

# Increased Transcription and Activity of Glutathione Synthase in Response to Deficiencies in Folate, Vitamin E, and Apolipoprotein E

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Oxidative stress is a major contributing factor in neurodegeneration and can arise from dietary, environmental, and genetic sources. Here we examine the separate and combined impact of deprivation of folate and vitamin E, coupled with dietary iron as a prooxidant, on normal mice and transgenic mice lacking apolipoprotein E (ApoE<sup>-/-</sup> mice). Both mouse strains exhibited increased levels of glutathione when deprived of folate and vitamin E, but a substantial further increase was observed in ApoE<sup>-/-</sup> mice. To determine the mechanism(s) underlying this increase, we quantified transcription and activity of glutathione synthase (GS). Both normal and ApoE<sup>-/-</sup> mice demonstrated increased GS activity when deprived of folate and vitamin E. However, transcription was increased only in ApoE<sup>-/-</sup> mice deprived of folate and vitamin E. These findings demonstrate that deficiency in one gene can result in compensatory up-regulation in a second relevant gene and, furthermore, indicate that compensation for oxidative stress can occur in brain tissue at epigenetic and genetic levels depending on the nature and/or extent of oxidative stress.

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**Key words:** folate; vitamin E; oxidative stress; glutathione synthase; apolipoprotein E; Alzheimer's disease

Oxidative damage represents a pivotal contributing factor for the neurodegeneration that accompanies Alzheimer's disease (AD) and can arise from environmental, nutritional, or genetic compromise (for reviews see Berr, 2002; Butterfield et al., 2002; Floyd and Hensley, 2002; Perry et al., 2002a,b). One dietary source of oxidative stress is folate deficiency, which has recently been shown to contribute to many age-related neurological and psychological disorders, including dementia, impaired cogni-

tion, depression, psychosis, and Parkinson's disease as well AD (Mizrahi et al., 2003; for reviews see Mattson and Shea, 2002; Shea and Rogers, 2002a; Shea et al., 2002a). Folate deficiency increases neuronal oxidative stress by several mechanisms, including increasing levels of the neurotoxin homocysteine, levels of which are related to the progression and severity of AD (Postiglione et al., 2001), decreasing endogenous antioxidants, and inducing DNA damage and depleting energy reserves (Kruman et al., 2001; Ho et al., 2002, 2003).

Folate deprivation also potentiates the deleterious impact of certain other risk factors for AD, including amyloid beta, glutamate, metal ion neurotoxicity, and the presence of the E4 allele of apolipoprotein E (Ho et al., 2001, 2002, 2003; Kruman et al., 2000, 2002; Shea and Rogers, 2002b; Shea et al., 2002b,c; White et al., 2001), the latter of which is independently associated with oxidative damage in AD (Hayek et al., 1994; Lowell et al., 1995; Ramassamy et al., 1999). Transgenic mice lacking ApoE (ApoE<sup>-/-</sup> mice) exhibit increased oxidative stress and, therefore, are a useful model for assessing the impact of a genetic predisposition to oxidative stress on neurodegeneration and its modulation by folate deficiency (see, e.g., Ramassamy et al., 1999, 2000, 2001; Huang et al., 2000; Veinbergs et al., 2000; Shea and Rogers, 2002b; Shea et al., 2002b,c). We have demonstrated that ApoE<sup>-/-</sup> mice, but not normal mice, displayed oxidative damage to brain tissue within 1 month of folate depriva-

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tion. Vitamin E deprivation alone did not induce detectable oxidative damage in this short-term trial; however, simultaneous deprivation of vitamin E potentiated the effects of folate deprivation on oxidative damage (Shea and Rogers, 2002b; Shea et al., 2002b,c). Brain tissue of both normal and ApoE<sup>-/-</sup> mice contains increased levels of glutathione when deprived of folate in the presence of the prooxidant iron; however, ApoE<sup>-/-</sup> mice deprived of folate displayed a further increase in glutathione beyond that in normal mice (Shea et al., 2002b). These findings suggested that both dietary and genetic deficiencies may foster a compensatory increase in glutathione. In efforts to determine the mechanism(s) underlying these differential increases, we monitored transcription and activity of glutathione synthase (GS) and glutathione levels in brain tissue of normal and ApoE<sup>-/-</sup> mice deprived of folate and vitamin E while being subjected to iron-induced oxidative stress.

