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Age-related changes in antioxidant enzymes and lipid peroxidation in brains of control and transgenic mice overexpressing copper-zinc superoxide dismutase

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Summary

The aim of our study was first to obtain a comprehensive profile of the brain antioxidant defense potential and peroxidative damage during aging. We investigated copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), seleno-dependent glutathione peroxidase (GSH-PX), glutathione reductase (GSSG-R) activities, endogenous and in vitro stimulated lipid peroxidation in 40 brains of control mice divided into 3 age groups: 2 months (young), 12 months (middle-aged) and 28 months (old). We found a positive correlation between age and activities of CuZnSOD ($r = 0.47$; $P < 0.01$) and GSH-PX ($r = 0.72$; $P < 0.0001$). CuZnSOD and GSH-PX activities are independently regulated during brain aging since temporal changes of these two enzymes do not correlate. No modification in MnSOD activity and basal lipid peroxidation was observed as a function of age. Nevertheless, stimulated lipid peroxidation was significantly higher at 12 months (6.53 ± 0.71 μmole MDA/g tissue) than at 2 months (5.69 ± 0.90) and significantly lower at 28 months (5.13 ± 0.33) than at 12 months.

Second, we used genetic manipulations to construct transgenic mice that specifically overexpress CuZnSOD to understand the role of CuZnSOD in neuronal aging. The human CuZnSOD transgene expression was stable during aging. The increased CuZnSOD activity in the brain (1.9-fold) of transgenic mice resulted in an enhanced rate of basal lipid peroxidation and in increased MnSOD activity in the 3 age groups. Other antioxidant enzymes did not exhibit modifications indicating the independence of the regulation between CuZnSOD and glutathione-related enzymes probably due to their different cellular localization in the brain.

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exon 2 of the human CuZnSOD transgene). These primers should bind to the human CuZnSOD transgene and lead to an amplified band of 210 bp. Specific amplification of the human CuZnSOD transgene was carried out by heating for 30 s at 94°C (denaturation), 1 min at 60°C (annealing) and 1 min at 72°C (polymerization) over 30 cycles. Then, 20 μl of each sample was subjected to electrophoresis on a 2% agarose Tris-borate gel, the gel was stained with ethidium bromide and the 210-bp DNA band was visualized by ultraviolet transillumination.

Enzyme assays

Preparation of samples

Control and transgenic mice of each age were killed by decapitation. Brains were promptly dissected and were homogenized in a Potter-Elvejhem homogenizer in 5 ml of phosphate buffer 50 mM pH 7.5. The resulting homogenates were next centrifugated at $100.000 \times g$ for 1 h, the supernatant was removed and used for measurement of enzymatic activities and for protein content determination according to the method of Lowry et al. (1951). All the above steps were carried out at +4°C.

Enzymatic determination of CuZnSOD and MnSOD

Total SOD activity was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Marklund and Marklund (1974). MnSOD activity was determined under the same conditions with addition of 3 mmole/l KCN for 15 min in the assay buffer to inhibit CuZnSOD activity. CuZnSOD activity was obtained by subtracting the MnSOD activity from the total activity. One unit of CuZnSOD and MnSOD activity is defined as the amount of the enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50%.

Enzymatic determination of GSH-PX

We measured GSH-PX activity by the modified method of Paglia and Valentine (1967) as described by Carmagnol et al. (1983), using *tert*-butyl hydroperoxide as substrate. GSSG produced by the action of GSH-PX and peroxide was reduced by GSSG-R and NADPH, the decrease in concentration of NADPH being recorded at 340 nm. The reaction mixture, maintened at 30°C, consisted of 1 mmole of *tert*-butyl hydroperoxide, 1.4 units of yeast GSSG-R (type III; Sigma Chemical Co., St. Louis, MO), 1.43 mmole of NADPH, and 1.5 mmole of KCN per liter of 100 mmole/l sodium phosphate buffer and ethylenedinitrilo tetraacetic acid disodium salt 3 mmole/l (pH 7.0). The assay kinetics were calculated by using a molar absorptivity for NADPH of 6.22×10^{-3} L/mole/cm at 340 nm. Enzyme activity was expressed in terms of nmole of NADPH oxidized per min per mg of protein.

Enzymatic determination of GSSG-R

GSSG-R activity was assayed according to the method of Calberg and Mannervik (1984). NADPH oxidation was monitored at pH 7.4 and 30°C, and enzyme activity was expressed in terms of nmole of NADPH oxidized per min per mg of protein.

