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ORIGINAL ARTICLE

Dietary Supplementation with 3-Deaza Adenosine, *N*-Acetyl Cysteine, and *S*-Adenosyl Methionine Provide Neuroprotection Against Multiple Consequences of Vitamin Deficiency and Oxidative Challenge

Relevance to Age-Related Neurodegeneration

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Abstract

Folate deprivation induces neurotoxicity that is potentiated by additional nutritional and genetic deficiencies including vitamin E and apolipoprotein E deficiency. These deficiencies collectively induce oxidative damage, cognitive impairment, and compensatory alteration in glutathione generation. Treatment with agents that regulate distinct portions of the methionine cycle, including the *S*-adenosyl homocysteine hydrolase inhibitor, 3-deaza adenosine, the methyl donor *S*-adenosyl methionine, and the antioxidant *N*-acetyl cysteine, provide neuroprotection against various aspects of neurotoxicity in normal and apolipoprotein E deficient mice and in cultured neuronal cells deprived of dietary folate and vitamin E and subjected to iron overload. Here it is demonstrated that simultaneous treatment with these agents provide superior neuroprotection by alleviating individual and overlapping neurotoxic consequences. These findings support combinatorial treatments with agents that compenate for differential insults in age-related neurodegenerative disorders. *doi: 10.1385/NMM:6:1:1*

Index Entries: Folate; homocysteine; 3-deaza adenosine; *S*-adenosyl methionine; *N*-acetyl cysteine; glutathione; neurodegeneration; oxidative stress; apolipoprotein E.

Introduction

The vitamin B folate is an obligate cofactor for the regeneration of methionine from homocysteine (HC) via the action of 5′,10′-methylene tetrahydrofolate reductase (MTHFR), which converts folate to 5-methyltetrahydrofolate (Stover, 2004). The B_{12}-dependent enzyme methionine synthase (MS)

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catalyzes a methyl transfer from 5-methyltetrahydrofolate to HC, thereby producing methionine and tetrahydrofolate (Banerjee and Matthews, 1990). Methionine is normally converted to *S*-adenosylmethionine (SAM; Cantoni, 1953). SAM is required in several transmethylation reactions, including methylation of proteins, phospholipids, neurotransmitters, and nucleic acids (Mudd and Cantoni, 1964). Transmethylation reactions involving SAM produce S-adenosylhomocysteine (SAH). SAH is then further hydrolyzed to HC via SAH hydrolase. HC is either remethylated back to methionine via the above folate- and B_{12}-dependent reactions, converted to cystathionine by cystathionine-β synthase, which leads to glutathione (GSH; Djurhuus et al., 1989) or exported from cells.

Perturbation in the methionine cycle because of dietary deficiency in folate, and/or compromise in its usage because of polymorphisms in MTHFR, contribute to a host of developmental, chronic, and age-related disorders (Frosst et al., 1995; Arrula et al., 1997; Shields et al., 1999; Brotto and Yang, 2000; Stern et al., 2000; Friso et al., 2002; Stover, 2004). One consequence of folate deficiency is increased HC (Fiskerstrand et al., 1997), which fosters increased HC export. Since folate deficiency prevents methionine regeneration, SAM levels are also reduced as a consequence of folate or B_{12} deficiency (Hyland et al., 1988; Surtees et al., 1991; Selhub et al., 1992), which compromises the transmethylation reactions described above.

Please check Fiskerstrand et al. (1997) is not cited in references.

Please specify where above.

