 weeks significantly increased and reached peak values at about 3 weeks. After 4

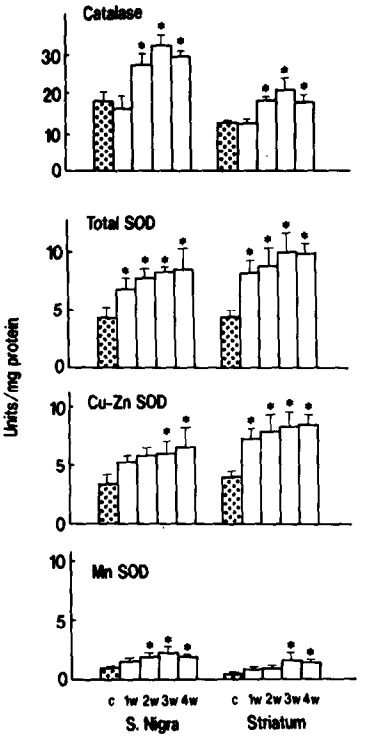

Fig. 1. Activities of CAT, total SOD and two different forms of SOD in the s. nigra and striatum of young male control rats (c) and rats treated with deprenyl for different time intervals. Deprenyl was continuously infused s.c. by osmotic minipumps. *Significantly different from corresponding control values (ANOVA + Scheffe's test, $P < 0.05$). Number of animals in each group was four except for the group treated with deprenyl for 2 weeks (N = 3).

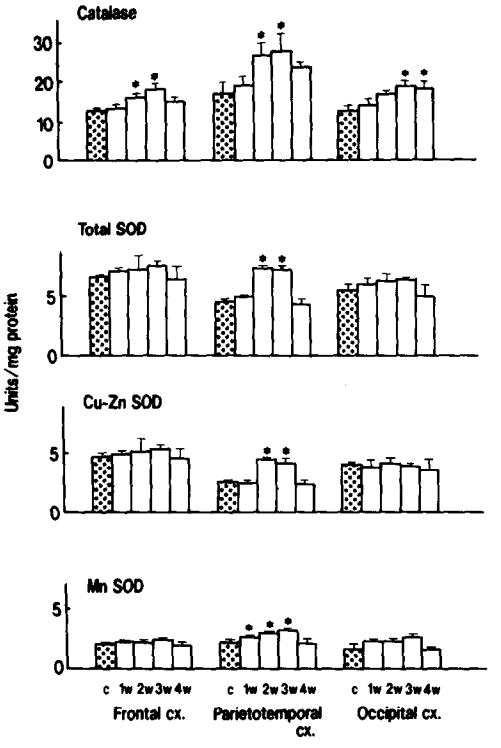

Fig. 2. Activities of CAT, total SOD and two different forms of SOD in three different cortical regions in young male control rats (c) and rats treated with deprenyl for different time intervals. Deprenyl was continuously infused s.c. by osmotic minipumps. *Significantly different from corresponding control values (ANOVA + Scheffe's test, $P < 0.05$). Cx, cortex. Number of animals in each group is the same as in Fig. 1.

weeks, the CAT activities tended to be slightly lower compared to the 3-week values in both tissues.

Figure 2 summarizes activity changes in three different cortical regions. Total SOD activities tended to be slightly higher at 2 or 3 weeks compared to respective control values. This was due to increases in both types of SOD activities. Unlike the other two tissues shown in Fig. 1, the tendency of SOD to increase in cortical regions was not as striking in deprenyl-treated animals and a statistically significant increase was seen only in the parietotemporal cortex from rats treated with deprenyl for 2 or 3 weeks. CAT activity also tended to increase for all three cortical regions, but only after 2 weeks of deprenyl infusion, as was observed for the other two tissues (s. nigra and striatum) shown in Fig. 1. Interestingly, again some values in rats treated for 4 weeks were lower than the 3-week values. Similarly, none of the SOD values at 4 weeks were significantly different from respective control values.

Figure 3 summarizes activity changes in the hippocampus and cerebellum, and the liver. Unlike changes in the tissues shown in Figs 1 and 2, enzyme activities in these three tissues did not increase significantly throughout the infusion period of 4 weeks.

DISCUSSION

We have shown previously that s.c. injection [1, 3] or infusion [2, 3] of deprenyl at a dose of 2 mg/kg/day for 3 weeks significantly increases SOD and CAT activities (but not that of GSH peroxidase) in the striatum of young male F-344 rats. Individual values at 3 weeks obtained in the present study are comparable to corresponding values found in our previous studies [2, 3] or values found in our initial study using s.c. injection of deprenyl [1]. The primary question, how and when these enzyme activities are changed during the course of deprenyl infusion, appears to be fairly well answered by our present results. In both the s. nigra and striatum, SOD activities started to increase significantly by 1 week of deprenyl infusion, and continued to increase further with time up to 4 weeks.