CuZnSOD, MnSOD, GSH-PX and GSSG-R activities were automatically measured in triplicate on a Cobas-Bio centrifugal analyzer (Hoffman-LaRoche, Basel, Switzerland) according to a modification of the methodology of Jaskot et al. (1983).

Measurement of basal and stimulated lipid peroxidation in brain tissue

Basal lipid peroxidation was estimated in the brain homogenates using a modified thiobarbituric acid (TBA) test of Ohkawa et al. (1979) as described by Ceballos-Picot et al. (1991). Stimulated lipid peroxidation was assayed by a modification of Dexter et al.'s (1989) methodology: incubation of brain homogenates at 37°C for 60 min with 0.01 mM $FeSO_4$ plus 0.25 mM ascorbic acid (in order to estimate the sensitivity to in vitro peroxidation), previous to performing the thiobarbituric assay. After cooling with ice, 1.5 ml of *n*-butanol was added and the mixture was shaken vigorously for 15 min. After centrifugation at $4000 \times g$ for 10 min, the absorbance of the organic layer (upper layer) was determined by using fluorometric measurement (excitation: 515 nm; emission: 553 nm) with an Aminco-Bowman spectrofluorometer. MDA levels were calculated rela-

tive to standard preparation from the hydrolysis of 1,1,3,3-tetramethoxypropane. Results were expressed as μmole of MDA per g of wet tissue. Each value was the average of a triplicate analysis.

Fatty acid determination

Brain lipids were extracted according to Folch et al. (1957). Total brain lipids were separated directly by one-dimensional thin layer chromatography using migrating solvent described by Vitiello and Zanetta (1978). Lipid spots were detected under UV light and brain phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were scraped into teflon-lined screw-capped tubes for methylation. Methyl esters were preparated in the presence of silica gel plate materials by the method of Lepage and Roy (1986).

The methyl esters were then analyzed by gas liquid chromatography using Carbowax 20 M coated-silica capillary column (length 25 m, internal diameter: 0.22 mm). The temperatures of injector and detector were 230°C and 250°C respectively, oven from 185°C to 225°C at 1.5°C/min after 12 min isotherm. The gas chromatograph Girdel 3300 was equipped with Ross injection devices and flame ionization detector coupled to integrator. Individual fatty acids were identified using a reference mixture of known fatty acid composition. Quantitation of fatty acids was based on the internal standard heptadecanoic acid and is reported in terms of mole% of total fatty acids.

Statistical methods

Analyses were conducted using the statistical analysis package Statview SE. Means of biological measures are presented with standard deviation (m ± SD). Animals were categorized in three groups according to their age: 2 months (young), 12 months (middle-aged) and 28 months (old). After testing for normality and variance homogeneity, the data were subjected to *t*-tests between each age group of control mice, each age group of transgenic mice and between transgenic and control mice. When the variance was heterogeneous, the Wilcoxon test was used. The 0.05 level was selected as the point of minimal statistical significance.

To examine the strength of relationship between two quantitative variables and/or between age and each variable we calculated correlation coefficients (Pearson noted *r*). Analysis of correlations was realized between all the variables in