Folate deficiency contributes to many neurological and psychological disorders including dementia, impaired cognition, depression, psychosis, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (for reviews, *see* Mattson and Shea, 2002; Shea and Rogers, 2002a; Shea et al., 2002a; Lushcinger and Mayeux, 2004). The neuronal system demonstrates a particular requirement for folate in that levels in spinal fluid are normally fourfold that of plasma; this increased level declines with age (Serot et al., 2001), and reduced folate levels are strongly associated with atrophy of the cerebral cortex (Snowdon et al., 2000). HC, the export of which increases following folate deficiency, is a potent neurotoxin that stimulates the *N*-methyl-D-aspartate receptor, increases calcium influx, oxidative stress, excitotoxicity, induces DNA breakage, increases neuronal apoptosis, potentiates amyloid-β and copper neurotoxicity, and is related to the severity and progression of AD (Kruman et al., 2000, 2002; Ho et al., 2001, 2003; Postiglione et al., 2001; White et al., 2001; Shea et al., 2002a; Tjattias et al., 2004). Diminished SAM levels, also resulting from folate deficiency, increase DNA breakage, neuronal apoptosis and degeneration (Hyland et al., 1988; Surtees et al., 1991; Selhub et al., 1992; Ho et al., 2003; Shea et al., 2004). SAM levels, as well as the activity of the enzyme responsible for its generation (methionine-S-adenosyltransferase), are decreased in the spinal fluid and brains of individuals exhibiting neurodegeneration compared with age-matched controls (Bottiglieri et al., 1990; Gomes et al., 1994; Morrison et al., 1996; Muller et al., 2001; Kennedy et al., 2004). Diminished SAM may underlie the gradual hypomethylation of DNA that accompanies aging (Seshadri et al., 2002). An optimal therapeutic approach to compensate for folate deficiency might therefore require antagonists for each of the above deleterious effects.

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Please check Kennedy et al., 2004 is not cited in references.

Our findings demonstrate that the deleterious effects of folate deprivation are potentiated by deficiency in apolipoprotein E. Deficiencies in apolipoprotein E (ApoE) function increase oxidative stress and are associated with AD (Kamboh, 2004). Transgenic mice homozygously lacking apolipoprotein E (ApoE–/–) undergo markedly more severe oxidative damage and cognitive impairment when maintained on a folate-deficient diet than do normal mice (Shea and Rogers, 2002b; Shea et al., 2002b; Mihalick et al., 2004). ApoE–/– mice also upregulate transcription of glutathione synthase (GS) and display increased GSH in brain tissue in an apparently unsuccessful attempt to compensate for the increased oxidative damage resulting from deficiency in folate and ApoE coupled with iron challenge (Tchantchou et al., 2004).

Prior studies have demonstrated the efficacy of several agents against one or more of the above neuropathological consequences of folate deprivation and HC exposure. These include (1) the SAH inhibitor 3-deaza-adenosine (DZA), which prevents conversion of SAH to HC (Jeong et al., 1999) and reduced production of HC in mice and in cultured neurons following folate deprivation (Ho et al., 2003; Shea et al., 2003); (2) the antioxidant and GSH precursor *N*-acetyl cysteine (NAC; Mitchell et al., 1973; Dringen et al., 1999; Ou et al., 1999; Pocernich et al., 2001), which increased GSH and reduced ROS in cultured neurons deprived of folate (Ho et al., 2003),

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and (3) SAM, which reduced apoptosis following HC treatment of cultured neurons (Ho et al., 2002). We examined whether treatment with a combination of one or more of these agents provided superior neuroprotection as compared with treatment with these agents individually.

