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Regional Difference of ROS Generation, lipid Peroxidaton, and Antioxidant Enzyme Activity In Rat Brain and Their Dietary Modulation

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One of the potential causes of age-related neuronal damage can be reactive oxygen species (ROS), as the brain is particularly sensitive to oxidative damage. In the present study, we investigated the effects of aging and dietary restriction (DR) on ROS generation, lipid peroxidation, and antioxidant enzymes in cerebrum, hippocampus, and cerebellum of 6-, 12- 18-, and 24-month-old rats. ROS generation significantly increased with age in cerebrum of *ad libitum* (AL) rats. However, no significant age-difference was observed in hippocampus and cerebellum. DR significantly decreased ROS generation in cerebrum and cerebellum at 24-months. On the other hand, the increased lipid peroxidation of AL rats during aging was significantly reduced by DR in all regions. Our results further showed that catalase activity decreased with age in cerebellum of AL rats, which was reversed by DR, although SOD activity had little change by aging and DR in all regions. In a similar way, glutathione (GSH) peroxidase activity increased with age in cerebrum of AL rats, while DR suppressed it at 24-months. These data further support the evidence that the vulnerability to oxidative stress in the brain is region-specific.

Key words : Brain, ROS generation, Lipid peroxidation, Antioxidant enzymes, Aging, Dietary restriction

INTRODUCTION

One of the potential causes of age-related neuronal damage is endogenous reactive oxygen species (ROS) that are generated from aerobic metabolism (Floyd et al., 1984). The brain is especially vulnerable to oxidative damage as a result of its high oxygen consumption rate, its abundant lipid content, and the relative paucity of antioxidant enzymes compared to other tissues (Agranoff, 1984; Sokoloff, 1977; Hill and Switzer, 1984; Zaleska and Floyd, 1975).

There are two possible causes for the age-related increases in oxidative stress, which is implicated in an imbalance between the formation and spread of ROS and antioxidant defenses. One is that ROS generation increase during aging and/or the other is that the level of antioxidative defenses weakens during aging. For example, studies in rats and mice indicated that the mitochondria-induced oxidative stress significantly increases during aging (Lykkesfeldt *et al.*, 1998; Onaran *et al.*, 1997). Susceptibility of tissue damage in response to exogeneous oxidative stress increased with age, which is related to a declined antioxidative capacity (Sohal and Orr, 1992). Thus, an age-related increase in pro-oxidants is coupled with decreased antioxidants resulting in redox imbalance.

Dietary restriction (DR) has been shown to alter normal aging process by modulating most age-associated functional declines (Masoro, 1989; Yu, 1990) as well as delaying the onset and/or retarding progression of a large number of age-related diseases (Masoro *et al.*, 1991; Shimokawa *et al.*, 1991). Recent studies have documented DR as a most effective modulator of oxidative stress and antioxidant defense systems (Koizumi *et al.*, 1987; Sohal *et al.*, 1994; Luhtala *et al.*, 1994; Dogru-Abbasoglu *et al.*, 1997; Hussain et al., 1995; Mo *et al.*, 1995). Yu and his group (Yu, 1990; Lee and Yu, 1990)

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proposed that the beneficial effect of DR was the maintenance of cellular homeostasis by keeping; i) intact membrane structure and function, ii) proper redox state of cellular balance, and iii) detoxification process of xenobiotics. Earlier, Koizumi *et al.* (1987) reported that the anti-oxidative action of DR by showing increased catalase activity and suppressed lipid peroxidation by DR in liver homogenate.

There have been several conflicting reports showing age-associated alterations in oxidative damage and antioxidant enzyme activity in brain (Dogru-Abbasoglu *et al.*, 1997; Hussain *et al.*, 1995; Mo *et al.*, 1995). The conflicting data may be related to the possibility that both the pro-oxidant generation and antioxidant defenses can be region-specific. In the present study, we investigated the effects of age and DR on ROS generation, lipid peroxidation (LPO), and antioxidant enzyme activities in cerebrum, cerebellum, and hip-pocampus of aged and dietary restricted Fischer 344 rats.