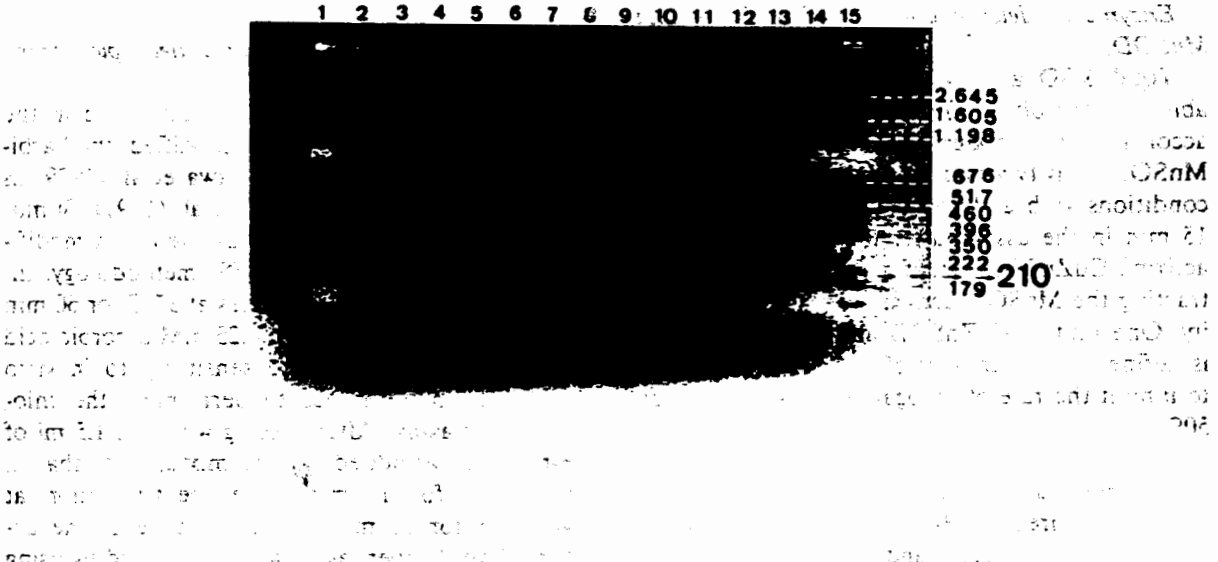

Fig. 1. Identification of transgenic mice by PCR. Lanes 1–5 and 7–9: positive transgenic offspring containing the human CuZnSOD gene. Lanes 6 and 10–11: negative offspring lacking the transgene. Lane 12: human DNA as a positive control. Lane 13: mouse DNA as a negative control. Lane 14: water. Lane 15: plasmid PGEM as a marker of size.

controls and transgenic mice. Only significant data are presented here.

Results

Seventy-five animals, 40 controls and 35 transgenic mice, were included in the present study. The mean age for each age group at time of inclusion was 2 months (13 controls and 11 transgenic mice), 12 months (12 controls and 14 transgenic mice) and 28 months (15 controls and 10 transgenic mice).

Mean values with standard deviations (m ± SD) of biological parameters are presented in Tables 1 and 2. CuZnSOD activity was the biological parameter with the smaller coefficient of variation, ratio of SD on mean (10.5%). Measure of basal lipid peroxidation according to the TBA test had the higher coefficient of variation (17.7%).

Identification of transgenic mice by PCR

The results of a typical experiment are shown in Fig. 1. Positive transgenic offspring containing the human CuZnSOD gene (lanes 1–5 and 7–9) yield a strong band of the correct size (210 bp), while DNA from animals lacking the transgene (lanes 6 and 10–11) generates no detectable signals.

Aging changes in the antioxidant enzyme activities in the central nervous system of control mice

To establish whether enzymes implicated in oxygen free radical metabolism were modified in

TABLE 1

AGE-RELATED CHANGES IN ANTIOXIDANT ENZYME ACTIVITIES IN THE BRAINS OF CONTROL AND TRANSGENIC MICE

	Age (months)			
	2	12	28	
CuZnSOD				
Control	43.2 ± 5.6 (n = 13)	50.3 ± 5.1 ^{a***} (n = 12)	50.3 ± 5.4 ^{a***} (n = 15)	A. $P < 0.01$
Transgenic	81.0 ± 8.3 ^{c****} (n = 11)	81.8 ± 9.3 ^{c****} (n = 11)	84.7 ± 7.0 ^{c****} (n = 10)	A. NS
MnSOD				
Control	35.6 ± 6.6 (n = 13)	35.8 ± 2.6 (n = 12)	32.4 ± 2.7 (n = 15)	A. NS
Transgenic	44.2 ± 7.9 ^{c***} (n = 11)	40.3 ± 6.1 ^{c**} (n = 13)	40.0 ± 2.9 ^{c****} (n = 10)	A. NS
GSH-PX				
Control	10.4 ± 1.9 (n = 13)	12.6 ± 1.8 ^{a**} (n = 12)	14.6 ± 1.4 ^{a****,b**} (n = 15)	A. $P < 0.0001$
Transgenic	11.8 ± 2.4 (n = 11)	12.4 ± 1.9 (n = 13)	14.4 ± 1.5 ^{a***} (n = 10)	A. $P < 0.01$
GSSG-R				
Control	41.0 ± 6.5 (n = 13)	40.2 ± 7.0 (n = 12)	41.7 ± 7.1 (n = 15)	A. NS
Transgenic	38.9 ± 7.3 (n = 11)	39.8 ± 4.5 (n = 14)	37.9 ± 4.5 (n = 10)	A. NS

Results are mean ± SD; n, number of mice; CuZnSOD and MnSOD: U/mg of protein; GSH-PX and GSSG-R: nmole of NADPH oxidized/min/mg of protein.