## MATERIALS AND METHODS

### Mice and Harvesting of Brain Tissue

Normal and ApoE<sup>-/-</sup> mice (Jackson Laboratories, Bar Harbor, ME; Piedrahita et al., 1992) were maintained for 1 month on a basal folate- and vitamin E-free chow ("AIN-76"; Purina/Mother Hubbard, Inc.; Shea and Rogers, 2002b; Shea et al., 2002b); chow and drinking water were provided ad libitum. For some groups, this basal diet was supplemented with folic acid (2 mg/kg total diet wet weight), vitamin E (as  $\alpha$ -tocopherol; 1 g/kg total diet wet weight), and iron (50 g/500 g total diet) as a prooxidant (Shea and Rogers, 2002b; Shea et al., 2002b,c). Our prior studies have confirmed that the presence of dietary iron alone does not increase oxidative damage in either normal or ApoE<sup>-/-</sup> mouse brain tissue provided that folate is present (Shea and Rogers, 2002b). For simplicity, supplementation with folic acid and vitamin E without iron is defined as the "complete diet"; treatment, with the prooxidant iron in the absence of folic acid and vitamin E was defined as the "deficient diet." Mice were sacrificed by cervical dislocation and brains rapidly removed. A portion (40–50 mg) of the frontal cortex was immediately frozen in liquid nitrogen, and the remainder of the brain was immersed in 50% glycerol and stored at  $-80^{\circ}\text{C}$  until use. We conducted relatively short-term (1 month) studies, because deprivation of folate for this period has previously been demonstrated to be sufficient to induce oxidative damage and a compensatory increase in glutathione (Shea and Rogers, 2002b; Shea et al., 2002b,c) yet may avoid potential systemic damage as well as the overall age-related neurodegeneration described for ApoE knockout mice (Pratico et al., 1999).

### RNA Extraction and RT-PCR

Total mRNA was extracted from frontal cortex by using an RNA isolation kit (Ambion, Inc.), which utilizes a modification of the method of Chomczynski and Sacchi (1987). Extracted mRNA was immediately stored at  $-20^{\circ}\text{C}$ .

Two different sets of forward and reverse primers: a short-fragment primer set (GSSFP1-GSSFP2; 5'-GAATGGAAGCTGCTTTGAGGCTGGG-3' and 5'-AGGCACTAGAACCTG-

CTGAAAG-3', respectively; product size 88 base pairs) and a long-fragment primer set (GSLFP1-GSLFP2; 5'-TTGCTGCTCCTAGCCACTTT-3' and 5'-GCCTCCTGAGTGAAGTCCAG-3', respectively; product size 391 base pairs) were used to amplify two different fragments flanking the 1,678–1,766 and 1,082–1,473 regions of the murine GS gene, respectively. To provide a normalization factor for different mice and different diets, the "housekeeping" gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified by using the primers GAPDHFP1-GAPDHFP2: 5'-ACCACAGTCCATGCCATCAC, and 5'-TCCACCACCCTGTTGCTGTA, respectively, flanking the 565–1017 region (product size 451 base pairs). Primer sequences were selected using the software Primer 3 available online at <http://www-genome.wi.mit.edu>. To verify the specificity of these sequences, all sequences were compared with GenBank by using the Blast algorithm. Standard PCR from genomic DNA confirmed the lack of nonspecific amplification of the GS short-fragment primers (not shown).

RT-PCR was performed with the Access RT-PCR system (Promega, Madison, WI), following the manufacturer's recommendations, with 2  $\mu\text{g}$  of purified mRNA (in 3  $\mu\text{l}$ ) and 0.5  $\mu\text{M}$  each of forward and reverse primers. This system allows reverse transcription of mRNA, followed by Tfl DNA polymerase-mediated amplification of the resultant cDNA in the same tube. Amplification was carried out for 35 cycles.

