Neurochemical Research, Vol. 19, No. 9, 1994, pp. 1131–1137

Evidence of an Oxidative Challenge in the Alzheimer's Brain

Louisa Balazs[1] and Michael Leon[2,3]

(Accepted March 14, 1994)

Alzheimer's disease may arise from or produce oxidative damage in the brain. To assess the responses of the Alzheimer's brain to possible oxidative challenges, we assayed for glutathione, glucose-6-phosphate dehydrogenase, catalase and superoxide dismutase in twelve regions of Alzheimer's disease and aged control brains. In addition, we determined levels of malondialdehyde to evaluate lipid peroxidation in these brain regions. Most brain regions showed evidence of a response to an oxidative challenge, but the cellular response to this challenge differed among brain regions. These data suggest that the entire Alzheimer's brain may be subject to an oxidative challenge, but that some brain areas may be more vulnerable than others to the consequent neural damage that characterizes the disease.

KEY WORDS: Alzheimer's disease; glucose-6-phosphate dehydrogenase; glutathione; superoxide dismutase; catalase; malondialdehyde; lipid peroxidation.

INTRODUCTION

The action of oxygen free radicals has been suggested as a possible mediator of the cellular damage in the affected regions of the Alzheimer's brain (1). Indeed, oxidative activity has been thought to play a role in normal brain aging (2). There are normally defense mechanisms present in all neurons that protect these cells from oxidative damage. Superoxide is formed during normal cellular oxidative reactions (3,4) and is rapidly converted by superoxide dismutase (SOD) to hydrogen peroxide, a powerful oxidant [3] that is turned into oxygen and water through the action of either catalase or glutathione (GSH). Under some conditions, however, the superoxide is not inactivated, and oxygen free radicals are formed. Oxygen free radicals formed in neurons can attack lipids, proteins and nucleic acids (5-7), any of which could induce cell death.

In support of this idea is the finding that an increase in plaques formed by amyloid in the normally aging mouse brain can be prevented by the introduction of antioxidants into the diet (8). Moreover, increasing oxidative stress in cultured neurons induces immunoreactivity for both ALZ-50 and paired helical filament (9), two proteins expressed in the Alzheimer's brain. Increased superoxide dismutase mRNA also was found in hippocampal pyramidal cells (10). Neurofibrillary tangles associated with neurons in Alzheimer's brains have increased superoxide dismutase immunoreactivity (12) and hippocampal pyramidal cells, which are greatly affected by Alzheimer's disease, are highly immunoreactive for antibodies to the enzyme (13). On the other hand, Marklund, et al. (11) reported minimal superoxide dismutase changes in regions of Alzheimer's brains.

Alzheimer's disease brains also have increased basal levels of lipid peroxidation (14,15), indicative of oxidative damage. When no change in basal levels have been found, an increase in stimulated lipid peroxidation has been reported (16). Subarrao et al. (15) also have shown increased peroxidation of Alzheimer's cortex in vitro in response to an oxidative challenge.

[1] Department of Pathology, University of Tennessee College of Medicine, Memphis, Tennessee 38163.
[2] Department of Psychobiology, University of California, Irvine, California 92717.
[3] To whom to address reprint requests.

0364-3190/94/0900-1131$07.00/0

Catalase is a cellular antioxidant and high levels of immunoreactivity to this enzyme have been found in the neuron-associated neurofibrillary tangles of Alzheimer's patients (12). These data suggest that these neurons may be responding to an oxidative challenge. GSH underlies four types of cellular defenses (17): 1) it reduces intracellular peroxides via GSH-peroxidase and GSH S-transferases; 2) it facilitates the non-enzymatic reduction of free radicals; 3) it maintains reduced disulfide bonds in cellular proteins via GSH S-transferases; and 4) it conjugates directly with toxins (17). GSH can prevent oxidative damage to cells by endogenous products of oxidative metabolism (17). Perry et al. (18) found no difference in GSH levels of Alzheimer's and control brains. Adams et al. (19), however, found increased GSH in Alzheimer's hippocampus and midbrain, but not in frontal cortex, putamen or caudate nucleus.

Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme in the hexose monophosphate shunt whose function is the generation of NADPH, a coenzyme that represents a major source of cytoplasmic reducing power (20). Cytoplasmic NADPH is involved in cellular defense systems, since a high NADPH:NADP ratio assures that GSH remains in its reduced and active form (21). G6PD is elevated in the frontal cortex of Alzheimer's patients, a finding consistent with increased oxidative activity (22). While these data support a role for oxidative factors in Alzheimer's disease, there are conflicting data regarding the evidence for oxidative action in the Alzheimer's brain.

Differing findings may be due in part to a limited number of both brain regions selected and cellular defense mechanisms measured. With that problem in mind, we determined the levels of GSH, malondialdehyde levels (MDA) as a measure of lipid peroxidation, as well as the activity of catalase, superoxide dismutase (SOD) and G6PD in 12 regions from Alzheimer's and aged control brains. The brain areas chosen for analysis were: olfactory bulb, nucleus basalis of Meynert, prepyriform gyrus, parolfactory gyrus, parahippocampal gyrus, hippocampus, amygdala, frontal cortex, motor cortex, sensory cortex, occipital cortex and cerebellum. These areas were selected for their differing levels of neural degeneration as a consequence of Alzheimer's disease.

EXPERIMENTAL PROCEDURE

Brain Tissue. We obtained a total of 7 Alzheimer's (4 male, 3 female) and 5 aged control (2 male, 3 female) brains from the Alzheimer's Disease Research Center at UCI. The Alzheimer's brains were taken from cases that were confirmed by postmortem brain neuropathological examination according to the CERAD criteria (28). The mean age of death for the Alzheimer's group was 74.8 years (range 63-89 years) and 74.0 years for the aged controls (range 61-86 years). The mean postmortem interval was 7.3 hr for the Alzheimer's and 5.8 hr for the controls. Neither parameter differed significantly between groups. No sex differences were found in the analysis.

Tissue Preparation. Postmortem human brain slices were snap frozen in liquid nitrogen and stored at −70°C. The brain regions of interest were dissected frozen and any pial blood vessels or meninges were removed prior to weighing in a Mettler analytical balance (sample wet weight range 15-40 mg). The tissue was quickly minced in the appropriate homogenization solution. Samples then were placed on ice and homogenized by inserting the probe of a sonic dismembranator three times, each for 15 sec with a 1-min cooling period.

MDA. We assayed the specimens for thiobarbituric acid (TBA) reactive lipid oxidation end products, using the method of Chan and Fishman (6). Samples were sonicated in 10 μl volume per mg tissue of 100 μM Tris and 100 μl of aliquots were used for the assay in duplicates. The MDA reaction mixture consisted of 1.1 ml 10% TCA, 0.5 mL PBS (pH 7.4) and 0.5 ml of 0.5% TBA pluse 100 μl tissue homogenate. A standard curve was constructed using known amounts of 1, 1,3,3-tetraethoxy-propane (TEP) with concentrations between 0.1 and 5.0 nM. The reaction mixture was incubated at 100°C for 15 min, then cooled down to ambient temperature and centrifuged at 10,000 *g* for 15 min. The supernatant was removed and the concentration of TBA reactive materials was measured with a Gilson Response spectrophotometer at 532 nm and 25°C. Mean absorbance values of the duplicates was calculated and the concentration of TBA reactive material present in the specimen was calculated from the standard curve. Data were expressed as nM of lipid peroxidation end products/mg protein. Protein concentration for this and all other assays in the original homogenates (in the case of MDA in 100 μM Tris) was measured by the method of Lowry et al. (25) using bovine serum albumin as standard.

Catalase. Catalase was assayed by a modification of the method of Cohen et al. (10). Briefly, the tissue was sonicated on ice in 10 μl per mg isotonic 10 mM phosphate buffer solution (PBS, pH 7.4) at 4°C and centrifuged at 7000 *g* for 10 min. The supernatant was collected and ethanol was added to a final concentration of 170 μM followed by a 30 min incubation on ice. After adding Triton-X 100 to a final concentration of 1%, the homogenates were mixed and diluted 10 fold with PBS. To initiate the enzymatic reaction, 500 μL of the diluted homogenate and 5.0 ml, 6 mM H_2O_2 (in 10 μM K-phosphate buffer; pH 7.0), were mixed and incubated on ice for 3 min before the reaction was stopped with 1 ml 6N H_2SO_4. The H_2O_2 unreacted with the tissue catalase was titrated by adding a standard excess of $KMnO_4$ (7 ml of 0.01 N $KMnO_4$), oxidization of which was measured spectrophotometrically at 480 nm wavelength. A standard curve was constructed by using commercially available catalase from bovine liver (Sigma) in the 0.15-4.37 U range. Samples were assayed in duplicates and their mean values calculated and used for evaluation. Enzyme activity was expressed in units/mg protein, where 1 unit of catalase reacts with 1 μM of H_2O_2 per minute.