A, aging effect (correlation between age and activity): NS, not significant; ^{a,b} Significant differences among age groups (Student's *t*-test or Wilcoxon test); ^{a} significantly different from 2 months, ^{b} significantly different from 12 months; ^{c} significant difference between transgenic and control mice: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

the brain as a function of age, we measured CuZnSOD, MnSOD, GSH-PX and GSSG-R activities in the whole brain of 40 control mice classified in 3 age groups, as reported in Table 1. The mean (±SD) CuZnSOD activity was higher at 28 months (50.3 ± 5.4 U/mg protein) compared to 2 months (43.2 ± 5.6) ($P < 0.01$) and was significantly higher at 12 months (50.3 ± 5.1) compared to 2 months ($P < 0.01$).

The mean (±SD) GSH-PX activity was significantly higher at 28 months (14.6 ± 1.4 nmole NADPH oxidized/min/mg protein) compared to 2 months (10.4 ± 1.9) ($P < 0.0001$); at 12 months (12.6 ± 1.8) compared to 2 months ($P < 0.05$) and at 28 months compared to 12 months ($P < 0.01$). No modification with age in MnSOD and GSSG-R activities was observed.

We found a positive correlation between age and CuZnSOD activity ($r = 0.47$; $P < 0.01$) and age and GSH-PX activity ($r = 0.72$; $P < 0.0001$).

Another positive correlation was found between CuZnSOD and GSSG-R activities when the 40 mice of the 3 groups were considered ($r = 0.34$; $P < 0.05$). Moreover, when this correlation was studied for each age group, a strong positive correlation was only found at 28 months ($r = 0.82$; $P < 0.001$).

TABLE 2

BASAL AND STIMULATED LIPID PEROXIDATION (AS MALONDIALDEHYDE) IN BRAIN HOMOGENATES FROM CONTROL AND TRANSGENIC MICE

	Age (months)		
	2	12	28
Basal peroxidation			
Control	0.22 ± 0.03 (n = 18)	0.22 ± 0.01 (n = 9)	0.25 ± 0.06 (n = 8)
Transgenic	0.25 ± 0.03 ^{c**} (n = 18)	0.24 ± 0.01 ^{c**} (n = 9)	0.29 ± 0.08 ^{c**} (n = 8)
Stimulated peroxidation			
Control	5.69 ± 0.89 (n = 13)	6.53 ± 0.71 ^{a**} (n = 9)	5.13 ± 0.33 ^{b**} (n = 8)
Transgenic	6.22 ± 1.02 (n = 12)	6.67 ± 0.63 ^{a**} (n = 9)	5.81 ± 0.60 (n = 9)

Results are mean ± SD; n, number of mice; basal lipid peroxidation: μmole MDA/g tissue; stimulated lipid peroxidation: μmole MDA/g tissue.

^{a,b} Significant differences among age groups (Student's *t*-test or Wilcoxon test): ^{a} significantly different from 2 months, ^{b} significantly different from 12 months; ^{c} significant difference between transgenic and control mice: * $P < 0.05$; ** $P < 0.01$.

Aging changes of antioxidant enzyme activities in the central nervous system of transgenic mice

Increased CuZnSOD activity due to the expression of the transgene was detected in the brain of transgenic mice and was similar in each group analyzed (Table 1). The mean (±SD) CuZnSOD activity was 81.0 ± 8.3 U/mg protein at 2 months (1.88-fold increased activity compared to the control group of 2 months; $P < 0.0001$); 81.8 ± 9.3 U/mg protein at 12 months (1.63-fold increased activity compated to the control group of 12 months; $P < 0.0001$); 84.7 ± 7.0 in 28 months (1.68-fold increased activity compared to the control group of 28 months; $P < 0.0001$).

The mean (±SD) GSH-PX activity was significantly higher at 28 months (14.4 ± 1.5 nmole NADPH oxidized/min/mg protein) compared to 2 months (11.8 ± 2.4 U/mg; $P < 0.01$) and a positive correlation between age and GSH-PX activity was observed ($r = 0.45$; $P < 0.01$).