### Electrophoresis, Sequencing, and Densitometric Analysis of RT-PCR Products

RT-PCR products of the GS and GAPDH genes amplified using the above-described GSLFP1-GSLFP2 and GAPDHFP1-GAPDHFP2 primer sets (Invitrogen Life Technologies, La Jolla, CA) were electrophoresed on a 2% agarose gel (90 V for 1 hr), followed by visualization with 0.2  $\mu\text{g}/\text{ml}$  ethidium bromide for 30 min, which allowed determination of their molecular masses to be the anticipated respective sizes of 391 bp and 451 bp. Gels were stained in 0.2  $\mu\text{g}/\text{ml}$  ethidium bromide for 30 min and visualized under ultraviolet light by using a Gel Doc 2000 machine from Bio-Rad (Hercules, CA) and Quantity One software. The RT-PCR product amplified from the short-fragment GSSFP1-GSSFP2 primer set was electrophoresed on a 20% polyacrylamide Bis-acrylamide gel (300 V for 8 hr), which yielded a species that migrated at the anticipated size of 88 bp. The 391-bp and 451-bp RT-PCR products were eluted from the gel, cloned, and sequenced via dye-terminator chemistry with an Beckman/Coulter CEQ-200X sequencer, which confirmed their identity as fragments of the GS and GAPDH genes, respectively.

### Densitometric Analyses

The density of RT-PCR products of GS and GAPDH was determined by using NIH Image software (1.62). The values obtained for GS were normalized according to those obtained for the GAPDH housekeeping gene for the respective mouse according to the formula [(GS densitometric units/GADPH densitometric units)  $\cdot$  GS densitometric units]. Normalization was carried out for each mouse individually, and the mean and SEM were then determined for all normalized values; the total number of samples was three or four mice derived from

two independent experiments, for a total of seven or eight mice. Statistical analyses were carried out using Student's *t*-test and ANOVA. For calculations of percentage change in various parameters, mean values obtained for normal mice maintained on the complete diet were defined as 1, and the percentages by which all other groups differed from these "control" values were determined.

### GS Activity Assay and Quantification of Glutathione

GS activity in brain tissue was determined as described elsewhere (Volohonsky et al., 2002), with modifications. In brief, brain tissue preserved in 50% glycerol at  $-80^{\circ}\text{C}$  was rinsed with phosphate-buffered saline and homogenized in 50 mM Imidazole (pH 7.5) containing 10 mM  $\text{MgCl}_2$ , at a final protein concentration of 0.25 g/ml. Homogenates were centrifuged at (10,000*g* at  $4^{\circ}\text{C}$ , 30 min), and the resulting supernatant was incubated with 0.5 mM Acivicin for 1 hr at room temperature. Aliquots (100  $\mu\text{g}$  in 20  $\mu\text{l}$ ) were added to 180  $\mu\text{l}$  of 100 mM Tris-HCl (pH 8.0) containing 50 mM KCl, 20 mM  $\text{MgCl}_2$ , 2 mM EDTA, 10 mM ATP, 2.5 mM dithiothreitol, 500  $\mu\text{M}$  Acivicin, 5 mM glycine, and 5 mM  $\gamma$ -glutamyl-cysteine. Aliquots (60  $\mu\text{l}$ ) of the final reaction mix were combined with 90  $\mu\text{l}$  of 10% SSA and the rest incubated at  $37^{\circ}\text{C}$  for 30 min. At the end of the incubation, another 60  $\mu\text{l}$  were added to 40  $\mu\text{l}$  10% SSA. The acidified mixtures were centrifuged at 10,000*g* for 5 min, and the glutathione levels were determined by high-performance liquid chromatography (HPLC) as described elsewhere (Shea et al., 2002b) in the resulting supernatants after 0 and 30 min of incubation. All chemicals were from Sigma-Aldrich (St. Louis, MO).