Materials and Methods

Normal and Transgenic Mice

ApoE–/– mice (B6.129P2Apoe^{tm1Unc}N11) approx 9 mo of age, and normal mice of the identical genetic background approx 9 mo of age and between 2 and 2.5 yr of age received basal, vitamin-free diets ("AIN-76; " Purina/Mother Hubbard, Inc) either supplemented with folic acid (40 mg/kg), and vitamin E (1 g/kg total diet wet weight) (defined as the "complete diet"), or lacking folate and vitamin E and containing iron (50 g/500 g total diet wet weight) as a prooxidant (defined as the "deficient diet; " Shea and Rogers, 2002b; Shea et al., 2002b) for 1 mo. Additional groups of mice maintained on the deficient diet received DZA (40 mg/kg diet wet weight), SAM, (80 mg/kg diet wet weight), and/or NAC (1 g/kg diet wet weight; Shea et al., 2003). We did not separately test the influence of DZA, SAM, or NAC supplementation on folate and vitamin E deprivation; however, previous studies have indicated that vitamin E deprivation alone did not induce compensatory increases in GSH or GS (Shea et al., 2002). Prior studies have shown that inclusion of dietary iron alone did not foster an increase in oxidative damage in either mouse strain in these 1-mo studies (Shea and Rogers, 2002b). Overall "end point" oxidative damage was monitored in total brain homogenates by quantifying thiobarbituric acid-reactive substances (TBARs; reported as µm/mg total protein) as described (Shea and Rogers, 2002a; Linsley et al., 2004). Cognitive performance was monitored by standard nonreward-based Y and reward-based T-mazes; Y-maze values are reported as percent alternation, and T-maze values are reported as percent passing (Mihalick et al., 2004). GSH levels were determined by high-performance liquid chromatography (HPLC) and are reported as µm/mg total protein (Shea et al., 2002). GS transcription was monitored by reverse transcription polymerase chain reaction (RT-PCR) of RNA isolated from brain and GS activity was determined in brain homogenates and are reported in relative densitometric units as described (Tchantchou et al., 2004). All chemicals were from Sigma-Aldrich. Two to four independent experiments were carried out for all analyses, with three to four mice of each strain in each group, for a total *n* of 6–16 for all values reported.

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Cell Culture

SH-SY-5Y human neuroblastoma cells were cultured in DMEM containing 10% fetal calf serum and were differentiated for 7 d with 10 µ*M* retinoic acid (Ekinci et al., 1999; Ho et al., 2003). To monitor the consequences of folate deprivation, cultures received DMEM lacking folate (Sigma) for 2 h at which point some cultures also received 25 m*M* DZA (Jeong et al., 1999; Ho et al., 2003; Shea et al., 2003), 100 µ*M* SAM, and/or 10 m*M* NAC (Ho et al., 2003). To monitor intracellular peroxide concentrations as an index of ROS, cultures received 10 µL/mL DFCD (2′7′-dichlorofluorescein diacetate; Kodak) for 20 min, after which cultures were visualized under fluorescein UV optics. Images were captured using a Dage CCL-72 camera operated by NIH Image via a Scion LG-3 frame grabber and stored as PICT files. Identical illumination and capture settings were used for all images. Multiple fields (5–10) at 20 × were captured from duplicate or triplicate cultures. Relative fluorescent intensity was quantified from stored images using NIH Image analysis software. Values for ROS are reported as arbitrary densitometric units (Ho et al., 2001). Statistical analyses were carried out by Student's *t*-test or analysis of variance. Values were considered statistically different if $p = 0.05$.

Results

Normal and ApoE–/– mice treated for 1 mo with a complete diet (containing folate and vitamin E and lacking the iron challenge) or with a "deficient" (the above basal diet lacking folate and vitamin E and containing high-dietary iron as a prooxidant; Shea and Rogers, 2002b) exhibit oxidative damage, cognitive impairment as ascertained by Y and T-maze tests, and undergo compensatory increases in GSH and GS transcription and activity (Fig. 2). Additional groups of mice receiving the deficient diet were supplemented with DZA, SMA, or NAC

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Table 1
Combined treatment with DZA, SAM, and NAC attenuates HC levels more effectively than DZA alone

Conditions	HC (μ*M*/mg protein)	% change
Normal mice, complete diet	28.7 ± 0.4	–
ApoE–/– mice, deficient diet	38.9 ± 1.5	35.6 ± 1.9*
ApoE–/– mice, deficient diet, DZA	34.0 ± 3.3	18.5 ± 2.3*
ApoE–/– mice, deficient diet, DZA, SAM, NAC	12.5 ± 8.1	–56.3 ± 0.7*

HC was quantified in homogenates of brain tissue by HPLC as described (Shea and Rogers, 2002b). Values are presented as raw data (mean ± standard deviation) and as the % change (± standard deviation) vs values obtained for normal mice maintained on the complete diet. Values statistically increased ($p < 0.05$) over those in normal mice on the complete diet are indicated by an asterisk; values statistically decreased are indicated by two asterisks.

individually or in combination. These agents demonstrated varied efficacy against each of these parameters.