As in control brains, no modification in MnSOD and GSSG-R activities was observed in the brain of transgenic mice according to age. Moreover, the correlation between CuZnSOD and GSSG-R activities observed in the control brains was not found in transgenic brains.

Effect of increased CuZnSOD activity on the antioxidant defense capacity in transgenic brains

The MnSOD activity was significantly higher in transgenic mice compared to control mice ($P < 0.0001$) when the 35 mice of the 3 groups were considered as well as in a separate analysis for each age group.

No significant modifications was observed for GSH-PX and GSSG-R activities between transgenic and control brains in each age group studied.

Basal and stimulated lipid peroxidation measurement in control and in transgenic brains according to age

The basal level of lipid peroxides (as measured by the MDA-TBA test), and the level of stimulated lipid peroxides production (as measured by

the MDA-TBA test after incubating tissue with a free radicals-generating system: $FeSO_4$ plus ascorbic acid) in 3 age groups of control and transgenic brains are reported in Table 2.

Basal lipid peroxidation was not changed as a function of age in the control brains.

However, stimulated lipid peroxidation was significantly increased at 12 months (6.53 ± 0.71 μmole MDA/g tissue) compared to 2 months (5.69 ± 0.90) ($P < 0.05$) and significantly decreased at 28 months (5.13 ± 0.33) compared to 12 months (6.53 ± 0.71) ($P < 0.05$). The same tendency was observed for transgenic mice according to age (Table 2).

Basal lipid peroxidation was significantly increased in the whole brains of transgenic mice compared to control brains at 2 months ($P < 0.01$), 12 months ($P < 0.01$) and 28 months ($P < 0.01$).

Fatty acid composition of transgenic mouse brains

The fatty acid composition of brain phospholipids from control and transgenic mice is set out in Table 3. The results reported are confined to phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, which are particularly rich in PUFAs. No relevant differences could be discerned between transgenic and control brains, particularly the composition of (n-3) and (n-6) PUFAs were not found to be abnormal.

Discussion

Age-related modifications in antioxidant enzyme activities and lipid peroxidation in control mice

The antioxidant enzymes CuZn and MnSOD, and glutathione-related enzymes (e.g., GSH-PX and GSSG-R) form a substantial defensive net-

