

Research report

# Folate quenches oxidative damage in brains of apolipoprotein E-deficient mice: augmentation by vitamin E

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## Abstract

We demonstrate that folate and vitamin E can compensate for the diminished oxidative buffering capacity of brains of apolipoprotein E-deficient mice. Normal and ApoE<sup>tm1U<sup>ne</sup></sup> homozygous ‘knockout’ mice were maintained for 1 month on a diet either lacking or supplemented with folate, vitamin E or iron as a pro-oxidant after which brain tissue was harvested and analyzed for thiobarbituric acid-reactive substances (TBARs) as an index of oxidative damage. Normal mice exhibited no significant difference in TBARs following iron challenge in the presence or absence of vitamin E, folic acid or both. Similarly, ApoE knockout mice exhibited no significant differences following dietary iron challenge in the presence or absence of vitamin E. However, ApoE knockout mice accumulated significantly increased TBARs following iron challenge when folic acid was withheld, and accumulated even more TBARs when both folic acid and vitamin E were withheld. These findings demonstrate that ApoE knockout mice during vitamin deficiency are less capable of buffering the consequences of dietary iron challenge than are normal mice. Since the apolipoprotein E4 allele, which exhibits diminished oxidative buffering capacity, is linked to Alzheimer’s disease (AD), these data underscore the possibility that critical nutritional deficiencies may modulate the impact of genetic compromise on neurodegeneration in AD.

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## 1. Introduction

Oxidative damage is at the core of neuronal degeneration in Alzheimer’s disease (AD) [21,23,40]. Oxidative damage initiates at the membrane following exposure to amyloid-beta, encompasses cytoskeletal protein and nucleic acid damage, and ultimately extends to regions beyond the amyloid-beta lesions themselves, indicating the presence of generalized oxidative damage [13,16,24,40,49]. Restoring or maintaining oxidative buffering capacity therefore represents one useful therapeutic approach to minimize neurodegeneration.

Apolipoprotein E (ApoE) is a cholesterol transport protein that is important in development and regeneration of the nervous system. The ApoE 4 allele is associated with a 3–4-fold increase in the prevalence of AD and with a younger mean age of onset [7], although the precise mechanisms underlying this association are not fully

resolved. Transgenic mice lacking ApoE demonstrate synaptic loss and cytoskeletal compromise [18]. Since ApoE normally redistributes lipid breakdown products [11,28,29,41], such deficiencies may be crucial under conditions of oxidative stress and resultant membrane compromise [8,9,31,32]. ApoE deficiency may increase the levels of free iron, which may trigger production of reactive oxygen species (ROS) [17].

Recent studies with ApoE-deficient mice demonstrated an increase in oxidative damage and a decrease in vitamin E levels [33], while vitamin E supplementation has been reported to reduce oxidative damage in these mice [47]. To examine more closely whether or not the deficiency in vitamin E contributed to increased oxidative damage in these mice, we compared the impact of deficiency in dietary vitamin E on the oxidative buffering capacity of brain tissue of ApoE-deficient mice versus normal mice. We also tested the impact of deficiency of dietary folate, which has also been correlated with neurodegeneration [36,37,41,45] and can buffer CNS oxidative damage [44]. Folate is required to control levels of the non-protein amino acid homocysteine (HC) (e.g. Ref. [27]), a potent

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neurotoxin that also potentiates beta-amyloid and glutamate neurotoxicity [9,14,45] and is itself associated with AD [35,36].

## 2. Materials and methods

Normal C57Bl/6J mice and ApoE<sup>tm1Une</sup> homozygous 'knockout' mice on a C57Bl/6J background ([26]; obtained from Jackson Laboratories, Bar Harbor, ME) received a vitamin-free, basal diet ('AIN-76'; Purina/Mother Hubbard, Inc, Richmond, IN.; [10,46]) lacking or supplemented with folic acid (4 mg/kg total diet wet weight, vitamin E (as alpha-tocopherol, 50 IU/kg), and/or a mixture of fish and corn oil (8 and 2% of the total diet wet weight, respectively) and iron (as ferric citrate; 4 g/500 g total diet wet weight) for 1 month (all additives obtained from Sigma–Aldrich, after which total central nervous system (CNS) tissue was harvested, homogenized, normalized according to total protein and aliquots of unfractionated homogenates were analyzed for thiobarbituric acid-reactive substances (TBARs) as an end-point index of oxidative damage by standard methodologies previously utilized to analyze CNS in AD [17] and in ApoE-knockout mice [33]. Mice were between 10 and 14 months of age. Data were pooled from four separate experiments, each of which contained three to four mice of mixed gender per diet; in certain experiments, genders were considered separately.