Hemoglobin. Since erythrocytes contain SOD and G6PDH, the possibility exists that any differences in SOD and G6PDH found between brain areas could be due to differences in the number erythrocytes (i.e. vascularization and perfusion) present in the tissue. To determine whether there was a correlation between SOD or G6PDH enzyme activities and the amount erythrocytes contaminating the tissue, we measured hemoglobin levels in the homogenates using a commercially available kit (Sigma) based on the method of Marklund (27).

G6PD. Analysis of G6PD activity in the tissue samples was done by the Cho and Joshi (29) modification of the method originally de-

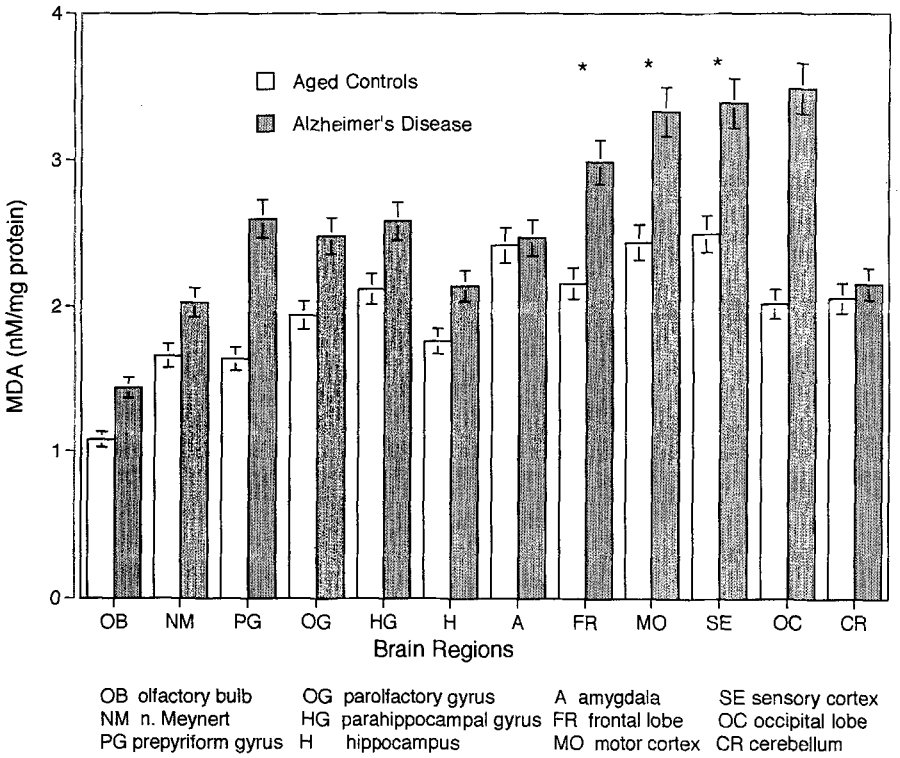

Fig. 1. Mean malondialdehyde levels (+/− SEM) for Alzheimer's and aged control brain areas. Stars indicate statistically significant differences ($p<0.05$) between groups.

veloped by Catalano et al. (28). G6PD activity was measured by following the reduction of NADP to NADPH that occurs as glucose-6-phosphate is converted to 6-phosphogluconate. Tissue was sonicated in 10 volumes per mg ice-cold 100 mM Tris HCl buffer (pH 7.4). Homogenates were then spun at 12,000 *g* for 5 min at 4°C. Fifty µl aliquots of the supernatant was added to 950 µl reaction mixture containing of 17.5 mM HEPES (pH 7.5), 2 mM glucose-6-phosphate, 0.9 mM NADP, 10 mM MgCl2, and 10 mM 2,3-diphosphoglycerate. NADPH production was followed on a Gilford Response spectrophotometer by measuring the increase in absorbance over a period of 3 min at 340 nm at 25°C. G6PD activity was expressed in mU/mg protein.