All three agents individually prevented the increase in TBARs associated with folate deprivation of ApoE–/– mice (Fig. 2). NAC and DZA, but not SAM, prevented the decline in cognitive impairment as ascertained by the Y-maze test. Conversely, SAM and DZA prevented the decline in cognitive impairment as ascertained by the T-maze test. These differing results reflect that these two assays probe related but distinct assays of cognitive performance (Lalonde, 2002; Mihalick et al., 2004, and references. therein).

We also tested the efficacy of these agents against the alterations in GSH and GS transcription and expression that accompany folate deprivation in ApoE–/– mice (Shea et al., 2003). NAC and SAM, but not DZA, prevented the accumulation in GSH; treatment with all three agents yielded an effect similar to either NAC or SAM. Similarly, NAC and SAM, but not DZA, each prevented the increase in transcription of GS; however, combined treatment with all three agents provided statistically increased neuroprotection versus that provided by NAC or SAM alone. NAC and DZA, but not SAM, alleviated the increase in GS activity; combined treatment with all three agents had a similar effect as did NAC or DZA alone.

One consequence of folate deprivation is increased HC, which itself contributes to neuronal oxidative stress. We therefore examined the consequences of treatment with these agents on HC levels. Increased levels of HC were observed in brain tissue of ApoE–/– mice maintained on either diet as compared to levels observed in normal mice maintained on the complete diet (Table 1). Maintenance of normal mice on the deficient diet did not increase HC levels in brains of normal mice (not shown). Consistent with our prior studies (Shea et al., 2004), supplementation of the deficient diet with DZA attenuated the increase observed in ApoE–/– mice by approx 50%. Supplementation with all three agents, however, reduced HC levels to less than half of those observed in normal mice maintained on the complete diet (Table 1).

Whereas normal mice at 9 mo of age do not exhibit signs of neuronal trauma when maintained on the deficient diet (e.g., Fig. 1), the deficient diet does increase oxidative damage and impairs performance

Table 1
Fig. 1.

Fig. 1. Effect of DZA, NAC, and SAM individually and in combination on neurodegenerative consequences of folate and vitamin E deprivation in normal and ApoE–/– mice. Normal and ApoE–/– mice approximately 9 mo of age were maintained for 1 mo on complete and deficient diets with and without supplementation with DZA, SAM, and/or NAC. Mice were tested for performance on the Y-maze (reported as % alternations; Mihalick et al., 2004), and the T-maze (reported as % passing; Mihalick et al., 2004) the following day, then sacrificed and homogenates of brain tissue were analyzed for TBARs (μm/mg total protein in brain homogenate), GSH (μm/mg total protein), and expression and activity of GS as described in Materials and Methods. Values are presented as raw data (mean ± standard deviation) and as the % change (± standard deviation) vs values obtained for normal mice maintained on the complete diet. Values differing statistically from normal mice on the complete diet are indicated by an asterisk.