HC was monitored in plasma according to Araki and Sako [2] with modifications. Aliquots of plasma (100  $\mu$ l) were combined with 30  $\mu$ l of 30  $\mu$ M cystamine (as an internal standard) and 10  $\mu$ l 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 *g* for 10 min. An 80  $\mu$ l volume of the resulting supernatant were combined with 160  $\mu$ l of 2 M boric acid containing 4 mM EDTA (pH10.5), followed by 80  $\mu$ l of 1.0 mg/ml SBDF [7-fluorbenzo-2-oxa-1,3-diazide-4-sulfonate] in the same buffer. Samples were incubated for 1 h at 60 °C, then were injected (50  $\mu$ l) into a Hewlett-Packard model 1090 HPLC equipped with a model 1046A fluorescence detector and a Hewlett-Packard 4.6 $\times$ 60 mm high-speed analytical column packed with 3  $\mu$ M ODs (C18) Hypersil silica. The isocratic mobile phase consisted of 2 volumes of methanol/98 volumes of 0.1 M phosphate buffer (pH 2.0). HC concentrations were then determined by comparison of peak height ratios to the cystamine internal standard.

## 3. Results and discussion

To test the efficacy of 1 month of folate deprivation, we quantified plasma HC. HPLC analysis of plasma demonstrated significantly ( $P<0.05$ ) increased in HC levels

following 1 month deprivation of dietary folate in the above normal mice: mice receiving folate had  $7.7\pm 1.4$   $\mu$ M plasma HC, mice deprived of folate had  $11.1\pm 1.8$   $\mu$ M; mean $\pm$ S.E.M.;  $n=3$  mice each). Since HC is continuously eliminated from plasma via liver and kidney metabolism and clearance [44], this increase represents a 43% net steady-state increase in HC; CNS tissue is therefore constantly bathed with markedly increased HC levels.

CNS of normal mice did not contain increased TBARs following iron challenge in the presence or absence of folate and/or vitamin E. ApoE knockout mice also did not display an increase in CNS TBARs in the presence of folate, nor in the absence of vitamin E, following iron challenge. However, ApoE knockout mice deprived of folate demonstrated significantly increased TBARs when challenged with iron; deprivation of both folate and vitamin E increased TBARs even further in these mice (Fig. 1; Table 2). These data indicate that vitamin E was not effective in the absence of folate, but did augment the ability of folate to quench CNS TBARs in ApoE knockout mice (Fig. 1). Since vitamin E is lipophilic, the inability of vitamin E to prevent oxidative damage in ApoE knockout mice deprived of folate may reflect the inability of vitamin E to quench cytosolic products derived from lipid peroxidation [12,22,25,43]. These considerations allow speculation as to why vitamin E demonstrated only limited efficacy in clinical trials [35]. In addition, our data reveal that folate deficiency for as little as 1 month resulted in significant oxidative damage in ApoE knockout mice, while deficiency of vitamin E alone for this period did not. Prolonged vitamin E deficiency (9–12 months) does result in oxidative damage in these mice [30,47]. While vitamin E is clearly essential, these comparisons suggest folate deprivation may have a more severe impact on CNS neurodegeneration than vitamin E deprivation.

The dietary regimen utilized herein specifies replacement of a portion of the water utilized to dissolve the basal diet powder with fish and corn oil to achieve a pro-oxidant state along with iron (above); no significant differences were observed in the presence or absence of folate and vitamin E for TBARs in CNS of either normal or ApoE knockout mice in the presence or absence of oil (Table 1), indicating that none of the changes observed following inclusion of dietary iron were derived from oil content alone but rather were due to the pro-oxidant state generated by dietary iron in the presence of oil [10]. The markedly differential TBARs observed among diets containing and lacking folate and vitamin E in ApoE knockout mice further demonstrate that TBARs are not derived simply by the presence of this level of dietary iron. The possibility remained that TBARs measured in CNS could be derived in part from residual blood within CNS vasculature. While this potential artifact could be addressed by perfusion of CNS tissue prior to sacrifice, we were concerned, however, about the possible induction of additional oxidative stress during the perfusion procedure.

