

## Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease

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**ABSTRACT** The relationship between Alzheimer disease (AD) and aging is not currently known. In this study, post-mortem frontal- and occipital-pole brain samples were obtained from 16 subjects with AD, 8 age-matched controls, and 5 young controls. These samples were analyzed both for protein oxidation products (carbonyl) and the activities of two enzymes vulnerable to mixed-function oxidation, glutamine synthetase and creatine kinase. Glutamine synthetase is more sensitive to mixed-function oxidation than creatine kinase. Carbonyl content rises exponentially with age, at double the rate in the frontal pole compared with the occipital pole. Compared with young controls, both aged groups (AD and age-matched controls) have increased carbonyl content and decreased glutamine synthetase and creatine kinase activities, which are more marked in the frontal than occipital pole in all instances. We conclude that protein oxidation products accumulate in the brain and that oxidation-vulnerable enzyme activities decrease with aging in the same regional pattern (frontal more affected than occipital). However, only glutamine synthetase activity distinguishes AD from age-matched controls: Because glutamine synthetase activity is differentially reduced in the frontal pole in AD, we suggest that AD may represent a specific brain vulnerability to age-related oxidation.

Aging can be defined as the nonfunctional alteration of structure or homeostatic capability in an individual organism as it lives (1). A particular consequence of the aging process, at the cellular level, is the accumulation of proteins covalently modified by specific mechanisms—e.g., mixed-function oxidation (MFO), deamidation, ubiquitin conjugation, and glycation (2–7). These steps have been reported to mark proteins for subsequent proteolysis.

MFO is of particular interest because of the site-specific nature of the modifications (8–13), tending to occur near the metal-containing catalytic site of particular enzymes—e.g., glutamine synthetase. Free-radical-mediated forms of MFO could be one of the important mechanisms in normal aging (14–16), causing protein structural alterations, loss of enzymatic function, or interference with regulatory protein interactions.

In this study, we provide evidence that the level of oxidatively modified protein is increased in the aging brain. We also demonstrate that in Alzheimer disease (AD), a disease strongly associated with aging, there is regional loss of glutamine synthetase activity. Glutamine synthetase is an enzyme particularly sensitive to MFO. We propose the hypothesis that AD is an alteration in the normal aging process in selectively vulnerable brain cells or brain areas

that are challenged by increased oxidation with advancing age.

### MATERIALS AND METHODS

**Brain Tissue Samples.** Brain specimens were obtained at autopsy from 16 patients with AD, 8 age-matched controls (AC), and 5 young controls (YC). Tissues were received from the Kentucky Medical Examiners Program and the University of Kentucky AD Research Center, under approved research protocols. All subjects with AD were studied clinically and showed progressive dementia without evidence of other neurologic illnesses. Control subjects had no history of dementia or other neurologic disease. Neuropathologic examination confirmed the absence of any significant gross or microscopic brain abnormalities in controls. The diagnosis of AD was confirmed by neuropathologic examination by using standard criteria (17). Specimens (1–5 g) were taken from the brain frontal pole (FP) and occipital pole (OP) of each subject and kept frozen at  $-70^{\circ}\text{C}$  until used. Experience in our laboratory has shown no significant effect on the carbonyl and enzyme assay when tissue is stored at  $-70^{\circ}\text{C}$  for up to 3 yr. The specimens were coded, and all subsequent analyses were performed blind to brain region, age, and disease status.

**Brain Sample Extracts.** FP and OP samples were minced and suspended in 10 mM Hepes buffer, pH 7.4/137 mM NaCl/4.6 mM KCl/1.1 mM  $\text{KH}_2\text{PO}_4$ /0.6 mM  $\text{MgSO}_4$ . To prevent artifactual proteolysis of oxidized proteins, the protease inhibitors leupeptin (0.5  $\mu\text{g}/\text{ml}$ ), pepstatin (0.7  $\mu\text{g}/\text{ml}$ ), phenylmethylsulfonyl fluoride (40  $\mu\text{g}/\text{ml}$ ), and aprotinin (0.5  $\mu\text{g}/\text{ml}$ ) were added to the mixture along with 1.1 mM EDTA. Cells were disrupted using a Vibra cell sonicator. Insoluble debris was pelleted in 1.0- to 1.5-ml aliquots at  $100,000 \times g$  for 5 min with a Beckman TL-100 refrigerated centrifuge. The pellet includes most nucleic acid and lipid. The clear supernatant was recovered and used for both carbonyl and enzyme assay.