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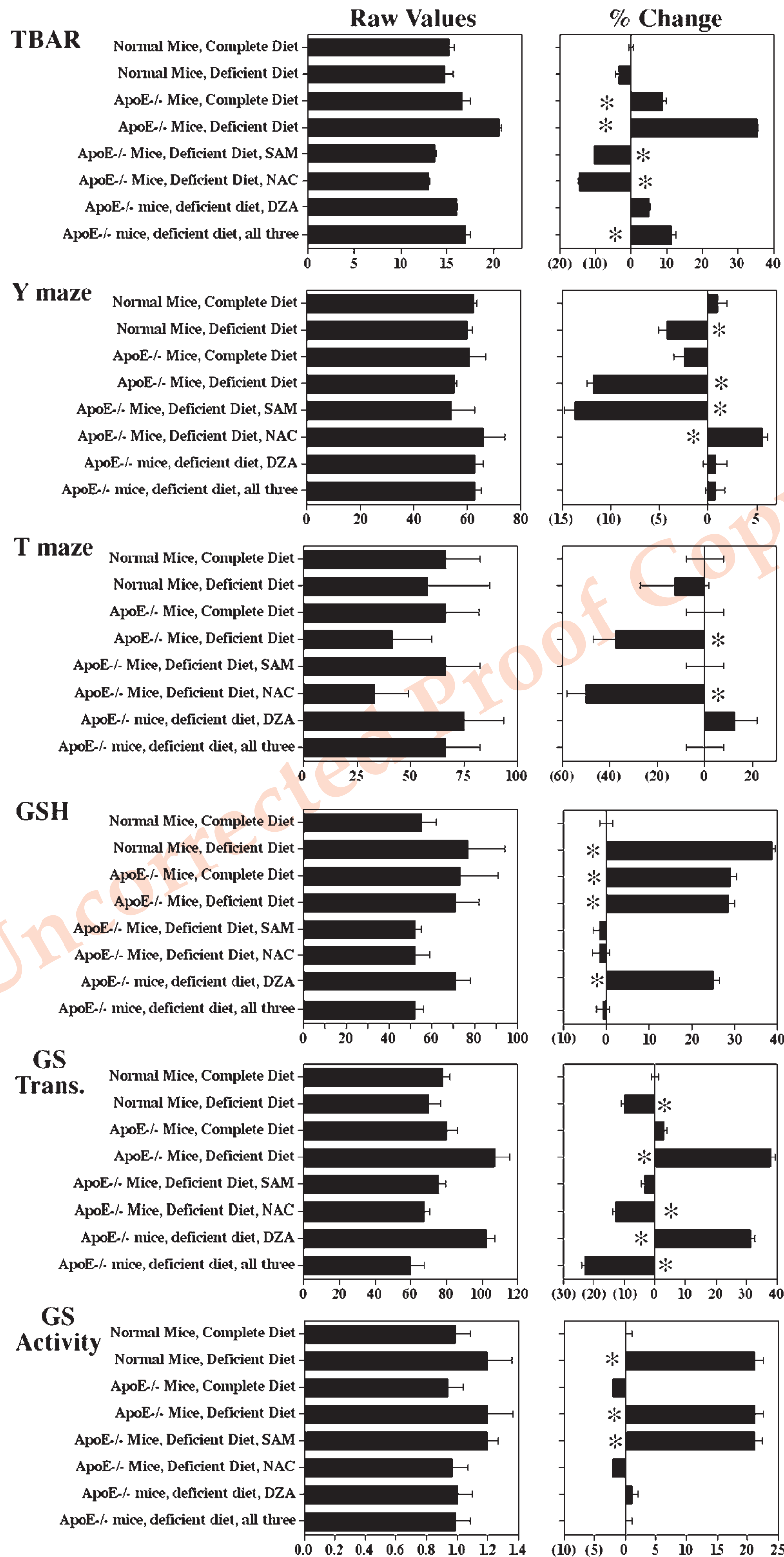

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Table 2
Combined treatment with DZA, SAM, and NAC prevents oxidative damage and impaired cognitive performance in normal aged mice

Conditions	TBAR	Y-maze	T-maze
Deficient diet	22.9 ± 8.7*	(10.8) ± 4.3*	(43.5) ± 16.0*
Deficient diet + DZA, SAM, NAC	(11.5) ± 14.2	8.7 ± 13.4	(34.8) ± 8.3*

Normal mice aged 2–2.5 yr were maintained on the complete, deficient, and deficient diets supplemented with DZA, SAM, and NAC for 1 mo, then subjected to Y and T-maze tests and brain tissue examined for oxidative damage via TBARs. Values are presented as the % change (± standard deviation) vs values obtained for normal mice maintained on the complete diet. Values statistically increased ($p < 0.05$) over those in normal mice on the complete diet are indicated by an asterisk.

Table 2

in the Y and T-mazes in normal mice aged 2–2.5 yr (Table 2). However, supplementation of the deficient diet with DZA, SAM, and NAC prevented the increase in oxidative damage and maintained normal performance in the Y-maze test. In contrast to the beneficial effect on 9-mo-old ApoE–/– mice (Fig. 1), however, these agents in combination did not improve the performance of normal aged mice in the T-maze test (Table 2). We did not test DZA, SAM, and NAC individually on aged mice.