GSH. The total GSH content expressed here is a sum of GSH, the mixed disulfide of GSH with cystein, and 2 × GSSG (oxidized glutathione), as defined by Perry et al. (18). The tissue was homogenized in 10 µl per mg ice-cold 1% 5-sulfosalicylic acid (SSA). Following centrifugation of the homogenate for 5 min at 10,000 × g, the supernatant was collected. Total GSH was determined using the glutathione disulfide reductase-DTNB (5,5'-dithiobis-2-nitrobenzoic acid) recycling assay [2,34]. In brief, 50 µl supernatant was added to 750 µL of 360 µM NADPH in TE buffer (150 mM Tris, 7.5 mM EDTA, pH 7.5) plus 100 µL 7.5 mM DTNB, and the final volume was adjusted to 1100 µL with 1% SSA. GSH reductase (0.5 units in 20 µl TE) was added to initiate the reaction. The rate of 2-nitro-5-thiobenzoic acid (TNB) formation was followed at 412 nm at 30°C and compared to rates obtained using known amounts of GSH prepared in the same solvent in the 0.2 mM - 2 mM range. Background values due to spontaneous TNB formation in the absence of added GSH were subtracted from the experimental values. All samples were assayed in duplicate and GSH concentration was calculated in nM/mg protein.

SOD. We assayed for SOD using the method of Kostyuk and Potapovich (19) by measuring SOD-induced protection of the oxidation of quercetin at room temperature elicited by UV light at 365 nm wavelength. The tissue was sonicated on ice three times for 15 sec in 10 µl per mg volume of physiological saline. The homogenate was centrifuged at 4°C for 15 min at 10,000 *g* and the supernatant was further diluted 10-fold with saline. The reaction mixture consisted of 0.8 mM N, N, N1, N1 - tetramethylethylenediamine (TMEDA), 0.08 mM EDTA dissolved in a total volume of 3 ml of 16 mM phosphate buffer (pH 10), plus 100 µl diluted sample supernatant. The reaction was initiated by the addition of 100 µl of quercetin stock solution (150 µg/ml in N, N - methylformamide), followed by a 30 min exposure to UV light. The unreacted quercetin was measured at 406 nm spectrophotometrically. A standard curve was constructed using commercially available SOD from bovine liver (Sigma) in the 0.4 to 3.2 U range. Samples were assayed in duplicate and SOD activity was calculated as U/mg protein.

Statistical Analysis. All data were subjected to a two-way ANOVA with repeated measures and individual Scheffe tests were performed post hoc.

RESULTS

MDA. Our assays reveal elevated levels of MDA in Alzheimer's brains relative to those found in controls