## RESULTS

We have previously reported increased levels of glutathione in brain tissue of normal and ApoE $^{-/-}$  mice following folate deprivation and iron challenge (Shea et al., 2002b). Because vitamin E deficiency augments the impact of folate deficiency on brain tissue (Shea and Rogers, 2002b), we tested here the impact of combined deficiency in folate and vitamin E and the effect of these dietary deficiencies on the lack of ApoE while mice were subjected to dietary iron. Normal mice displayed and approximately 15% increase in glutathione in brain tissue when maintained on the deficient diet (i.e., in the absence of folate and vitamin E coupled with iron challenge) vs. levels in brains of mice maintained on the complete diet (containing folate and vitamin E and lacking iron). ApoE $^{-/-}$  mice maintained on the complete diet displayed an approximately 20% increase relative to normal mice on the complete diet. However, when ApoE $^{-/-}$  mice were maintained on the deficient diet, they displayed an approximate 36% increase. These data indicate that compensatory increases in glutathione can be caused by both dietary and genetic influences and, moreover, that the combined impact of both invokes an additive response. These differential increases in glutathione are similar to those obtained following folate deficiency and iron challenge but in the presence of vitamin E (Shea et al., 2002b). To determine the mechanism(s) responsible for these

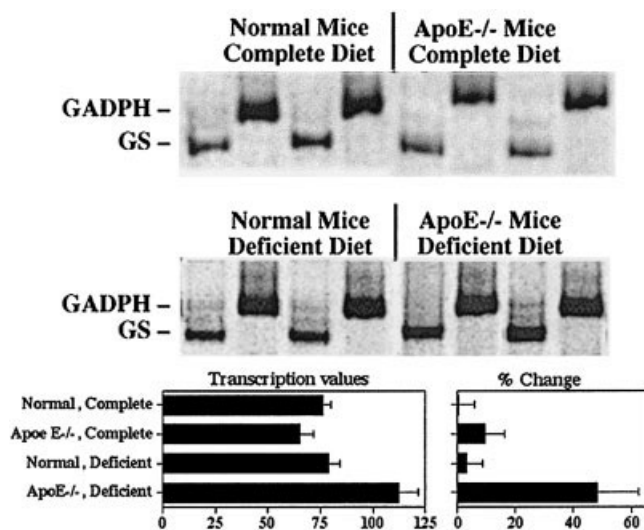


Fig. 1. Transcription of GS is increased in ApoE $^{-/-}$  mice maintained on the deficient diet. Representative gels of RT-PCR products using the long-fragment primers for GS and the primers for GADPH as described in Materials and Methods; products from two separate normal and ApoE $^{-/-}$  mice maintained on each diet as indicated. The short-fragment GS primers gave virtually identical results (not shown). The accompanying graphs present mean values ( $\pm$ SEM) obtained from densitometric analyses of two separate gel electrophoretograms each of RT-PCR products generated using both sets of GS primers each and the GAPDH "housekeeping" gene from a total of seven or eight normal and seven or eight ApoE $^{-/-}$  mice on each diet from two separate experiments. To facilitate comparison, we also present the percentage increase in transcription compared with values obtained from normal mice maintained on the complete diet (the latter of which were defined as 1 for these calculations). Note that transcription increased only in ApoE $^{-/-}$  mice and only when they were maintained on the deficient diet.

compensatory responses, we next examined activity and transcription of GS in mice maintained under the above-mentioned complete and deficient diets.

Transcription of GS in normal mice maintained on the deficient diet, or ApoE $^{-/-}$  mice maintained on the complete diet, did not differ from that in normal mice maintained on the complete diet (Fig. 1). However, transcription of GS was elevated by  $48\% \pm 14.7\%$  when ApoE $^{-/-}$  mice were maintained on the deficient diet ( $P < .03$  vs. normal mice on the complete diet; ANOVA). These data indicate that the combined impact of dietary (folate and vitamin E) and genetic (ApoE) deficiencies induced increased compensatory expression of GS, whereas neither of these dietary and genetic deficiencies did so individually.