TABLE 3

FATTY ACID COMPOSITION OF BRAIN LIPIDS FROM CONTROL AND TRANSGENIC MICE

Fatty acid	Control mice			Transgenic mice		
	PC	PE	PS	PC	PE	PS
Saturated						
14:0	0.31 ± 0.09	0.26 ± 0.06	0.27 ± 0.16	0.33 ± 0.05	0.21 ± 0.06	0.51 ± 0.12
16:0	48.16 ± 4.78	7.58 ± 0.84	4.27 ± 0.65	48.16 ± 1.74	7.82 ± 1.06	5.43 ± 1.33
18:0	14.53 ± 0.56	27.16 ± 1.84	44.00 ± 3.90	14.11 ± 0.69	28.74 ± 3.86	42.40 ± 4.24
20:0	0.23 ± 0.03	0.34 ± 0.07	0.52 ± 0.08	0.25 ± 0.02	0.33 ± 0.04	0.52 ± 0.04
Monounsaturated						
16:1 (n-9)	0.49 ± 0.05	0.34 ± 0.08	0.48 ± 0.29	0.64 ± 0.09	0.34 ± 0.08	0.57 ± 0.17
16:1 (n-7)	0.46 ± 0.06	0.25 ± 0.05	0.17 ± 0.05	0.53 ± 0.05	0.26 ± 0.04	0.28 ± 0.02
18:1 (n-9)	19.21 ± 1.93	9.88 ± 1.23	11.75 ± 1.43	19.38 ± 0.47	10.36 ± 1.37	11.85 ± 2.31
18:1 (n-7)	5.46 ± 0.54	1.82 ± 0.26	1.14 ± 0.13	5.71 ± 0.35	1.95 ± 0.33	1.13 ± 0.22
20:1 (n-9)	0.80 ± 0.06	1.91 ± 0.18	1.10 ± 0.10	0.75 ± 0.04	1.93 ± 0.32	1.00 ± 0.17
20:1 (n-7)	0.34 ± 0.05	0.32 ± 0.01	0.20 ± 0.02	0.36 ± 0.04	0.34 ± 0.04	0.19 ± 0.04
Polyunsaturated						
18:2 (n-6)	0.57 ± 0.14	0.49 ± 0.14	0.40 ± 0.13	0.82 ± 0.23	0.74 ± 0.15	0.44 ± 0.22
18:3 (n-6)	0.12 ± 0.04	0.15 ± 0.11	0.22 ± 0.02	0.12 ± 0.03	0.14 ± 0.07	0.23 ± 0.05
20:3 (n-6)	0.28 ± 0.09	0.53 ± 0.06	0.47 ± 0.07	0.26 ± 0.07	0.51 ± 0.03	0.48 ± 0.16
20:4 (n-6)	4.24 ± 1.08	13.54 ± 0.53	3.85 ± 0.73	4.22 ± 0.31	13.44 ± 0.29	3.31 ± 0.46
22:4 (n-6)	0.35 ± 0.09	5.67 ± 0.28	3.00 ± 0.25	0.36 ± 0.08	5.30 ± 0.13	2.73 ± 0.22
22:5 (n-6)	1.22 ± 0.09	0.86 ± 0.22	0.99 ± 0.22	1.04 ± 0.32	0.83 ± 0.24	0.88 ± 0.09
20:5 (n-3)	0.17 ± 0.03	0.08 ± 0.05	0.65 ± 0.10	0.16 ± 0.02	0.06 ± 0.05	0.63 ± 0.09
22:5 (n-3)	0.22 ± 0.10	0.29 ± 0.04	0.40 ± 0.08	0.16 ± 0.02	0.27 ± 0.05	0.40 ± 0.21
22:6 (n-3)	2.64 ± 0.86	28.40 ± 1.90	26.02 ± 2.86	2.55 ± 0.38	28.31 ± 1.90	26.35 ± 2.10
Total (n-3)	3.03 ± 0.99	28.77 ± 1.87	27.07 ± 2.74	2.87 ± 0.42	28.69 ± 1.89	27.39 ± 1.86
Total (n-6)	6.78 ± 1.53	21.35 ± 0.68	8.90 ± 1.10	6.82 ± 1.04	20.94 ± 0.46	8.22 ± 0.67

PC, phosphatidylcholine, PE, phosphatidylethanolamine, PS, phosphatidylserine.

Values are expressed as mean mole % of total fatty acids ± SD (five brains from each group were analyzed).

work against oxidative stress. In this report, the activities of CuZnSOD, MnSOD and glutathione-related enzymes were simultaneously measured in the brains of mice as a function of age. Our data clearly showed an increase in CuZnSOD and GSH-PX activities as a function of age, whereas MnSOD and GSSG-R activities were unchanged. Thus, the alterations of brain antioxidant enzyme activities with aging differ between enzymes. Furthermore it is clear that the cellular antioxidant system is enhanced rather than weakened in senescent brain.

Our data showing increased CuZnSOD activity with age are compatible with the other results in rats (Bracco et al., 1986; Cand et Verdetti, 1989; Desavagayam et al., 1989; Mavelli et al., 1982). However, these findings contradict other authors who report no age-related increase in rats (Barja de Quiroga et al., 1990; Cao Danh et al., 1983; Kellog et Fridovich, 1976; Kurobe et al., 1990; Reiss and Gershon, 1976) or in mice (Cao Danh et al., 1983; Kellogg and Fridovich, 1976; Reiss and Gershon, 1976), or even a decrease in rats (Benzi et al., 1989; Bracco et al., 1986; Geremia et al., 1990; Gupta et al., 1991; Lebel et Bondy, 1991; Massie et al., 1979; Mizuno et Ohta, 1986; Semsei et al., 1991) or mice (Massie et al., 1979). It was demonstrated that the rate of mitochondrial $O_2^{\cdot-}$ generation is correlated with aging (Sawada et al., 1987; Sohal et al., 1990). Thus, the observed increase in CuZnSOD activity in the central nervous system of mice during aging may constitute a brain protection against superoxide anion elevation. Moreover, cytochrome oxidase activity increases in the brains of aging rats (Vanella et al., 1989), this suggests a close relationship between CuZnSOD activity and the rate of oxygen reduction by brain mitochondria. We found no changes in MnSOD activity with age. A possible explanation for the different CuZn and MnSOD activity profiles during aging is that the two enzymes are differently regulated in the brain. Few data are available for MnSOD activity in brain: an increase of MnSOD activity in the brain of old rats and mice has been reported (Cao Danh et al., 1983; Bracco et al., 1986; Scarpa et al., 1987) although another group showed a decrease (Ledig et al., 1982).