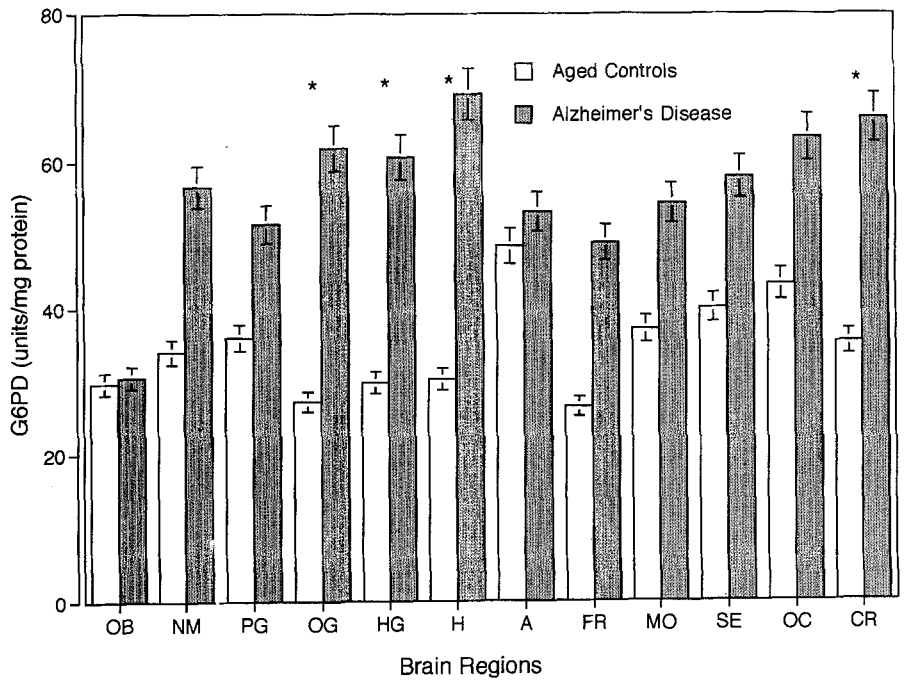

Fig. 2. Mean glucose-6-phosphate-dehydrogenase activity (+/− SEM) for Alzheimer's and aged control brain areas.

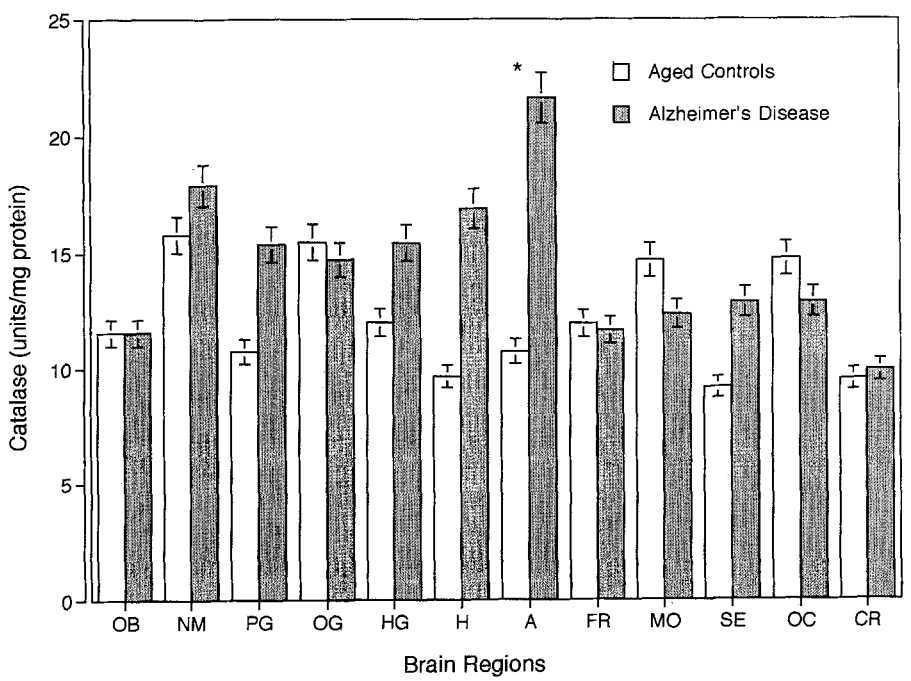

Fig. 3. Mean catalase activity (+/− SEM) for Alzheimer's and aged control brain areas.