We next examined the activity of GS. Unlike transcription of GS, which was increased only in ApoE $^{-/-}$  mice maintained on the deficient diet, GS activity was increased in brain tissue of both normal and ApoE $^{-/-}$  mice maintained on the deficient diet vs. normal mice on the complete diet; however, a larger increase was observed

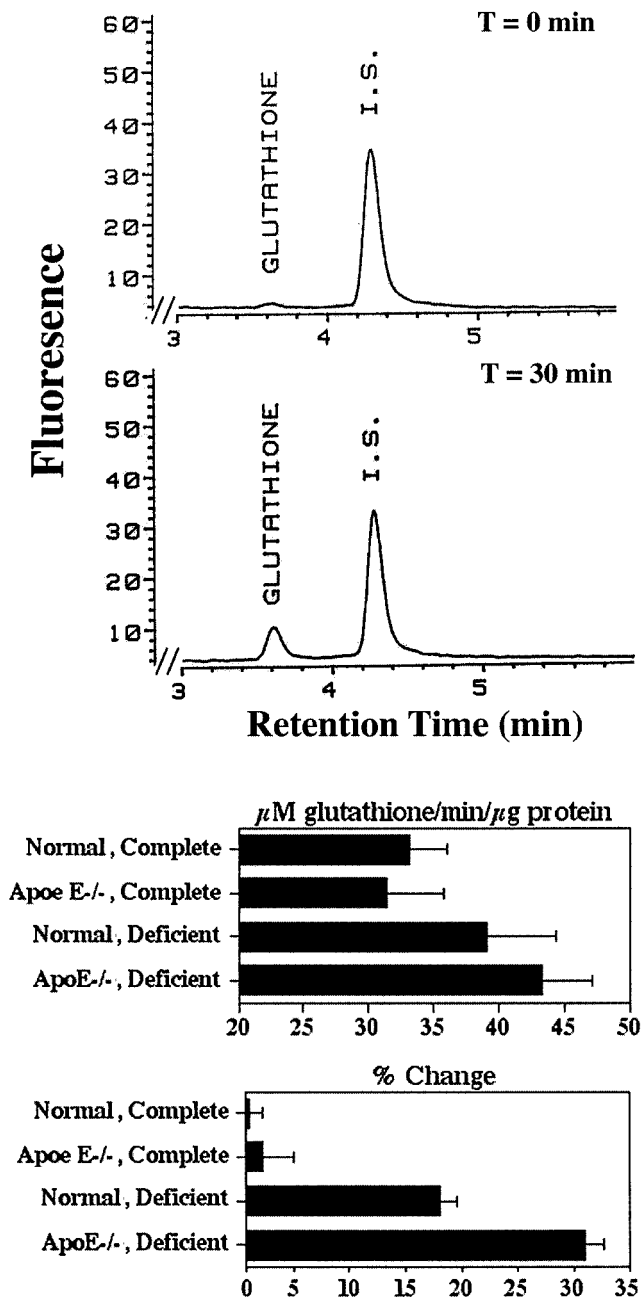


Fig. 2. Activity of GS is increased in both normal and ApoE<sup>-/-</sup> mice following dietary deficiency. Panels show the relevant portion of representative chromatograms obtained with HPLC from brain tissue of ApoE<sup>-/-</sup> mice maintained on the deficient diet. "I.S." denotes an internal standard included for normalization and demarcation of retention time. The accompanying graph presents the mean increase in activity for seven or eight normal and seven or eight ApoE<sup>-/-</sup> knockout mice on each diet from two separate experiments. Also presented is the percentage increase in activity ( $\pm$ SEM) compared with values obtained from normal mice maintained on the complete diet (the latter of which were defined as 1 for these calculations). Note the increase in GS activity in both normal and ApoE<sup>-/-</sup> mice maintained on the deficient diet.