MATERIALS AND METHODS

Materials

2',7'-Dichlorofluorescin diacetate (DCFDA) was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Specific pathogen-free (SPF) male Fischer 344 rats, which were received from Charles River Laboratories (Kingston, NY, USA), were fed a diet of the following composition:21% soybean protein, 15% sucrose, 43.65% dextrin, 10% corn oil, 0.15% α-methionine, 0.2% choline chloride, 5% salt mix, 2% vitamin mix and 3% Solka-Floc. All rats were fed *ad libitum* (AL) until 6 weeks of age; then, they were divided into two groups: *ad libitum*-fed control and diet-restricted (DR) group. Food intake of DR was restricted by 60% of the food intake of the AL control group. Detailed procedures for the maintenance of SPF status and the implement of DR were previously reported (Lee and Yu, 1990). Rats of 6, 12, 18, and 24 months of age were used in this study.

Tissue preparation

Rat cerebrum, hippocampus, and cerebellum were weighed and homogenized in 7-volumes of ice-cold 50 mM phosphate buffer (pH 7.4) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 80 mg/L trypsin inhibitor, and 1 μM leupeptin. The crude nuclear fraction was removed by centrifugation at 900 g for 15 min at 4°C. The resulting supernatant was centrifuged at 12,000 g for 15 min at 4°C to prepare mitochondrial and postmitochondrial fraction. A postmitochondrial fraction contained cytosol and microsome.

Assay for ROS generation

Production of ROS was measured as previously described (Thomas *et al.*, 1992). Briefly, postmitochondrial fractions were loaded with 25 μM DCFDA (diluted from a stock solution of 12.5 mM in ethanol) for 10 min. The initial fluorescence value of the dye-loaded samples was recorded and samples were then incubated for 30 min. The formation of the fluorescent probe DCF was then monitored at excitation and emission wavelengths of 485 nm and 530 nm, respectively, by Fluorescence Plate Reader (BIO-TEK Instruments, Inc., Winooski, USA). Production of ROS was expressed as the amount of formed DCF/mg protein/min.

Lipid peroxidation (LPO) mesurement

A. Basal thiobarbituric acid-reactive substance (TBARS) assay

LPO was determined using the TBARS assay (Laganiere and Yu, 1987). Assay mixture (1.2% TBA solution : 8.1% SDS solution : 20% acetic acid = 20 : 4 : 30) 0.5 ml was added to a brain postmitochondrialfraction, and then the reaction mixture was boiled at 94°C for 30 min. After cooling, TBARS was extracted by butanol and the entire mixture was centrifuged at 600 g for 10 min. Fluorescence intensity of the butanol fraction was measured at excitation wavelength of 530 nm and emission wavelength of 590 nm, respectively, by Fluorescence Plate Reader (BIO-TEK Instruments). TBARS concentrations were calculated according to a malondialdehyde (MDA) standard calibration curve.

B. Induced TBARS assay

After inducing LPO by incubating a brain homogenate with NADPH and ADP/FeSO4 at 37°C for 20 min, 0.5 ml of assay mixture (1.2% TBA solution:8.1% SDS solution : 20% acetic acid = 20 : 4 : 30) was added to it, and then the reaction mixture was boiled at 94 for 30 min. After cooling, TBARS was extracted by butanol and the entire mixture was centrifuged at 600 g for 10 min. Fluorescence intensity of the butanol fraction was measured at excitation wavelength of 530 nm and emission wavelength of 590 nm, respectively, by Fluorescence Plate Reader (BIO-TEK Instruments). TBARS concentrations were calculated according to a malondialdehyde (MDA) standard calibration curve.

Cu/Zn-SOD activity

Cu/Zn-SOD activity was measured using the xanthine/xanthine oxidase and cytochrome c reduction assay (McCord and Fridovich, 1969). The postmitochondrial fractions were added to assay mixture containing 200

mM potassium phosphate buffer (pH 7.4), 200 mM KCl, 10 mM EDTA, 0.5 mM xanthine, 0.126 U xanthine oxidase, and 0.1 mM cytochrome c. The assay was carried out in the presence of 1.5 mM KCN. The optical density change at 550 nm was monitored at 25°C.

Catalase activity

Catalase activity was measured according to a modified method of Beer *et al.* (Baudhuin *et al.*, 1964). The postmitochondrial fractions were added to the assay mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 30 mM H_2O_2 in a final volume of 1 ml. Changes in the optical density at 240 nm were spectrophotometrically monitored at 30°C.