It is also clear that both types of SOD significantly increased with time. In contrast to SOD activities, the CAT activity stayed totally unchanged at 1 week, starting to increase only after 2 weeks of deprenyl infusion and reaching a peak at 3 weeks. The direct

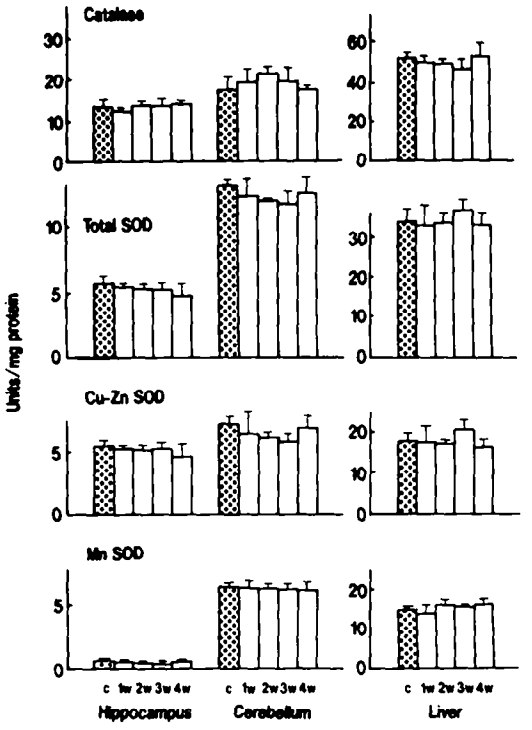

Fig. 3. Activities of CAT, total SOD and two different forms of SOD in the hippocampus and cerebellum, and the liver of control rats (c) and rats treated with deprenyl for different time intervals. Deprenyl was continuously infused s.c. by osmotic minipumps. Number of animals in each group is the same as in Fig. 1.

mechanism whereby deprenyl increases the activities of these enzymes remains unknown. However, the results of the present study, showing that it takes 3 weeks to achieve the highest activities for both enzymes and that there exists at least a 1-week interval between the increases in SOD and CAT activities, may hold a clue to the mechanism(s) underlying this phenomenon.

It is well known that the activities of these antioxidant enzymes including GSH Px are increased very rapidly by a hypoxia/anoxia stress, especially when followed by reoxygenation [11–14]. Enzyme activities are also increased in response to hyperoxia [15]. All of these changes occur within a few hours or several days at most [11–15]. Furthermore, to our knowledge, there has been no information published suggesting that deprenyl causes pharmacological effects related to oxygen crisis such as circulatory disturbances. The rather long time course of deprenyl's effect of increasing antioxidant enzymes as well as the lack of information suggesting the possible effect of deprenyl in causing an oxygen crisis suggests that it is highly unlikely that the enzyme activity increases observed in our studies with deprenyl is related to an oxygen crisis.

An approximately 1-week interval between the starts of increase in SOD and CAT activities is interesting but also difficult to explain. It is possible that deprenyl's effect on CAT simply takes a longer time than on SOD. Alternatively, it also appears possible that deprenyl works directly only on SOD and that the increase in CAT activity is an adaptation to the increase in SOD. This possibility would seem to explain the time interval of 1 week. However, as was shown in our data, CAT activities are usually much more abundant than activities of SOD. Accordingly, an overproduction of H_2O_2 by an increase in SOD activities may not easily lead to the increase in CAT activity in simple theoretical terms. However, there is little information available in terms of the kinetic and dynamic aspects of chain reactions mediated by these enzymes. In particular, practically nothing is known regarding *in vivo* situations where the possible microcompartmentation for reactions may further complicate the situation. Accordingly, the above possibility cannot be excluded at the moment.

In contrast to the changes observed in the striatum and s. nigra, changes in the cerebral cortices were not so remarkable and some activity changes during deprenyl treatment were not significantly different from respective control values. However, the tendency toward an increase is not equivocal, particularly in the parietotemporal cortex at 2–3 weeks, where significant increases were observed in the activities of both SOD and CAT. Interestingly, enzyme activities tended to be lower at 4 weeks compared with respective values at 3 weeks, and any SOD activity values were significantly no more different from respective control values at this time.

A similar tendency toward decrease at 4 weeks was also seen for CAT activity in the parietotemporal cortex as well as the s. nigra and striatum, although the differences between 3 and 4 week values were minor in the latter two tissues. Whether the activities of enzymes tend to decline after 4 weeks of treatment remains to be clarified, since some enzyme (especially SOD) activities are at their maximum values at this period in certain regions such as the s. nigra and striatum. Furthermore, the physiological significance of the tendency of activity to decrease after 4 weeks in cortical regions remains unknown. However, if the enzyme activity increase observed in these experiments is due to so-called enzyme induction, such a tendency to decrease during long term exposure to an inducer is possible. It has been reported that during long term exposure to phenobarbital, some enzyme activities of the hepatic microsomal monooxygenase system that were at first significantly induced by phenobarbital returned to control levels, while some others remained at higher levels [16].

Deprenyl has been shown to prolong the life span of rats at least by two independent groups [4, 17]. In our own study, a significant increase in the life span of deprenyl-treated rats in comparison to saline-treated rats was also confirmed [18], although the magnitude of the increase was far smaller than that reported by Knoll [4], but higher than that reported by Milgram *et al.* [17]. Such an increase in life span was suggested to be related to an increase in antioxidant enzyme activity [4], as supported by the present study. For such an experiment on rat life span, the drug must be administered for a long

period of 2–3 years. Accordingly, it would be important to examine changes in these activities in animals treated for a much longer period than has been studied up to now in order to link enzyme activity modulation by this drug to its possible effect on the life span of animals. Studies are in progress in our laboratory to clarify these important issues.

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