in ApoE<sup>-/-</sup> mice than in normal mice on this diet (Fig. 2). Activity observed in ApoE<sup>-/-</sup> mice maintained on the deficient diet ( $30.7\% \pm 11\%$ ) differed significantly from that in all other conditions ( $P < .05$ ; ANOVA). Normal mice maintained on the deficient diet also displayed a marked (17.9%) increase in GS activity (Fig. 2, Table I). However, because of the relatively wide variance among samples from individual mice (15.8%), this value did not achieve statistical significance ( $P < .159$ ; ANOVA); indeed, considerable variation was observed for both mouse strains maintained on both diets (Table I). Nevertheless, the increase in GS activity in normal mice maintained on the deficient diet was more than half (60%) of the increase observed in ApoE<sup>-/-</sup> mice maintained on this diet, suggesting that dietary deficiencies in folate and vitamin E affect GS activity. By contrast, ApoE<sup>-/-</sup> mice maintained on the complete diet did not display an increase in activity compared with normal mice maintained on the complete diet ( $P < .43$ ). The larger increase in activity in ApoE<sup>-/-</sup> mice maintained on the deficient diet vs. GS activity in normal mice on the deficient diet is likely to be derived from the unique increase in transcription of GS in ApoE<sup>-/-</sup> mice maintained on the deficient diet, coupled with the diet-induced increased activity in both mouse strains. These data collectively suggest that deficiencies in folate and vitamin E exert a more profound effect on GS activity than does the absence of ApoE, insofar as ApoE<sup>-/-</sup> mice maintained on the complete diet did not show an increase in GS activity vs. normal mice on the complete diet.

### DISCUSSION

The findings of the present study provide insight into compensatory mechanisms for neuronal oxidative stress. They further demonstrate that the nervous system can up-regulate transcription of a second gene of relevant function to compensate for the absence of another. In addition, our findings demonstrate distinct compensatory responses in the glutathione pathway following dietary or genetic compromise as follows. Increased activity of GS was observed in both normal and ApoE<sup>-/-</sup> mice when maintained on the deficient diet. The overall increase in GS activity in ApoE<sup>-/-</sup> mice maintained on the deficient diet beyond that of normal mice on the deficient diet was likely derived both from increased activity and from increased transcription. At least part of the increase in activity was apparently in response to diet-induced oxidative stress rather than oxidative stress resulting from the lack of ApoE, in that 1) no change in activity was observed in ApoE<sup>-/-</sup> mice maintained on the complete diet and 2) normal mice maintained on the deficient diet also showed an increase in activity, albeit less than that of ApoE<sup>-/-</sup> mice on the deficient diet. Increased transcription was not a response to diet-induced oxidative stress, insofar as normal mice maintained on the deficient diet did not demonstrate increased transcription. By contrast, a significant increase in transcription of GS was observed only in ApoE<sup>-/-</sup> mice and only when they were maintained on

**TABLE I. Dietary and Genetic Influences on GS, Glutathione, and Oxidative Damage in Brain Tissue\***

Mouse strain and diet	Percentage change			
	GS transcription	GS activity	Glutathione	TBARs <sup>a</sup>
Normal mice, complete diet	1 ± 5.3	1 ± 8.8	1 ± 2.2	1 ± 14.6
ApoE <sup>-/-</sup> mice, complete diet	9.7 ± 6.8	2.3 ± 13.6	19.5 ± 12.7	69 ± 46
Normal mice, deficient diet	3.5 ± 5.4	17.9 ± 15.8	14.8 ± 1.2	42 ± 55
ApoE <sup>-/-</sup> mice, deficient diet	48 ± 14.7	30.7 ± 11	36.2 ± 1.0	308 ± 46

\*Values in all cases are presented as the mean percentage increase (±SEM) compared with values obtained from normal mice maintained on the complete diet (the latter of which were defined as 1 for these calculations). Values for transcription (obtained as arbitrary densitometric units) were pooled from those obtained for both GS primer sets. In all cases, n = a 7–8 normal and 7–8 ApoE<sup>-/-</sup> mice from two separate experiments. GS activity is defined as micromolar glutathione produced per minute per microgram total protein. Values for glutathione are expressed as nanomoles per milligram total protein. <sup>a</sup>TBAR data are from Shea and Rogers (2002b) and are included herein for comparative purposes only; TBARs are in micromolar per milligram total protein.