Glutathione (GSH) peroxidase activity

GSH peroxidase activity was measured according to method of Laurence and Burk (1976). The postmitochondrial fractions were added to assay mixture containing 100 mM potassium phosphate buffer (pH 7.0), 10 mM NaN_3, 10 mM GSH, 1.5 mM NADPH, 36 mU of GSH reductase, and 5 mM H_2O_2. The optical density changes at 340 nm were spectrophotometrically monitored at 25°C.

Statistical analysis

Data were shown as mean±SEM. Results were analyzed statistically by Super ANOVA and values of $p < 0.05$ were considered statistically significant.

RESULTS

Effects of aging and DR on ROS generation

The generation of ROS in postmitochondrial fraction from cerebrum, hippocampus, and cerebellum were presented in Table I. ROS generation increased with age in cerebrum with a significant rise at 24 months of age compared to 6 months of age in AL rats. However, no such significant difference was noted in hippocampus and cerebellum with age. DR significantly decreased ROS generation in cerebrum at 24 months of age and in cerebellum at 18 and 24 months of age. On the other hand, interestingly, mitochondrial ROS generation showed no significant change by age and DR in all regions tested (unshown data).

Table I. Effect of aging and dietary restriction on ROS generation in postmitochondrial fraction

Age(Months) \ Groups		ROS generation [formed DCF (pmol/mg protein/min)]		
		Cerebrum	Hippocampus	Cerebellum
6	AL	7.07±0.23	9.07±0.52	14.17±0.33
	DR	7.33±0.28	8.54±0.61	13.11±0.69
12	AL	7.46±0.11	9.55±0.36	13.19±0.55
	DR	7.16±0.31	9.11±0.60	11.92±0.59
18	AL	7.94±0.27[a]	9.45±0.67	13.36±0.38
	DR	7.40±0.14	8.85±0.31	7.54±0.33[c]
24	AL	8.41±0.19[b]	9.85±0.46	15.37±0.85
	DR	7.23±0.15[c]	9.30±0.17	7.70±0.16[c]

ROS generation was measured by DCF formation with a fluorescent probe, DCFDA. Each value is the mean±S.E. of six rats. Statistical significance: [a]$p<0.05$ and [b]$p<0.001$ vs. 6 month-old rats of *ad libitum* group, and [c]$p<0.001$ vs. counterpart of *ad libitum* group, respectively. DCF, dichlorofluorescein.

Effects of aging and DR on basal and induced LPO

We also measured effects of age and DR on LPO as a marker of oxidative damage in cerebrum, hippocampus, and cerebellum. Under basal condition, there was no

Table II. Effect of age and dietary restriction on basal lipid peroxidation

Age(Months) \ Groups		TBARS concentration (pmol/mg protein)		
		Cerebrum	Hippocampus	Cerebellum
6	AL	173.2±19.9	306.1±25.5	250.2±38.3
	DR	196.1±16.7	260.6±36.6	242.4±14.6
12	AL	189.8±18.1	296.1±15.8	239.1±18.8
	DR	205.1±25.4	236.5±23.3	193.9±14.6
18	AL	207.3±33.4	277.0±38.9	246.2±27.3
	DR	170.3±21.1	364.3±42.0	194.3±24.2
24	AL	223.9±15.6	293.2±42.8	247.1±29.1
	DR	154.1±8.30[a]	228.6±21.7	161.0±15.4[b]

Lipid peroxidarion was determined by using TBA assay, after preincubation with NADPH and the mixture of ADP, $FeSO_4$, and NaOH at 37°C for 20 min. Each value is the mean ± S.E. of six rats. Statistical significance: [a]$p<0.01$ and [b]$p<0.001$ vs. counterparts of AL group, respectively. TBARS, thiobarbituric acid-reactive substance.

Table III. Effect of aging and dietary restriction on induced lipid peroxidation

Age(Months) \ Groups		TBARS concentration (pmol/mg protein)		
		Cerebrum	Hippocampus	Cerebellum
6	AL	970.9±164.7	2235.5±212.7	1282.7±162.4
	DR	857.1±212.8	1744.7±157.8	1920.0±149.4
12	AL	1684.2±221.0[a]	2972.4±138.0[a]	1760.2±260.6
	DR	920.3±232.5	1639.4±285.5[d]	1879.7±271.2
18	AL	2280.1±133.9[c]	3055.7±403.8	2546.±8170.6[c]
	DR	647.4±102.2[e]	1166.9±280.9[d]	2155.2±188.3
24	AL	2027.0±261.7[b]	3087.4±273.7[a]	2693.4±247.3[c]
	DR	363.6±106.1[e]	807.3±134.8[e]	1342.6±219.7[d]