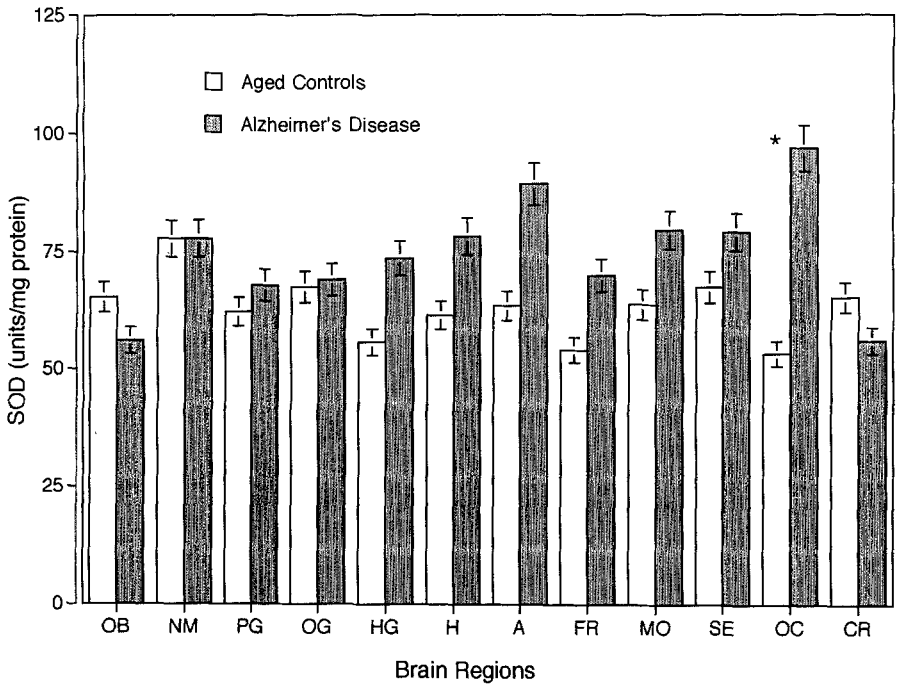

Fig. 4. Mean superoxide dismutase activity (+/− SEM) for Alzheimer's and aged control brain areas.

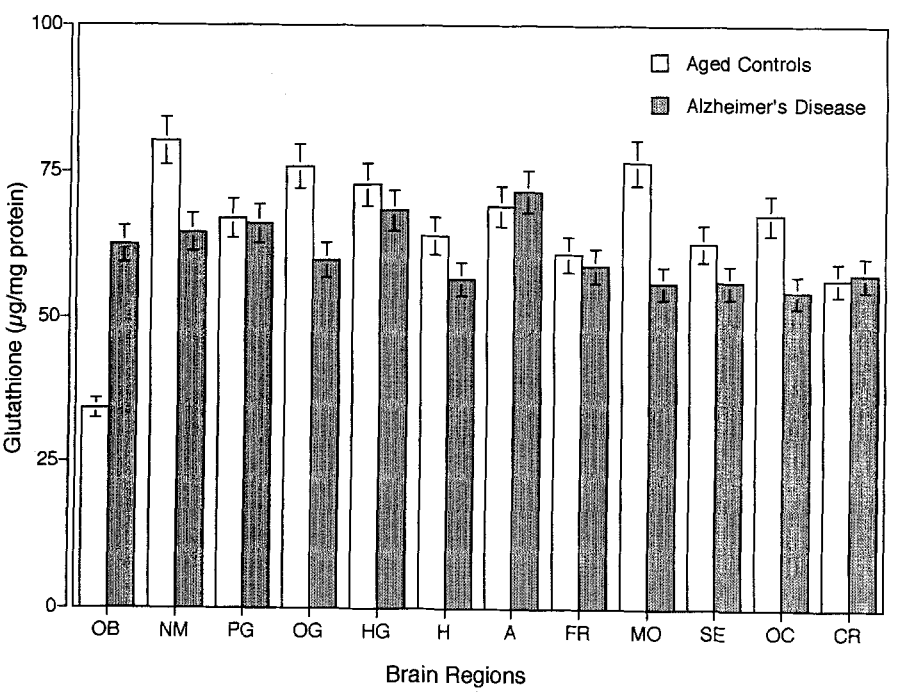

Fig. 5. Mean glutathione levels (+/− SEM) for Alzheimer's and aged control brain areas.

($F_{1,11}$ = 20.94, $p<0.001$), reflecting an increased lipid peroxidation in Alzheimer's brains. Individual comparisons indicate that MDA increased in motor cortex, sensory cortex and occipital cortex in Alzheimer's brains relative to their aged controls. The increased levels of MDA in other brain regions do not reach statistical significance.

G6PD. There is an overall increase in G6PD activity in Alzheimer's brains relative to controls ($F_{1,11}$ = 19.234, $p<0.001$). Individual comparisons reveal statistically significant increases in the hippocampus, parolfactory gyrus, parahippocampal gyrus and cerebellum of Alzheimer's brains relative to controls.

Catalase. Alzheimer's brains have higher catalase activity than control brains ($F_{1,11}$ = 4.857, $p<0.05$), but only the amygdala of Alzheimer's brains have a statistically significant increase in catalase activity relative to controls when individual comparisons are made.

SOD. There is an overall increase in the activity of this enzyme in the Alzheimer's brains relative to control brains ($F_{1,11}$ = 7.71, $p<0.02$). Individual comparisons revealed increases only in the Alzheimer's occipital cortex. No correlation was found between hemoglobin levels in the brain tissue and SOD activity.