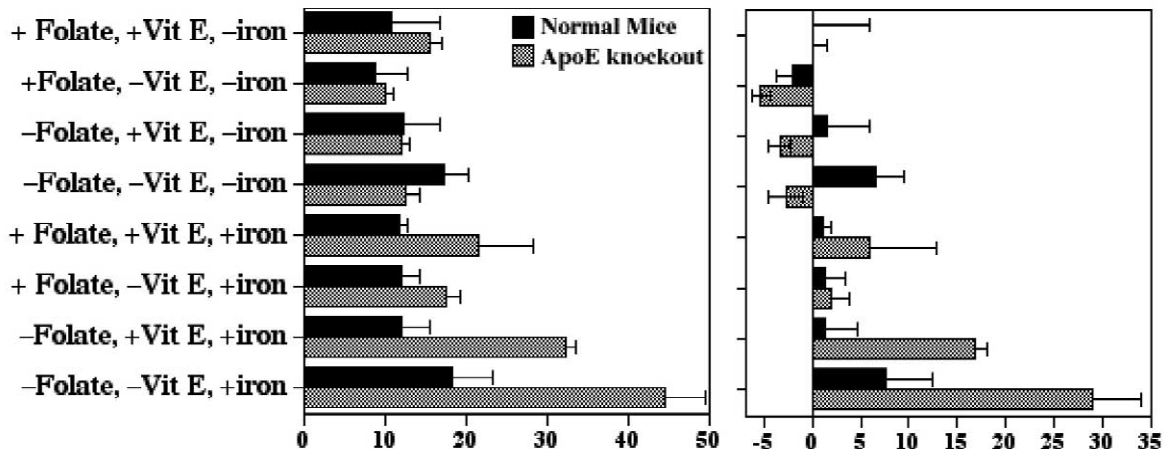


Fig. 1. TBARs in normal and ApoE-knockout mice following dietary challenge with iron in the presence and absence of folate and vitamin E. Normal and ApoE ‘knockout’ mice received a basal diet (‘AIN-76’; Purina)±8% fish oil+2% corn oil, iron (as ferric citrate; 4 g/500 g total wet weight of AIN-76 basal diet mixture), folic acid (2 mg/500 g total diet weight) and vitamin E (as alpha-tocopherol oil; Sigma; 1 g/kg total diet weight)) for 1 month (diet and water ad libitum). Total brain tissue was harvested and analyzed for TBARs. The graph on the left presents total TBARs measured in each sample; the graph on the right presents values for all conditions after subtracting the value obtained for normal mice fed a complete diet (i.e. containing folate and vitamin E) in the absence of iron challenge, in order to better indicate the extent of increase above TBARs resulting from normal metabolism. Values represent the mean±standard error of the mean in μM/total mg protein compiled from three independent experiments, with *n*=3–4 for each diet per experiment (total *n*=12 for each diet for all three experiments).

To address this issue, we therefore instead quantified TBARs in liver tissue from the same mice (Table 3). TBARs were elevated in liver of both normal and ApoE knockout mice when challenged with iron in absence of folate and vitamin E, in contrast to the above data demonstrating that CNS TBARs were elevated only in ApoE knockout but not normal mice following iron challenge. These data indicate that the values observed in CNS tissue are unlikely to be derived from contaminating plasma, otherwise liver of ApoE knockout and normal mice should have demonstrated similar differences in TBAR levels as did CNS under various dietary conditions.

These findings demonstrate that ApoE knockout mice subjected to vitamin deficiency are less capable of buffering the consequences of oxidative challenge from dietary iron than are normal mice. The differences between CNS and liver also demonstrate that CNS tissue is unique. Notably, our data are derived from total CNS tissue, not

just ‘at-risk’ areas for oxidative damage and AD neurodegeneration; accordingly, the damage in key areas such as hippocampus and cortex are likely to be even more elevated following folate and vitamin E deprivation. This possibility is supported by a prior study utilizing TBAR analyses of hippocampus of ApoE-deficient mice, which demonstrated increased oxidative damage, coupled with reduced levels of vitamin E and reduced activity of superoxide dismutase [33]. TBARs represent an ‘end-point’ measurement of oxidative damage and has been utilized in analysis of oxidative damage in AD [17]; also of interest may be to monitor sequential oxidative damage

Table 1  
Dietary oil did not alter CNS TBARs

Diet				TBARs Mean±S.D.	P value vs. control*
Folate	Vitamin E	Water	Oil		
+	+	+	-	13.4±6.0	-
+	+	-	+	12.4±2.9	0.8
-	+	+	-	14.1±3.7	0.8
-	+	-	+	13.2±2.4	0.9
-	-	+	-	16.4±4.4	0.9
-	-	-	+	16.6±2.4	0.4

Mice (*n*=3 to 4 per group) were subjected to the indicated diets for 1 month, after which TBARs were quantified in CNS tissue. Values were compared by Student’s *t*-test. \* Control is defined as the diet containing folate, vitamin E and water.

