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**Original Contribution**

APOLIPOPROTEIN E DEFICIENCY PROMOTES INCREASED OXIDATIVE STRESS AND COMPENSATORY INCREASES IN ANTIOXIDANTS IN BRAIN TISSUE

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Abstract—The epsilon 4 allele of the apolipoprotein E gene (ApoE) is associated with Alzheimer's disease (AD). The extent of oxidative damage in AD brains correlates with the presence of the E4 allele of ApoE, suggesting an association between the ApoE4 genotype and oxygen-mediated damage in AD. We tested this hypothesis by subjecting normal and transgenic mice lacking ApoE to oxidative stress by folate deprivation and/or excess dietary iron. Brain tissue of ApoE-deficient mice displayed increased glutathione and antioxidant levels, consistent with attempts to compensate for the lack of ApoE. Folate deprivation and iron challenge individually increased glutathione and antioxidant levels in both normal and ApoE-deficient brain tissue. However, combined treatment with folate deprivation and dietary iron depleted antioxidant capacity and induced oxidative damage in ApoE-deficient brains despite increased glutathione, indicating an inability to compensate for the lack of ApoE under these conditions. These data support the hypothesis that ApoE deficiency is associated with oxidative damage, and demonstrate a combinatorial influence of genetic predisposition, dietary deficiency, and oxidative stress on oxidative damage relevant to AD. © 2002 Elsevier Science Inc.

Keywords—Free radicals,

INTRODUCTION

The epsilon 4 allele of the apolipoprotein E gene (ApoE) is linked with an increase in, and an earlier age of onset, of sporadic and familial Alzheimer's disease (AD) [1]. Oxidative damage is elevated in the frontal cortex of AD patients, and the extent of this damage correlates with the presence of the E4 allele of ApoE; lipid peroxidation was significantly elevated in AD cases homozygous for the ApoE4 allele vs. age-matched controls or AD cases homozygous for E3 [2]. In addition, activities of enzymatic antioxidants including catalase and glutathione peroxidase were also higher in AD cases with at least one epsilon 4 allele of ApoE, while superoxide dismutase activity was unchanged [2]. Supplementation with certain agents such as Ginkgo biloba extract and the neu-

rosteroid dehydroepiandrosterone were able to protect control individuals and AD cases bearing one E4 allele and an E3 allele against lipid peroxidation, but were unable to protect E4 homozygotes. These findings prompted Ramassamy and colleagues [2] to advance the hypothesis that the ApoE4 genotype and reactive oxygen-mediated damage are linked in the frontal cortex of AD patients.

Studies with transgenic mice lacking ApoE suggest that deficiencies in this protein may lead to synaptic loss and cytoskeletal compromise [3]. ApoE-deficient mice also demonstrate increased susceptibility to lipid peroxidation under conditions that promote oxidative stress [4]. Since ApoE is normally secreted by astrocytes following neuronal injury to redistribute lipid breakdown products [5–8], deficiency in ApoE function may be crucial following accumulation of reactive oxygen species (ROS) and resultant membrane compromise [9]. Increased levels of glutathione are present in the central nervous system (CNS) of ApoE-deficient mice, which may represent an attempt to

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compensate for increased ROS due to diminished ApoE function [9].

We tested herein the hypothesis [2] that deficiencies in ApoE function are associated with increased oxidative stress in CNS. This was carried out by comparing the responses of transgenic mice lacking ApoE with those of normal mice of the identical genetic background to dietary oxidative stress induced by folate deprivation [10–12] and by inclusion of excess iron within their diet [13].

MATERIALS AND METHODS

Normal C57Bl/6J mice and ApoE^{tm1Une} homozygous knockout mice on a C57Bl/6J background (Jackson Laboratories, Bar Harbor, ME, USA) [14] were maintained for 1 month on a basal diet (AIN-76, Purina, St. Louis, MO, USA) [14,15] with and without folic acid (2 mg/500 g total diet weight) and/or 8% fish oil, 2% corn oil, and iron (as ferric citrate; 4 g/500 g total wet weight of AIN-76 basal diet mixture; diet and water ad libitum). As in our prior studies, substitution of this amount of oil for water in the absence of iron did not influence oxidative damage [16,17] or CNS antioxidant levels (not shown). Total brain tissue was harvested and frozen at -80°C until use. We utilized relatively short-term (1 month) studies since this period has previously been demonstrated to be sufficient to induce oxidative damage [13, 16,17], and to avoid potential systemic damage as well as the overall age-related neurodegeneration described for ApoE $-/-$ mice [18].