**Oxidized Protein (Carbonyl) Assay.** The protein concentration of the soluble protein fractions was determined according to the Pierce BCA method. The protein carbonyl content was measured by first forming labeled protein hydrazones derivatives using 2,4-dinitrophenylhydrazide. These derivatives were sequentially extracted with 10% (vol/vol) trichloroacetic acid followed by treatment with ethanol/ethyl acetate, 1:1 (vol/vol) and reextraction with 10% trichloro-

Abbreviations: MFO, mixed-function oxygenation, as used here is a general term referring to both enzymatic and nonenzymatic (metal-catalyzed) oxidations involving activated oxygen species; AD, Alzheimer disease; AC, age-matched control(s); YC, young control(s); FP, frontal pole; OP, occipital pole.

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acetic acid. The resulting precipitate was dissolved in 6 M guanidine hydrochloride. The difference spectrum between a 2,4-dinitrophenylhydrazide-protein in guanidine hydrochloride and a guanidine hydrochloride-protein blank was used to calculate nmol of 2,4-dinitrophenylhydrazide incorporated per mg of protein. The reference absorptivity of 21.0  $\text{mM}^{-1}\text{cm}^{-1}$  for aliphatic hydrozones was used. Results are reported as a mean of triplicate blinded arrays for each sample.

**Enzyme Activity Assays.** Glutamine synthetase activity was determined according to the method of Rowe *et al.* (18) and Miller *et al.* (19). Corrections were made for nonspecific glutaminase activity by comparing total activity in the presence and absence of adenosine diphosphate and arsenate. Creatine kinase activity in tissue extracts was determined by using standard methods (ref. 20, pp. 55–56). All assays are performed as a blinded triplicate and averaged for each sample.

**Statistics.** We used a repeated measures analysis of variance for a between-groups (AD vs. AC vs. YC) and within-groups (FP vs. OP) comparison of means. A *P* value <0.05 was considered significant.

### RESULTS

Clinical and autopsy data are given in Table 1. The AD and AC groups were comparable in age. There was a difference in postmortem interval between AD ( $3.7 \pm 0.3$  hr) and the two control groups (AC,  $8 \pm 1.7$ ; YC,  $6.9 \pm 2.0$  hr). However, there was no correlation between postmortem interval and any of the protein measures (carbonyl level, glutamine synthetase activity, or creatine kinase activity). Within each group, there was no correlation between age and any protein measure when computed for FP and OP separately. In the AD group, there was no correlation between disease duration and any protein measure.

**Protein Oxidation.** There was a positive correlation between age and brain carbonyl content in OP ( $r^2 = 0.45$ ) and FP ( $r^2 = 0.62$ ) in the combined AC and YC groups. Semilogarithmic plots (Fig. 1) show that the carbonyl increase with age occurs at double the rate in the FP ( $2.03 \times 10^{-2} \cdot \text{yr}^{-1}$ ;  $r^2 = 0.70$ ) compared with the OP ( $0.95 \times 10^{-2} \cdot \text{yr}^{-1}$ ;  $r^2 = 0.49$ ). The difference in rates is significant ( $P < 0.01$ ). In both AD and AC groups, FP oxidation was higher than OP (Fig. 2; Table 2); there was no regional difference in protein oxidation in the YC. Carbonyl content in both the FP and OP was comparable between the AD and AC groups; but in both regions in AD and AC group the oxidized protein values were elevated in comparison with the YC group.

**Glutamine Synthetase and Creatine Kinase Activity.** In YC group there is no significant difference between the FP and OP in activities of either glutamine synthetase or creatine kinase ( $3.79 \pm 0.27$  and  $4.22 \pm 0.34$  for FP;  $5802 \pm 284$  and

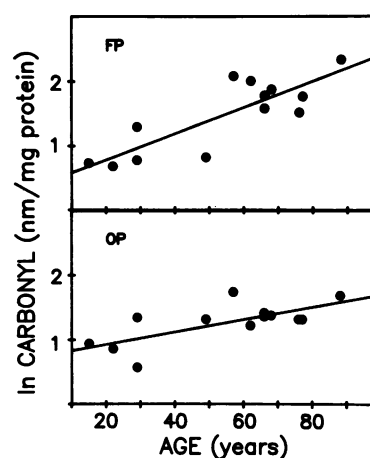


FIG. 1. Semilogarithmic regression plots of oxidized protein vs. subject age for all control samples (YC and AC groups). Slope of the plot representing rate of carbonyl accumulation in FP with age is approximately twice that in OP ( $P < 0.01$ ).

$5316 \pm 450$  units/mg of protein for OP, respectively). However, the levels of glutamine synthetase and creatine kinase in both FP and OP of the aged groups (AD, AC) were very much lower than in the YC group (Figs. 3 and 4; Tables 2 and 3). In both the AD and AC groups, the level of creatine kinase in the FP was slightly lower than in the OP.

There was no difference between the levels of glutamine synthetase activity in the FP and OP of the AC group. In contrast, the level of glutamine synthetase activity in the FP region of the AD group is appreciably lower than that in OP (Fig. 3). Glutamine synthetase activity in OP did not differ significantly between the AD and AC groups (Fig. 3).