Table 2  
Statistical analysis of oxidative damage in normal and ApoE-knockout mice following dietary challenge with iron in the presence and absence of folate and vitamin E

Diet			P values	
Folate	Vit. E	Iron	Normal mice	ApoE knockout mice
+	+	-	-	-
+	-	-	0.599	0.830
-	+	-	0.716	0.730
-	-	-	0.169	0.643
+	+	+	0.790	0.113
+	-	+	0.083	0.166
-	+	+	0.965	<b>0.000</b>
-	-	+	0.779	<b>0.002</b>

Mice were treated and tissue harvested as in Fig. 1 and Materials and methods. CNS tissue from mice receiving various diets to tissue was statistically compared to tissue from normal or ApoE knockout mice, respectively, receiving dietary folate and vitamin E, without iron. Statistically significant comparisons (*P*<0.05; Student’s *t*-test) are presented in boldface.

Table 3  
TBARs ( $\mu\text{M}$ ) from liver of normal and ApoE knockout mice

Diet			Regular mice	ApoE knockout
Folate	Vit. E	Iron	Mean	Mean
+	+	–	3.11 $\pm$ 0.7	4.0 $\pm$ 1.3
+	+	+	3.8 $\pm$ 0.6	4.3 $\pm$ 1.3
–	–	–	7.3 $\pm$ 5.0*	11.3 $\pm$ 4.5*
–	–	+	12.8 $\pm$ 4.5**	11.7 $\pm$ 4.8**

Mice ( $n=3$  or 4 per group) received basal diets containing or lacking folate, vitamin E and/or iron as indicated. Livers were homogenized, adjusted for total protein and TBARs quantified as described in Materials and methods.

\* Significantly different from the values obtained for mice receiving folate and vitamin E in the absence of iron ( $P<0.05$ , Student's  $t$ -test).

\*\* Significantly different from the identical diet in the absence of iron ( $P<0.05$ , Student's  $t$ -test).

to lipids, proteins and nucleic acids (e.g. Refs. [16,36,40,49]). Importantly, while TBARs provide an index of oxidative damage, we cannot distinguish whether or not increased TBARs are derived from increased generation of oxidative species, decreased elimination of oxidative species, or both. Given that increased TBARs are observed under certain conditions in ApoE knockout mice, which lack the normal functions of apolipoprotein E to re-distribute lipid breakdown products, it is reasonable to assume that at least some of the resulting increased TBARs are derived from failure to eliminate oxidative species.

Several limitations are inherent in the present analyses. For example, while TBARs represent a useful 'end point' analyses of oxidative damage, they can be derived from a combination of lipid, protein and DNA oxidative damage. The neuroprotective effect of vitamin E suggests that at least transient lipid peroxidation has occurred, but this has not been measured directly. It would be useful to conduct additional analyses to quantify the nature and extent of transient oxidative products that are generated under different dietary conditions utilized herein. In addition, we have examined total brain tissue, and not only do we lack information on distinct 'at-risk' and 'non-at-risk' brain regions, but we further have no clear indication of the relative contribution of neurons or glia to oxidative damage. Finally, we have no direct indication of these dietary regimens on potential compromise of cerebral vasculature, which could impact neuronal oxidative stress. However, we note that the distinct patterns observed between liver and brain argue against these data arising solely from vasculature complications.

Our findings demonstrate that a genetic deficiency as profound as a complete absence of ApoE can be alleviated by dietary measures. Similarly, a previous study has demonstrated that vitamin E supplementation can counteract spatial learning deficits in ApoE knockout mice [47]. It is therefore certainly reasonable to consider that a similar dietary regimen could compensate for the deficient antioxidant properties inherent in the ApoE 4 allele [6,22].

Our data and that of prior studies [30,47] may provide a partial explanation as to why certain ApoE alleles are associated with increased prevalence and earlier onset of AD, yet do not exhibit 100% penetrance [7].

It remains possible that additional latent genetic deficiencies related to AD, as well as other neuronal disorders involving oxidative stress, may manifest only when coupled with dietary compromise. For example, there are multiple variants of methylenetetrahydrofolate reductase (MTHFR, for which folate is an obligate cofactor [34,50]), deficiencies in which may compromise neuronal homeostasis even in the presence of what is considered sufficient dietary folate. However, no linkage was noted between one MTHFR polymorphism and ApoE4 in AD patients in a single population [4]. Errors in folate metabolism that may remain latent in isolation may be deleterious in the presence of additional genetic compromise. Further investigations of the interplay between nutrition and genetic compromise may provide insight into the etiology and pathogenesis of AD.

#### 4. For further reading

[1]; [3]; [5]; [15]; [19]; [20]; [38]; [39]; [42]; [48]

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