Glutathione levels were quantified in homogenates of CNS according to Araki and Sako [19] with modifications. Homogenates (100 μl) were reduced by mixing with 30 μl of 30 μM cystamine (as an internal standard) and 10 μl of tri-carboxyethylphosphine (100 mg/ml in 0.05 M HCl). Samples were vortexed, incubated at room temperature for 10 min, then centrifuged at $10,000 \times g$ for 10 min. Eighty microliters of the resulting supernatant were combined with 160 μl of 2 M boric acid/4 mM EDTA (pH 10.5), followed by 80 μl of 1.0 mg/ml SBDF [7-fluorbenzo-2-oxa-1,3-diazide-4-sulfonate] in boric acid/EDTA buffer. Samples are mixed, incubated for 60 min at 60°C , equilibrated to reach temperature, and 50 μl was injected into a Hewlett Packard model 1090 HPLC used with a 1046A fluorescence detector and a Hewlett Packard 4.6×60 mm high-speed analytical column (Hewlett Packard, Palo Alto, CA, USA) packed with 3 μM ODs (C18) Hypersil silica. The mobile phase consisted of methanol/0.1 M phosphate buffer pH 2.0 (2/98 by volume). Glutathione concentrations were then determined by comparison of peak height ratios of glutathione to the internal standard cystamine in the sample compared to peak heights obtained for a cystamine standard curve. Samples were derived from three to four normal

and three to four ApoE $-/-$ mice for each dietary condition from two or more independent experiments (total $n \geq 6$ for each diet for both experiments).

Oxidative damage in homogenates of CNS tissue was quantified by analysis of thiobarbituric acid-reactive substances (TBARs) as an index of endpoint oxidative damage by standard methodologies previously used to analyze oxidative damage in brain tissue in AD [20,21] and in brain tissue of mice during normal aging [22] and of the ApoE $-/-$ mice used herein [2,16,17]. Briefly, CNS homogenates containing 50 μg of total protein were mixed with 1 μM copper sulfate in 5 mM HEPES buffer (total volume 400 μl). Samples then received 1 ml of a 0.375% TBA/15% trichloroacetic acid in 0.25 N HCl, incubated for 30 min at 90°C , and clarified by centrifugation (1500 rpm for 10 min). The resulting supernatants were aspirated and fluorescence quantified in a fluorescent spectrophotometer (excitation 520 nm, emission 553 nm) by comparison with a standard curve of tetramethoxypropane in HCl. Samples were derived from three to four normal and three to four ApoE $-/-$ mice for each dietary condition, from three to four separate experiments (total $n \geq 12$ for each diet for all three experiments).

Total antioxidant capacity was quantified in homogenates of CNS tissue from normal and ApoE $-/-$ mice following the above dietary regimen according to Miller *et al.* [23]. Briefly, CNS samples (normalized for total protein) were diluted with 1.5 volumes of phosphate-buffered saline containing 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) and the chromogenic peroxidase metmyoglobin in the presence of hydrogen peroxide. This generates the radical cation ABTS⁺ and resultant absorbance was read at 600 nm. The degree of quenching of radical generation in individual samples, indicative of the presence of antioxidant activity, was quantified by comparison with a standard curve of 1 mM Trolox and expressed as “Trolox equivalent antioxidant capacity.” Samples were derived from three to four normal and three to four ApoE $-/-$ mice for each dietary condition, from two independent experiments (total $n \geq 6$ for each diet for both experiments).

RESULTS

Consistent with prior studies [9], CNS of ApoE-deficient mice demonstrated an approximate 20% increase in glutathione levels over levels found in CNS of normal mice (Fig. 1). It has been suggested that the increased glutathione found in CNS of ApoE-deficient mice represents an attempt to compensate for increased ROS due to diminished ApoE function [9]. We reasoned that, if this were the case, ApoE-deficient mice may respond to additional oxidative stress by further increasing glutathi-

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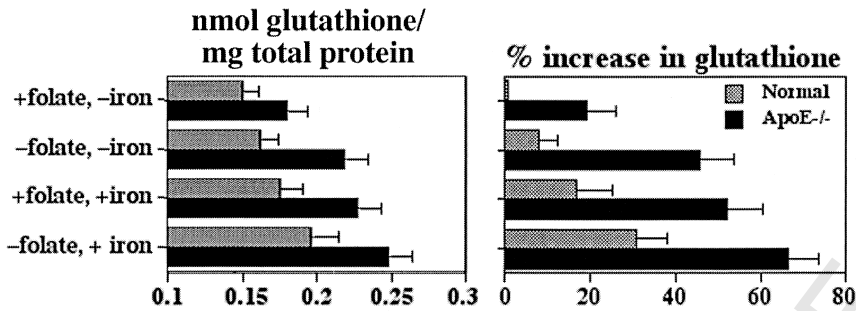