the deficient diet, suggesting that the combined impact of diet-induced and genetically induced oxidative stress was required to induce an increase in transcription. The magnitude of this combined impact was reflected by a synergistic increase in TBARs in brain tissue of ApoE<sup>-/-</sup> mice maintained under the deficient diet. As shown previously (Shea and Rogers, 2002b), an increase in oxidative damage to brain tissue resulted from both diet-induced and genetically induced oxidative stress (Table I). However, a synergistic increase in oxidative damage resulted from the combined impact of both diet-induced and genetically induced oxidative stress as follows. Maintenance of normal mice on the deficient diet induced an approximate 40% increase in TBARs, whereas the lack of ApoE function induced an approximate 70% increase. If diet-induced and genetically induced oxidative damage were merely additive, then an approximate 100% increase in TBARs would be anticipated in brain tissue of ApoE<sup>-/-</sup> mice when maintained on the deficient diet; by contrast, the combined impact of the absence of ApoE and the deficient diet resulted in a 300% increase in TBARs in brain tissue (Shea and Rogers, 2002b; see also Table I), indicating a synergistic deleterious impact of these dietary and genetic deficiencies. Notably, this marked increase in TBARs not only confirms the synergistic impact on brain tissue of diet-induced and genetically induced oxidative stress but further demonstrates that, despite induction of increases in both activity and transcription of GS (Figs. 1, 2), and the resultant additive increase in glutathione (Table I), ApoE<sup>-/-</sup> mice maintained on the deficient diet were unable to compensate fully for this synergistic increase in oxidative damage (Table I).

These findings highlight the fact that distinct compensatory responses (i.e., increased expression and, independently, increased activity) in an antioxidant-generating enzyme can be invoked depending on the nature and extent of oxidative stress. The combined efficacy of these responses was reflected by steady-state levels of glutathione, in that both diet-induced and genetically induced oxidative stress individually elevated glutathione levels, whereas the combined impact of both induced an apparent additive increase (Table I; see also Shea et al., 2002b).

Inasmuch as an increase in glutathione was observed in ApoE<sup>-/-</sup> mice maintained on the complete diet despite no increase in activity or transcription of GS, it would be of interest to determine the activity and/or transcription of other enzymes that regulate glutathione steady-state levels, such as glutathione peroxidase and glutathione reductase, and/or additional upstream enzymes (e.g., glutamate-cysteine ligase) that regulate glutathione production. In this regard, an increase in glutathione peroxidase and catalase, but not in superoxide dismutase, was observed in individuals with AD who were homozygous for E4 (Ramassamy et al., 1999, 2000). It would also be of interest to ascertain whether pathways regulating other endogenous antioxidants are similarly affected. Notably, the model system used here utilizes extreme examples of dietary deficiency (i.e., a complete lack of dietary folate and vitamin E rather than a more modest reduction) and ApoE deficiency (i.e., transgenic mice lacking all ApoE function rather than those homozygous for the relatively weak but functional E4 isoform; Growdon, 2001). The extent of these deficiencies is likely to have contributed to the inability of ApoE<sup>-/-</sup> mice maintained on the deficient diet to quench the full extent of oxidative damage observed in brain tissue; further such studies utilizing less profound dietary and genetic deficiencies that more closely resemble the extent of deficiencies faced by humans would be of interest. However, individuals with AD homozygous for the E4 allele showed a decrease in glutathione vs. individuals homozygous for the E3 allele of ApoE (Ramassamy et al., 2000); it may be that severe conditions, perhaps derived from multiple oxidative insults, such as those utilized herein, are required to stimulate increased production of glutathione. Notably, the differential and additive impact of dietary and genetic deficiencies on oxidative damage and compensatory responses in glutathione production highlight the fact that certain genetic predispositions may remain latent pending dietary deficiencies.

Only folate deficiency, and not vitamin E deficiency, has been reported to induce a compensatory increase in glutathione levels in both normal and ApoE<sup>-/-</sup> mice (Shea et al., 2003). Levels of glutathione in brain tissue of

normal and ApoE<sup>-/-</sup> mice following simultaneous folate and vitamin E deprivation, coupled with iron challenge, were similar to those observed following iron challenge in the absence of folate and but in the presence of vitamin E (Shea et al., 2002b). These findings indicate that, although deficiency of vitamin E potentiates oxidative damage to brain tissue resulting from folate deficiency (Shea and Rogers, 2002b), it cannot stimulate a further compensatory increase in glutathione beyond that resulting from folate deprivation alone. Glutathione levels can therefore apparently be regulated by folate-dependent mechanisms and are independent of vitamin E.