GSH. There is no overall difference in GSH levels between Alzheimer's and control brains. No individual regions have increased levels of GHS when compared to controls.

DISCUSSION

The Alzheimer's brain shows clear indications of being subject to an oxidative challenge. Lipid peroxidation is elevated in the Alzheimer's brain, along with increase in the collective cellular defense mechanisms represented by the increases in G6PD, catalase and SOD activity, although there is a marked heterogeneity in the degree of response in different brain areas.

These data may account for differences in reports on oxidative changes in Alzheimer's disease, since the selection of any one brain region or oxidative measure may not reveal an oxidative challenge throughout the brain. Since we did not measure all known cellular defense mechanisms for free radical oxidation, it is possible that an area such as the nucleus basalis of Meynert would evidence a response to an oxidative challenge. It is also possible that this area, which has increased cell loss [33], shows little sign of oxidative response because the affected cells are gone. There also may be defense mechanisms that we have not measured that would reflect oxidative patterns in such brain regions.

What these data do suggest is that there is a widespread oxidative challenge within the Alzheimer's brain. Indeed, there is a marked similarity between these findings and those assaying for these systems in the red blood cells of Alzheimer's patients. Perrins, et al. [34] found that while the glutathione system is unaffected by the disease, both superoxide dismutase and catalase increases in Alzheimer's patients. SOD is also elevated in Alzheimer's muscle tissue [35]. The similarity of the response in both brain and other tissues suggests that the disease may arise in all cells in the body, but that some parts of the brain are particularly vulnerable to its deleterious action.

ACKNOWLEDGMENTS

THIS work was supported by the Orange and Los Angeles Counties ADRC, LEAD award AG07918 to C. Cotman, as well as grants AG07546 and MH45353 to M. L. We thank Pauline Yahr for her suggestions for data analysis.

REFERENCES

1. Volcier, L., and Crino, P. B. 1990. Involvement of free radicals in dementia of the Alzheimer's type: a hypothesis. Neurobiol. Aging 11:567–571.
2. Freeman, B. A., and J. D. Crapo. 1982. Biology of disease: Free radicals and tissue injury. Lab. Invest. 47:412–426.
3. Haliwell, B., and Gutteridge, J. M. C. 1989. Free Radicals in Biology and Medicine. 2nd ed., Oxford, Clarendon Press.
4. Nohl, H., and Hegner, D. 1978. Do mitochondria produce oxygen radicals in vivo? Eur. J. Biochem. 82:563–567.
5. Davies, K. J. A. 1987. Protein damage and degradation by oxygen radicals. J. Biol. Chem. 262:9895–9901.
6. Lin, W. S., Wong, F., and Anderson, R. 1987. Role of superoxide in radiation-killing of Escherichia coli and in thymine release from thymidine. Biochem. Biophys. Res. Comm. 147:778–786.
7. Melho-Filho, A. C., and Menighini, R. 1984. In vivo formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Hager-Weiss reaction. Biochim. Biophys. Acta. 781:56–63.
8. Harman, D., Eddy, E. E., and Noffsinger, J. 1976. Free radical theory of aging: Inhibition of amyloidosis in mice after antioxidants; possible mechanism. Amer. Geriatrics Soc. 24:203–210.
9. Blass, J. P., Baker, A. C., Ko, L. W., and Black, R. S. 1990. Induction of Alzheimer antigens by an uncoupler of oxidative phosphorylation. Arch. Neurol. 47:864–869.
10. Ceballos, I., Javoy-Agid, F., Hirsch, E. C., Dumas, S., Kamoun, P. P., Sinet, P. M., and Agid, Y. 1989. Localization of copper-zinc superoxide dismutase mRNA in human hippocampus by in situ hybridization. Neurosci. Lett. 105:41–46.
11. Marklund, S. L., Aldolfsson, R., Gottfries, C. G., and Winbald, B. 1985. Superoxide dismutase isoenzymes in normal brains and in brains from patients with dementia of Alzheimer type. J. Neurol. Sci. 67:319–325.
12. Pappolla, M. A., Omar, R. A., Kim, K. S., and Robakis, N. K. 1992. Immunohistochemical evidence of antioxidant stress in Alzheimer's Disease. Am. J. Pathol. 140:621–628.