Fig. 1. Glutathione levels in CNS of normal and ApoE^{-/-} mice under various dietary regimens. Normal and ApoE^{-/-} mice received a basal diet containing or lacking iron and folic acid for 1 month. Total brain tissue was harvested and analyzed for total glutathione by HPLC as described in Materials and Methods. Values represent the mean \pm SEM nmol glutathione/mg total protein (left panel) or the mean percentage increase vs. \pm SEM in glutathione under the indicated dietary regimen vs. normal mice receiving folate but no iron. Data are compiled from two or more independent experiments; $n = 3-4$ mice per diet per experiment.

one beyond those observed for normal mice subjected to the same oxidative stress. To test this possibility, we therefore subjected normal and ApoE-deficient mice to folate deprivation and addition of excess iron to their diet, each of which induces oxidative stress [11,13]. Individually, deprivation of folate and dietary iron each increased glutathione levels in CNS of both normal and ApoE-deficient mice. In combination, folate deprivation with dietary iron further increased glutathione levels in both normal and ApoE-deficient mice. Notably, however, CNS of ApoE-deficient mice displayed a markedly greater increase than did CNS of normal mice under all conditions tested (Fig. 1).

We next attempted to determine whether or not the above increases in glutathione levels, and in particular the consistently higher levels observed in ApoE-deficient mice, indeed represented attempts to compensate for oxidative stress, and whether or not any such compensation was effective. To accomplish this, we analyzed TBARs in CNS of these mice as an overall endpoint index of oxidative damage [2,17,20]. Normal mice exhibited no significant difference in TBARs following iron challenge with or without folate (10.9 ± 6 vs. 12.3 ± 4.6 μmol TBARs/mg total protein with and without folate, respectively, in the absence of iron; 12.1 ± 2.2 vs. 18.4 ± 5 μmol /mg total protein with and without folate, respectively, in the presence of iron). Similarly, CNS of ApoE-deficient mice displayed statistically identical levels of TBARs with or without folate deprivation in the absence of iron (15.5 ± 6 vs. 12.2 ± 1 μmol /mg total protein, respectively). An apparent trend toward increased TBARs was observed in CNS of ApoE-deficient mice receiving dietary iron in the presence of folate (21.5 ± 6.8 μmol /mg total protein), but these values did not differ statistically ($p < .166$; Student's *t*-test) from those obtained for normal mice receiving folate in the absence of iron (above). However, ApoE-deficient mice displayed statistically increased

TBARs when challenged with dietary iron in the absence of folate (44.5 ± 5 μmol /mg total protein, $p < .05$ vs. all other conditions). These findings demonstrate that ApoE-deficient mice are less capable of buffering oxidative challenge than are normal mice. These findings also suggest that the increased levels of glutathione observed in CNS of ApoE-deficient mice following dietary challenge with iron and folate deficiency were incapable of compensating for the lack of ApoE function.

To test whether or not we had overwhelmed the endogenous antioxidant capacity of ApoE-deficient mice, we compared the total antioxidant activity in CNS homogenates in a cell-free assay. In the absence of any dietary challenge, CNS of ApoE^{-/-} mice exhibited a significantly increased oxidative buffering capacity vs. that of normal mice (0.79 ± 0.08 nmol Trolox equivalents/L of ApoE^{-/-} CNS homogenate vs. 0.53 ± 0.06 nmol/l of normal CNS homogenate; $p < .05$, Student's *t*-test), indicating that the lack of ApoE activity fostered an increase in one or more endogenous antioxidants. Folate deprivation fostered an additional increase in antioxidant in normal CNS (0.65 ± 0.09 nmol/l CNS homogenate). Challenge with dietary iron in the presence of folate further increased oxidative buffering capacity in both normal ($0.57 \pm .034$ nmol Trolox equivalents/L CNS homogenate) and ApoE^{-/-} mice (0.78 ± 0.1 nmol/l CNS homogenate), indicating endogenous antioxidants were upregulated in CNS of both mouse strains in response to oxidative stress. Combined treatment with dietary iron in the absence of folate fostered an even greater increase in antioxidant capacity in normal CNS (0.69 ± 0.25 nmol/l CNS homogenate). However, ApoE-deficient mice receiving iron in the absence of folate displayed less CNS antioxidant capacity than under any other condition for these mice (0.67 ± 0.14 nmol/l CNS homogenate). These findings suggest that the combined influence of iron challenge and folate deprivation depleted the antioxidant capacity of ApoE-

deficient mice. These data support the hypothesis that ApoE deficiency is associated with increased oxidative stress [2].