We have not conclusively determined in the present study whether the increased transcription of GS observed is derived specifically from a combination of dietary and genetic deficiencies or instead could be derived from an overall increase in oxidative burden regardless of the source. Of interest in addressing this issue would be to determine whether a substantial further increase in dietary oxidative stress would result in increased transcription of GS. Similarly, it would be of interest to delete or otherwise suppress the activity of one or more additional genes that regulate oxidative burden and monitor whether this is accompanied by increased transcription of GS in the absence of any dietary oxidative challenge. Apart from these caveats and future directions, the findings of the present study demonstrate that the central nervous system can respond to oxidative stress at both the genetic and the epigenetic levels (i.e., increased expression of the antioxidant-generating enzyme and increased activity, respectively). Notably, even combined up-regulation in GS transcription and activity was incapable of quenching the increased oxidative damage resulting from oxidative challenge of ApoE<sup>-/-</sup> mice deprived of folate and vitamin E, as evidenced by TBAR analyses (Shea and Rogers, 2002b). One study suggests, however, that glutathione alone may be unable to protect cells against oxidative damage (Spector et al., 1987). Similarly, recent studies indicate that consumption of vitamins C and E was not effective against AD (Luchsinger et al., 2003). Treatment with multiple antioxidants and/or antioxidant-promoting compounds may be required to provide sufficient neuroprotection to delay or reduce neurodegeneration (see, e.g., Dhitavat et al., 2001; Shea et al., 2002c).

These data highlight the potential deleterious impact of a combination of dietary and genetic risk factors for neurodegeneration; whereas the nervous system may be able to compensate for either dietary or genetic risk factors individually, it may not be able to compensate for the potential synergistic impact of a combination of both diet-induced and genetically induced oxidative damage. One interpretation of these data is that a genetic predisposition for AD, such as ApoE deficiency (Growdon, 2001; Rebeck et al., 2002), may remain latent pending the added burden of an age-related deficiency in nutrition (Gray, 1989). In addition, polymorphisms in one or more enzymes of the methionine cycle that impair folate usage

may also contribute to the onset or progression of AD. Polymorphisms in 5,10-methylenetetrahydrofolate reductase (MTHFR; the folate-dependent enzyme required for the conversion of HC to methionine; Mattson and Shea, 2001) that exhibit decreased activity are present in as much as 20% of some populations (Brotto and Yang, 2000). Diminished activity of this enzyme also reduces production of tetrahydrofolate (required for DNA synthesis) and reduced adenosylmethionine (required for DNA methylation; Stern et al., 2000). Notably, a 36% increase in MTHFR polymorphisms has recently been reported among young people (Reyes-Engel et al., 2002). The investigators considered that increased maternal dietary folate (confirmed in their samples) allowed for an increase in fetal viability despite latent deficiencies in MTHFR. Although no direct evidence exists to date indicating that a deficiency in MTHFR activity is a risk factor for AD in isolation (Chapman et al., 1998; Brunelli et al., 2001; Postiglione et al., 2001; Prince et al., 2001; Zuliani et al., 2001), MTHFR deficiency and the presence of ApoE4 may represent synergistic risk factors for AD (Regland et al., 1999). This is a plausible extension of the known association of diminished folate with AD, in that individuals homozygous for the deficient MTHFR polymorphisms are at particular risk for other folate-related neural defects when plasma folate is at the "low end" of the normal range (Sheilds et al., 1999). Moreover, individuals affected with AD who were also homozygous for the deficient MTHFR polymorphisms showed elevated HC vs. nonhomozygous AD patients despite similar folate and B12 levels in both groups (Postiglione et al., 2001). As with the above-mentioned considerations for the potential latency of ApoE deficiency, these findings highlight the potential for an increased latency of critical genetic deficiencies in folate metabolism that may be manifested only with age-related nutritional decline. This possibility is supported by the demonstration that the impact of deficiencies in MTHFR activity is further augmented by diminished dietary folate (Frosst et al., 1995; Shields et al., 1999).

Such latent genetic conditions, perhaps derived from more than one genetic locus, would undoubtedly contribute to the difficulty in determining the etiology of AD. Further studies of the combined impact of multiple AD risk factors is warranted.

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