Lipid peroxidarion was determined by using TBA assay, after preincubation with NADPH and the mixture of ADP, $FeSO_4$, and NaOH at 37°C for 20 min. Each value is the mean ± S.E. of six rats. Statistical significance: [a]$p<0.05$, [b]$p<0.01$, and [c]$p<0.001$ vs. 6-month-old rats of AL group, and [d]$p<0.01$ and [e]$p<0.001$ vs. corresponding aged rats of AL group, respectively. TBARS, thiobarbituric acid-reactive substance.

clear age-dependent change in LPO in all regions (Table II). In cerebrum, TBARS level was lower in DR rats than in AL rats at the age of 24-months (Table II). However, following induction of LPO with ADP/FeSO4 and NADPH, 24 month-old AL rats was significantly higher than that of 6 month-old-rats in all the regions (Table III). However, the TBARS concentration of DR rats was significantly lower in cerebrum at 18- and 24-months, hippocampus at 12-, 18- and 24-months, and cerebellum at 24-months than in the control AL group (Table III).

Effects of aging and DR on antioxidant enzyme activity

To examine whether age-related increase in ROS generation and LPO are related to declined antioxidative defenses, the activities of three principal antioxidant enzymes, SOD, catalase, and GSH peroxidase, were assayed. SOD activity changed little by aging and DR in all three regions of the brain (Table IV). Although catalase did not show any significant change in cerebrum and hippocampus, cerebellar catalase activity decreased in 24-month-old rats compared to 6-month-old rats, and DR reversed this age-associated decrease (Table V). In contrast, cerebral GSH peroxidase showed an increase activity with age in AL rats, but DR lowered this increase at 24 months (Table VI).

DISCUSSION

Recently, there has been heightened interest in the role of oxidative stress in various neurologic disorders including cerebral ischemia-reperfusion, head injury, Parkinson's disease, amyotrophic lateral sclerosis, Down's syndrome, and Alzheimers disease (Coyle and Puttfarcken, 1993). However, how ROS generation and antioxidative defence are altered during aging and modulated by DR in different regions of brain have not well explored.

Table IV. Effect of aging and dietary restriction on Cu/Zn-SOD activity.

Age(Months) \ Groups		Cu/Zn-SOD activity (mU/mg protein)]		
		Cerebrum	Hippocampus	Cerebellum
6	AL	11.46±2.13	20.25±2.48	16.87±2.24
	DR	12.96±1.64	19.46±1.71	12.56±0.90
12	AL	12.95±1.72	19.39±1.38	13.63±0.81
	DR	10.84±1.52	18.42±1.57	12.47±1.07
18	AL	13.55±2.34	17.60±1.18	12.21±0.78
	DR	12.62±3.20	16.23±2.36	13.74±1.65
24	AL	11.93±1.77	21.76±1.58	13.80±1.71
	DR	13.16±2.18	19.55±1.65	13.11±1.31

The values for the activities represent the mean±S.E. of six rats.

Table V. Effect of aging and dietary restriction on catalase activity.

Age(Months) \ Groups		Catalase activity (mU/mg protein)]		
		Cerebrum	Hippocampus	Cerebellum
6	AL	3.81±0.46	6.06±1.25	10.39±0.84
	DR	3.49±0.38	5.05±0.66	9.24±0.58
12	AL	3.79±0.24	6.28±0.66	10.57±0.70
	DR	3.66±0.30	5.45±0.69	9.67±0.59
18	AL	3.08±0.38	5.01±0.53	10.01±0.73
	DR	3.81±0.62	5.64±0.70	9.58±0.38
24	AL	3.23±0.35	4.56±0.54	7.41±0.54[a]
	DR	3.11±0.24	5.17±0.22	10.41±0.41[b]

The values for the activities represent the mean±S.E. of six rats. Statistical significance: [a]$p<0.05$ vs. 6-month-old rats of *ad libitum* group and [b]$p<0.001$ vs. 24-month-old rats of ad libitum group, respectively

Table VI. Effect of aging and dietary restriction on GSH-peroxidase activity.