13. Delacourte, A., Defossez, A., Ceballos, I., Nicole, A., and Sinet, P. M. 1988. Preferential localization of copper/zinc superoxide dismutase in the vulnerable cortical neurons in Alzheimer's disease. Neurosci. Lett. 92:247–253.
14. Chia, L. S., Thompson, J. E., and Moscarello, M. A. 1984. X-ray diffraction evidence for myelin disorder in brain from humans with Alzheimer's disease. Can Biochem. Biophys. Acta Ser. Biomembr. 775:308–312.
15. Subarao, K. V., Richardson, S., and Ang, L. C. 1990. Autopsy samples of Alzheimer's cortex show increased peroxidation in vitro. J. Neurochem. 55:342–345.
16. Hajimohammadreza, I., and Brammer, M. 1990. Brain membrane fludity and lipid peroxidation in Alzheimer's disease. Neurosci. Lett. 112:333–337.
17. Meister, A., and Anderson, M. E. 1983. Glutathione. Ann. Rev. Biochem. 52:711–760.
18. Perry, T. L., Yong, V. W., Bergeron, C., H. S., and Jones, K. 1987. Amino acids, glutathione, and glutathione transferase activity in the brains of patients with Alzheimer's disease. Ann. Neurol. 21:331–336.
19. Adams, J. D., Jr., Klaidman, L. K., Odunze, I. N., Shen, H. C., and Miller, C. A. 1991. Alzheimer's and Parkinson's disease. Brain levels of glutathione, glutathione disulfide, and Vitamin E. Mol. Chem. Neuropathol. 14:213–226.
20. Lehninger, A. L. 1975. Biochemistry. Worth: New York.
21. Kosower, N. S., and Kosower, E. M. 1978. The glutathione status of cells. Int. Rev. Cytol. 54:109–160.
22. Martins, R. N., Harper, C. G., Stokes, G. B., and Masters, C. L. 1986. Increased cerebral glucose-6-phosphate dehydrogenase activity in Alzheimer's disease may reflect oxidative stress. J. Neurochem. 46:1042–1045.
23. Mirra, S. S., Heyman, A., McKeel, D., Sumi, S. M., Crain, B. J., Brownlee, L. M., Vogel, F. S., Hughes, J. P., van Belle, G., and Berg, L. 1991. The consortium to establish a registry for Alzheimer's disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology. 41:479–486.
24. Chan, P. H., and Fishman, R. A. 1980. Transient formation of superoxide radicals in polyunsaturated fatty acid-induced brain swelling. J. Neurochem. 35:1004–1007.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randal, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.
26. Cohen, G., Dembiec, D., and Marcus, J. 1970. Measurement of catalase in tissue extracts. Analyt. Biochem. 34:30–38.
27. Marklund, S. 1979. A simple specific method for the determination of the hemoglobin contents of tissue homogenates. Clin. Chim. Acta. 92:229–234.
28. Catalano, E. W., Johnson, G. F., and Solomon, H. M. 1975. Measurement of erythrocyte glucose 6-phosphate dehydrogenase activity with a centrifugal analyzer. Clin. Chem. 21:134–138.
29. Cho, S., and Joshi, J. G. 1988. Effect of long-term feeding of aluminum chloride on hexokinase and glucose 6-phosphate dehydrogenase in the brain. Toxicology 48:61–69.
30. Anderson, M. E. 1985. Determination of glutathione and glutathione disulfide in biological samples. Meth. Enzymol. 113:548–555.
31. Tietze, F. 1969. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 27:502–522.
32. Kostyuk, V. A., and Potapovich, A. L. 1989. Superoxide-driven oxidation of quercetin and a simple sensitive assay for determination of superoxide dismutase. Biochem. Int. 19:1117–24.
33. Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., and DeLong, M. R. 1982. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237–1239.
34. Perrins, R., Briancon, S., Jeandel, C., Artur, Y., Minn, A., Penin, F., and Siest, G. 1990. Blood activity of Cu/Zn superoxide dismutase, glutathione peroxidase and catalase in Alzheimer's disease: a case-control study. Gerontology 36:306–313.
35. Zemlen, F. P., Thienhaus, O. J., and Bosman, H. B. 1989. Superoxide dismutase activity in Alzheimer's disease: possible mechanism for paired helical filament formation. Brain Res. 476:160–162.