The normalized ratio of glutamine synthetase to creatine kinase activity in each sample was analyzed for within-group and between-groups comparisons. Only the AD group showed a significantly decreased ratio in the FP vs. the OP ( $P < 0.001$ ). Between-groups comparisons by brain region did not yield statistically significant differences. A summary of significance values for all comparisons is given in Tables 2 and 3.

### DISCUSSION

**Aging.** Our general hypothesis is that the MFO of proteins and the failure of cells to remove these damaged proteins are important processes in normal aging. More specifically, aging may, in part, result from decreased activity in critical enzymes damaged by oxidation or failure to remove and replace these enzymes. That protein oxidation products accumulate with aging is demonstrated in this study by the marked difference in protein carbonyl content between young and old

Table 1. Characterization of study groups by number, age, postmortem interval, male-to-female ratio, and disease duration (for AD group)

Study group	<i>n</i>	Age, yr	PMI, hr	M/ F	Disease duration, yr
AD	16	$74.3 \pm 2.3$ (52–86)	$3.7 \pm 0.3$ (2–7.5)	13:5	$10.6 \pm 1.6$ (2–20)
AC	8	$69.9 \pm 3.1$ (57–88)	$8.0 \pm 1.7$ (3–16.5)	6:2	
YC	5	$28.8 \pm 5.7$ (15–49)	$6.9 \pm 2.0$ (3–12)	4:1	

Age, postmortem interval (PMI), and disease duration are reported as means  $\pm$  SEMs; ranges are given in parentheses. M/F, male-to-female ratio.

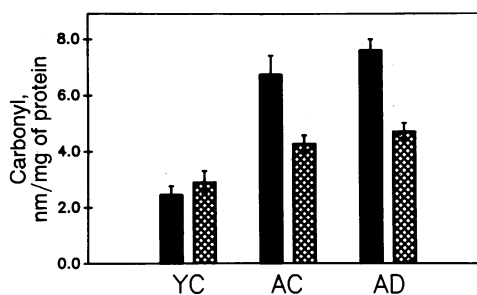


FIG. 2. Comparison of FP (■) and OP (▨) carbonyl content in AD, AC, and YC groups. There is marked increase in both frontal and occipital carbonyl in the two aged groups (AD, AC) compared with the YC group.

Table 2. Significance table for between groups (AD, AC, YC)

Comparison	FP, <i>P</i> value			OP, <i>P</i> value		
	Carbonyl	GS	CK	Carbonyl	GS	CK
AD vs. AC	0.0725	0.0036	0.0838	0.3606	0.6588	0.4868
AD vs. YC	0.0001	0.0001	0.0001	0.0025	0.0001	0.0002
AC vs. YC	0.0001	0.0001	0.0001	0.0296	0.0001	0.0001

GS, glutamine synthetase; CK, creatine kinase.

human brains. Increases in carbonyl content and associated enzyme activity loss with age have been observed in human circulating erythrocytes, cultured fibroblasts (16), and in an animal model of brain aging (21). There is ample evidence that the predominant mechanism of protein carbonyl formation is MFO (22). Moreover, there is evidence of free-radical-mediated MFO in the formation of protein carbonyl *in vitro* (23, 24), and *in vivo* (25, 26).

If it is granted that protein carbonyl MFO products accumulate in aging brain, is this change significant? Oxidative modification of an enzyme, for example, does not necessarily imply loss of function; the modification can conceivably occur in a region of the enzyme remote from catalytic or regulatory sites, with trivial direct functional effects. Even were oxidative modification to affect enzyme function, this alteration could be compensated for by mechanisms of increased synthesis or up-regulation of activity. On the other hand, loss of a function such as enzyme activity is a reasonable index of a significant aging effect. Our study demonstrates such a loss of critical enzyme activity. The potential for MFO to produce significant aging effects may lie in the site-specific nature of the oxidative modifications, occurring at functional sites—e.g., adjacent to the metal-containing catalytic site of glutamine synthetase (23). These oxidations, therefore, can selectively strike regions concerned with critical functions. Our data show a loss of glutamine synthetase- and creatine kinase-specific activity that parallels the regional increases in carbonyl content. In the present study, enzyme activities were lowest in the FP in the AC group, where carbonyl content is greatest. There were no regional differences in enzyme activities in the YC group. Further research is needed to show whether the observed decreases are due to oxidation alone or possibly other age-related factors.

**AD.** AD is the most common dementing disease of late life, affecting  $\approx 4$  million individuals in the United States. A striking feature is the logarithmic age-specific increase in prevalence with age, reaching as high as 47% of the population over 85 yr (27). Our hypothesis is that AD is a consequence of the inability to compensate for MFO of proteins, one of the normal processes of aging, thus linking the pathophysiology of the disease directly to the aging process.