Age(Months) \ Goups		GPX activity (mU/mg protein)		
		Cerebrum	Hippocampus	Cerebellum
6	AL	40.08±1.73	47.70±2.38	59.11±1.97
	DR	42.36±1.66	48.75±1.14	58.48±4.41
12	AL	43.80±3.76	50.98±1.74	68.28±2.71
	DR	42.77±2.30	48.55±1.65	60.77±4.35
18	AL	44.55±1.45	51.22±1.10	68.20±5.35
	DR	40.66±1.77	49.68±2.21	62.12±2.64
24	AL	48.51±1.75[a]	50.64±2.35	60.91±1.78
	DR	40.34±1.87[b]	49.70±3.11	59.36±4.24

The values for the activities represent the mean±S.E. of six rats. Statistical significance: [a]$p<0.01$ vs. 6-month-old rats of *ad libitum* group and [b]$p<0.01$ vs. 24-month-old rats of ad libitum group, respectively.

In the present study, the fluorescent dye DCFDA was used as a quantitative mean to assess the ROS generation rate. DCFDA has been used as a sensitive probe for cellular, mitochondrial, cytosolic, and microsomal production of ROS such as H_2O_2, O_2^-, $^{\cdot}OH$ and *etc* in many tissues (Lee and Yu, 1990; Thomas *et al.*, 1992; Kang *et al.*, 1997). In the postmitochondrial fraction, ROS generation increased with age in cerebrum of AL rats, while no significant difference was observed in hippocampus and cerebellum with age. DR markedly decreased ROS generation in cerebrum at 24 months of age and in cerebellum at 18 and 24 months of age. These data indicate that aging and DR have the differential effects on each region of brain, which is related to regional specificity including composition of neuron and glia.

LPO shows more consistent age-related phenomenon, thus have been used as a reliable biomarker of oxidative tissue damage (Gutteridge, 1995). In the present study, LPO induced by Fe^{2+}/ADP and NADPH increased with

age in all regions, and at the same time DR reduced significantly this increase, although no significant age-related change in basal LPO was detected. The inhibitory DR's action was clearly exhibited on LPO and DR's effect was greater in aged rats than young rats suggesting that DR's efficacy becomes more effective during senescence. These results are in line with the finding that DR animals have lower highly polyunsaturated fatty acids as shown by peroxidazability index (Laganiere and Yu, 1993).

On the other hand, the study of Lebel *et al.* (1991) showed that the generation rate of ROS decreased with age in the brain, owing to increase in antioxidant enzyme activities. Rao *et al.* (1990) reported that DR increased one or more of selected antioxidant enzymes in the liver, brain cortex, heart and kidney of rats and furthermore the authors showed reduced LPO in that four tissues. And the authors commented that the magnitude of the effect on antioxidant enzymes was differentially affected by DR. In the present study, catalase activity decreased with age in cerebellum of AL rats, which was reversed by DR, although SOD activity had little change by aging and DR in all regions. In a similar way, GSH peroxidase activity increased with age in cerebrum of AL rats, while DR suppressed it at 24-month. These results suggest that each region of brain has the different susceptibility to oxidative stress during aging and DR can reduce age-associated increase in peroxidative damage.

The interesting finding of the current study is that although in hippocampus, no significant age- and DR-related alterations in ROS generation and antioxidant enzyme activity were observed, LPO increased with age and DR reversed this increase. Antioxidant defense compounds such as catalase, GSH peroxidase, α-tocopherol and GSH are shown to be lower in brain than other organs (Rao *et al.*, 1990) and the indole melatonin has also been suggested to be a highly important antioxidant in brain (Beyer *et al.*, 1998; Miller et al., 1996). Recent study reported that melatonin prevents the delayed death of hippocampal neurons induced by enhanced excitatory neurotransmission (Skaper *et al.*, 1998). Therefore, these results may be due to regional specificity of hippocampus and other factors such as melatonin might be involved in age- and DR- associated change in LPO. The regional differences observed in this present study may well be related to changes in membrane lipid composition and/or age-dependent changes in anti-oxidative defenses in various brain regions. So far, there are no systematic studies on regional differentiation of antioxidant defenses and oxidative damages of aged brain.

In conclusion, although each region showed varied differential reponses to aging and DR for ROS generation, but LPO was consistently modulated by aging and DR. It was further shown that the increased LPO is not due to a decrease in antioxidant enzyme activities.

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