The patterns of changes in glutathione-related enzyme activities in the mouse brain are of particular interest: GSH-PX activity showed a significant positive correlation with increasing age, whereas GSSG-R activity did not vary with age. Vitorica et al. (1984) also found increasing GSH-PX activity with age in the soluble fraction of mitochondria. Other studies were unable to detect any variation in GSH-PX activity in the aging rat brain (Cand and Verdetti, 1989) or mouse brain (Hazelton and Lang, 1985), even a decrease has been reported (Sohal et al., 1990). For GSSG-R, a small but significant increase with age in rat brain has been reported (Hothersall et al., 1981; Sohal et al., 1990) and confined to some brain areas such as the hippocampus and the cerebellum (Mizuno and Ohta, 1986). A decrease has also been reported (Benzi et al., 1988). In brain, the GSH-PX/GSSG-R-catalyzed GSH-GSSG cycle plays a key role in metabolizing H_2O_2 and organic peroxides. The observed increase of GSH-PX activity with age suggests an increase in H_2O_2 formation in the cytoplasm and/or mitochondria, an adaptative phenomenon (Ceballos et al., 1988), and could underlie the age-related increase in the rate of prooxidant generation such as H_2O_2 as a candidate for being a biomarker of aging (Sohal, 1991). Concentrations of GSH are partly regulated by GSH-PX and GSSG-R. GSSG-R reduces GSSG to GSH. The increased activity of GSH-PX together with the unchanged GSSG-R activity could lead to a reduced GSH/GSSG ratio with age and could explain the age-associated decrease in GSH concentration reported in rat brain (Benzi et al., 1989; Ravindranath et al., 1989). Both CuZnSOD and GSH-PX activities increase with age. However, there was no direct correlation between the two activities. The strong positive correlation we have observed between CuZnSOD and GSSG-R in the 28 month control group has no evident biological explanation but was also observed in human erythrocytes of subjects aged 65 years and over (Berr et al., in preparation).

Since lipid peroxidation is one of the more deleterious effects of oxygen radical-induced damage, TBA-reactive products, which are indicative of malondialdehyde levels, were also assayed. There was no modification in basal lipid peroxidation in middle-aged and aged control

mice compared to young adults. These data show that accumulation of peroxidation products does not occur in old tissues. The age-correlated increase in CuZnSOD and/or GSH-PX activities presumably play a role in protection against basal lipid peroxidation. For example, the increase of GSH-PX may counteract the increased production of lipid peroxides that are precursors of MDA. Comparable results were obtained by Barqua de Quiroga et al. (1990) in rat brain. Nevertheless, other studies have given contradictory results in which an increase in basal lipid peroxidation is described (Geremia et al., 1990; Gupta et al., 1991; Mizuno and Ohta, 1986; Ravindranath et al., 1989; Sawada and Carlson, 1987) or even a decrease (Cand and Verdetti, 1989). However, the MDA production rate stimulated in vitro was clearly higher in middle-aged (12 months) than in young adult (2 months) or aged animals (28 months). Interpretation of the causes of these changes in MDA production stimulated in vitro is difficult because the TBA test used to collect the data can be influenced by factors such as vitamin E or PUFA content of membranes. Nevertheless, in vitro MDA data suggest that the susceptibility of brain tissue to free radical-induced peroxidation is highest during middle age. That sensitivity decreases in old mice probably reflects the reduced concentration of peroxidizable lipids (lowered PUFA substrate content) in the old brain (Eddy and Harman, 1975). A decreased lipid peroxidative potential was also observed in rat brain during aging (Devasagayam, 1989).