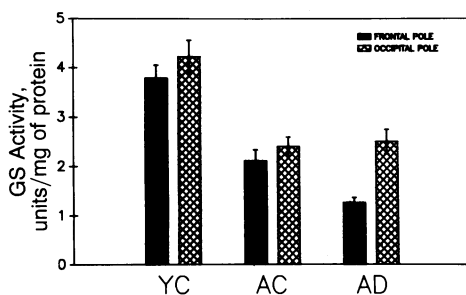


FIG. 3. Comparison of FP and OP glutamine synthetase (GS) activity in AD, AC, and YC. Corresponding regions have markedly decreased glutamine synthetase activity in the two aged groups (AD, AC) compared with the YC group. Frontal glutamine synthetase activity is significantly decreased in AD compared with AC.

A corollary of this hypothesis is that critical enzymatic functions are lost as a result of MFO.

We have observed that frontal cortical glutamine synthetase activity is significantly decreased in AD relative to age-matched controls. More importantly, there is a regional loss of glutamine synthetase activity in the FP. The loss of glutamine synthetase activity may significantly impair a number of neuronal and nonneuronal systems. Glutamine synthetase is a key enzyme in cellular nitrogen regulation (28) and is subject to a complex system of feedback controls (29). Decreased uptake of glutamate due to decreased glutamine synthetase activity could result in neurotoxic effects (30) of abnormally prolonged *N*-methyl-D-aspartate receptor activation (31) despite normal potassium-mediated release of this compound (32). Glutamine synthetase is critically involved in the regulation of intracellular ammonia and maintenance of cellular acid-base balance (33). Changes in intracellular buffering capacity for either ammonia or pH could have lethal consequences for selected populations of neurons. For example, decrease in intracellular pH has been proposed to delocalize intracellular iron, which can eventually result in production of oxygen free radicals and further protein oxidation (34).

In contrast to glutamine synthetase, although FP creatine kinase is reduced in both AD and AC groups, it does not distinguish between the two conditions. Thus, there is a differential loss of glutamine synthetase activity in FP in AD. This difference may be due to characteristic regional neuropathology: the homotypical cortex of the association areas of the frontal lobes are severely involved in AD, whereas motor, somatosensory, and primary visual areas are minimally affected (35). The OP specimens used in this study were from Brodmann area 17 of the visual cortex.

Neuronal degeneration in FP is unlikely to account for the differential glutamine synthetase activity loss because a substantial portion of glutamine synthetase activity is found in glial cells. Furthermore, total frontal lobe glutamine synthetase, as determined by radial immuno-diffusion, is not different in AD than in their AC (36). However, this immuno-diffusion study does not distinguish between active and inactivated enzyme. The demonstration of significantly decreased glutamine synthetase activity in AD supports the hypothesis of an accumulation of oxidized (inactivated) glutamine synthetase. A nonspecific or generalized loss of

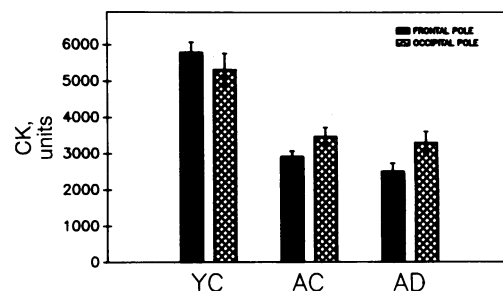


FIG. 4. FP and OP creatine kinase (CK) activity compared in AD, AC, and YC groups. CK activity is significantly reduced in the two aged groups (AD, AC). The two aged groups do not differ in creatine kinase activity in either region.

Table 3. Significance table for within groups (FP vs. OP)

	P values		
	AD	AC	YC
Carbonyl	0.0001	0.0001	0.5121
Glutamine synthetase	0.0001	0.3576	0.2826
Creatine kinase	0.0003	0.0524	0.1641

Comparisons used repeated-measure analysis of variance.

enzyme activity has not been observed in AD [e.g., ribonuclease activity (37, 38) and oligopeptidase activities (39) are preserved]. We conclude that the loss of glutamine synthetase activity seen in the FP in AD is best accounted for by the known vulnerability of glutamine synthetase to MFO (23). This defect could be due to (i) excess generation of free radicals or other oxidant species (40); (ii) decrease in intracellular protectants—e.g., free-radical scavenger superoxide dismutase, catalase, and glutathione peroxidase (41–43); (iii) abnormal regulation of protein synthesis, or (iv) decreased activity of specific proteases that attack oxidized enzymes (1). Further work is needed to confirm that excess oxidative modification of protein is functionally important in aging and that uncompensated oxidative modification is characteristic of AD.

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