DISCUSSION

The findings of this present study, along with those of Huang and colleagues [9], provide experimental evidence in supporting the association of increased oxidative stress with the ApoE genotype in AD [2,24]. They extend the findings of prior studies by demonstrating that increased oxidative stress resulting from ApoE deficiency can be enhanced by dietary deficiencies and oxidative challenge [16,17].

ApoE-deficient mice displayed increased CNS glutathione vs. normal CNS, and these levels increased even further in response to oxidative stress. Glutathione levels, and enzymes responsible for glutathione generation (e.g., cystathionine β -synthase), are increased under conditions of oxidative stress [25]. Moreover, experimental elevations in glutathione in AD brain were capable of reducing oxidative damage [26]. Similarly, overexpression of glutathione peroxidase increased the resistance of neuronal cells to amyloid- β neurotoxicity [27]. However, the increases in glutathione and overall antioxidant levels were nevertheless incapable of compensating completely for the absence of ApoE function, as evidenced by a selective increase in oxidative damage ApoE-deficient, but not normal, CNS tissue following iron challenge in the absence of folate. These data are consistent with prior indications that oxidative stress can overwhelm the ability of glutathione to quench ROS [28].

Folate deficiency has long been implicated in cardiovascular disorders; only recently has it become apparent that folate deficiency also contributes to neurological and psychological disorders including dementia, impaired cognition, depression, psychosis, and AD [29–34]. Low serum folate levels are strongly associated with atrophy of the cerebral cortex [35]. In addition, while levels of folate in cerebral spinal fluid are normally 3- to 4-fold higher than in blood, spinal fluid levels of folate are significantly lower in AD patients [36]. Folate supplementation has further been shown to reverse dementia in some cases [37]. Folate deficiency mediates its neurotoxicity in part by increasing levels of homocysteine [38], a nonprotein amino acid that overstimulates NMDA receptors, potentiates glutamate and amyloid- β neurotoxicity, and induces DNA breakage and lipid peroxidation [10,12,39,40]. Folate deficiency and resultant homocysteine accumulation compromise glutathione peroxidase activity [11,41], and reduced tissue levels of vitamins C, E, and A [42]. Recently, folate deficiency has been shown to elevate homocysteine and place neu-

rons at risk in mouse models of AD and Parkinson's disease [43–45]. Our findings indicate that, while folate deficiency increases glutathione levels and oxidative buffering capacity in normal mice, it has a substantially more profound effect on these parameters in the absence of ApoE function. These findings point toward an interplay of nutritional deficiencies and genetic predisposition to oxidative stress [46–48].

The full range of factors contributing to the onset and development of AD has not been resolved but is likely to be broad. Contributing factors include genetic predisposition [1], Abeta accumulation [49], oxidative stress [50–52], deficiencies in endogenous antioxidants [53], calcium mismetabolism and excitotoxicity [49,54], energy depletion [55–59], and nutritional compromises [16,17, 60–64]. The data of the present study extend the prior demonstration of profound interrelated effects of genetic predisposition, dietary deficiency, and oxidative stress on CNS oxidative damage relevant to AD [16]. Notably, the E4 allele of ApoE exhibits approximately 60%, rather than complete, penetrance in AD [1], leaving open the possibility that additional factors beyond the ApoE genotype influence the nature and progression of AD. The findings of our study, along with those of Duan *et al.* [43,44], suggest that deficiencies in folate metabolism may augment neurodegeneration, especially in combination with other deleterious conditions such as diminished ApoE function. In addition, while we used dietary iron as a generic pro-oxidant [13,15], it has been speculated that iron may be a contributing factor in AD [65]. Finally, while our findings support the notion that compromised nutrition, oxidative stress, and genetic predisposition may exert a combined deleterious influence on neuronal health, we tested the influence of a complete lack of folate (rather than a moderate reduction), coupled with a complete absence of ApoE activity (rather than the reduced activity characteristic of ApoE4 vs. other alleles). Accordingly, establishing the nature and extent of any link between nutritional compromise (including that of folate deficiency) and genetic predisposition to AD is likely to be difficult [64].

Nevertheless, the results of this study demonstrate that genetic and nutritional deficiencies can exert a combinatorial influence on neurodegeneration, especially under conditions that promote oxidative stress, and therefore suggest that further consideration of the combined role of nutritional and genetic compromise in the onset and progression of AD are warranted. Our studies have been confined to endpoint analyses of oxidative damage and antioxidant potential; it would be of interest to determine which particular oxidative species are generated during the course of oxidative challenge, whether or not a distinct profile or sequence of oxidative species are generated following oxidative challenge in ApoE-defi-

cient vs. normal mice, and which particular antioxidants are generated in response to oxidative challenge.

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