Influence of CuZnSOD transgene expression on the antioxidant enzyme regulation and lipid peroxidation

Despite the increased CuZnSOD activity in the brains of transgenic mice, GSH-PX activity was not modified in the 3 age groups studied when compared to the corresponding age group control. This contrasts with what has been observed in DS erythrocytes (Sinet et al., 1975), in lymphoid cells and fibroblasts (Anneren et al., 1987; Frisher et al., 1981) and in transfected cells expressing the human CuZnSOD gene (Ceballos et al., 1988), but corresponds to findings in DS fetal brain (Brooksbank and Balazs, 1984). The reason for the discrepancy between the results in the brains of transgenic mice and in the transfected cells, for the same human CuZnSOD transgene, could be a different cellular localization for CuZnSOD and GSH-PX enzymes in the brain. We have demonstrated in a previous report a specific neuronal localization of endogenous CuZnSOD and human CuZnSOD in the brain of transgenic mice particularly in the pyramidal neurons of the CA1–CA4 fieds of Ammon's horn (Ceballos-Picot et al., 1991). GSH-PX and glutathione-S-transferase proteins have both been observed in astrocytes and oligodendrocytes in rats (Cammer et al., 1989; Ushijima et al., 1986). These data underline the heterogeneous cellular distribution of the enzymes implicated in oxygen free radical detoxification and may form a basis for selective and/or regional expression of free radical toxicity in the brain.

The expression of the human CuZnSOD gene in the brain of transgenic mice resulted in increased MnSOD activity. MnSOD enzyme was localized in mitochondria in the rat brain (Akai et al., 1990), the increased CuZnSOD activity in CuZnSOD transgenic mice presumably leads to an augmentation in the steady-state superoxide concentrations inside the mitochondria, which may in turn trigger MnSOD expression. Increasing activity of MnSOD has been suggested to be due to an enhancement in the respiration state in aged rats (Chen et al., 1972). In mitochondria, $O_2^{\cdot-}$ is generated mainly from the membrane-borne respiratory chain, possibly at the NADH-ubiquinone reductase and ubiquinone-cytochrome *c* reductase steps (Chance et al., 1979). The increase MnSOD activity in transgenic mice might be a defense response to protect mitochondria from oxidative damage. Alternatively the increased MnSOD activity could lead to enhanced production of H_2O_2 inside the mitochondria and, as a consequence, to increased lipid peroxidation of mitochondrial membranes, since we have demonstrated the absence of adaptive increase in GSH-PX activity in brains of transgenic mice. In favor of this, a markedly higher rate of H_2O_2 generation in mitochondria is inversely correlated with life expectancy (Sohal, 1991). Oxidative damage to mitochondrial DNA can occur and fragments of oxidatively modified mitochondrial DNA

have been implicated in cancer and aging (Richter, 1988).

To further delineate the relationship between peroxidative stress and increased CuZnSOD activity without an adaptive rise in GSH-PX in the brain of transgenic mice, basal lipid peroxidation was determined and found to be significantly higher in total homogenates as compared to controls. This result was consistent with that reported for human CuZnSOD transfected cells (Elroy-Stein et al., 1986), as well as in blood platelets from human CuZnSOD transgenic mice (Schickler et al., 1989). During physiological aging in control mouse brain, an increased production of H_2O_2 is neutralized by a age-correlated increase in GSH-PX activity and there is no lipid peroxidation. Alternatively, in the brain of transgenic mice, increased activity of CuZnSOD activity could lead to an increase in the steady-state levels of H_2O_2, resulting in formation of $OH^{\cdot}$ which may enhance basal lipid peroxidation.

There was no difference between the PUFA composition in brains of transgenic and control mice. This observation is not incompatible with the increased basal lipid peroxidation observed in transgenic mice as compared to control mice, because the dietary fatty acids could repair and achieve the characteristic pattern of brain lipids (Chaudière et al., 1987). However, the composition of PUFAs in cerebral phosphoethanolamine and phosphoserine from DS patients was found to be markedly abnormal, with lower arachidonyl and higher docosahexaenoyl proportions and an elevated (n-3/n-6) ratio of PUFAs (Brooksbank and Balazs, 1985). Thus genes triplicated in human trisomy 21 other than CuZnSOD could account for these differences.

Further studies are required to determine how these antioxidant enzymes are controlled intracellularly. The ways in which stable adaptations might occur are through heritable changes in the cell's ability to transcribe certain genes selectively, to diminish cellular turnover of specific mRNAs, or to prolong the intracellular lifespan of the enzymes themselves. Anyway, our results suggest that regulation of antioxidant enzyme activities is necessary for cell integrity and that a deregulation of this complex antioxidant system may disturb the steady-state equilibrium of oxyradicals within cells, resulting in oxidative damage to biologically important molecules. Extended biochemical and ultrastructural studies of the brain of transgenic mice described here should clarify whether CuZnSOD excess participates in the accelerated aging in Down's syndrome, specially with regard to Alzheimer-like neuropathology.

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