Folate deficiency and HC affect vasculature as well as neurons (Candito et al., 1997; Shea et al., 2002a; Mattson and Shea, 2003). In order to determine whether or not DZA, SAM, and NAC exerted their beneficial effects on neurons themselves, we evaluated these agents individually and in combinations on ROS generated during folate deprivation in cultured SH-SY-5Y neuroblastoma cells, which represent a useful model for assay of folate deprivation and HC toxicity (Ho et al., 2001, 2002, 2003). Folate deprivation statistically ($p < 0.05$) increased ROS. Treatment with each agent prevented this increase, and moreover, statistically reduced ROS below levels observed in cultures receiving folate. Simultaneous treatment with SAM and DZA was not any more effective than either agent alone. Simultaneous treatment with NAC and DZA, NAC and SAM, and all three agents progressively significantly reduced ROS. Maximal protection was provided when all three drugs were applied together (Fig. 2).

Fig. 2.

Discussion

Here it is we examined that the neuroprotective efficacy of combinatorial agents that affect different portions of the methionine cycle on the consequences of folate and vitamin E deprivation, coupled with induction of iron-mediated oxidative stress. Each of these agents has been previously demonstrated to reduce various aspects of neurotoxicity in mice and in cultured neurons or neuroblastoma under these conditions; DZA inhibited HC formation, SAM prevented apoptosis and NAC increased GSH and prevented ROS formation (Ho et al., 2002, 2003; Shea et al., 2003). We examined diverse but overlapping parameters of oxidative stress-induced damage in mice, including ROS, cognitive performance (with two maze tests that themselves examine related but distinct aspects of memory; King et al., 1999; Corcoran et al., 2002; Mihalick et al., 2004), and compensatory upregulation of GSH and GS. No single agent was superior to the others in all of these assays. All three agents effectively prevented the increase in oxidative damage in ApoE–/– mice when tested individually; combined treatment with these agents did not provide any further neuroprotection. In the remainder of the assays, one or two of the agents was neuroprotective, and additional combined treatment with all three agents did not exhibit superior neuroprotection. However, the particular agents that demonstrated superior performance varied among tests, such that each of the three agents tested herein exceeded the other two in at least one test. Since the tests utilized herein assayed related but distinct facets of neurodegeneration, our findings underscore how treatment with a combination of agents directed against diverse aspects of neurodegeneration is likely to provide neuroprotection exceeding that obtainable from any one agent. Unexpectedly, combined treatment was superior in reduction of

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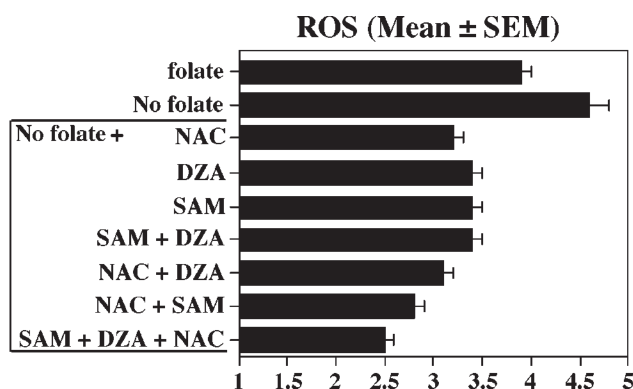

Fig. 2. Combined treatment with DZA, NAC, and SAM provides maximal protection against the consequences of folate deprivation. Differentiated SH-SY-5Y cells were cultured for 2 h in the presence or absence of folate, or in the absence of folate and supplemented with DZA, NAC, and/or SAM as indicated then analyzed for ROS. Folate deprivation statistically ($p < 0.05$) increased ROS. Treatment with each agent prevented this increase, and moreover, statistically reduced ROS below levels observed in cultures receiving folate. Simultaneous treatment with SAM and DZA was not any more effective than either agent alone. Simultaneous treatment with NAC and DZA, NAC and SAM, and all three agents progressively reduced ROS. Maximal protection was provided when all three drugs were applied together. Values represent the mean (± standard error of the mean) relative density (in arbitrary densitometric units with cultures receiving folate considered as "1").

HC levels than was treatment with only DZA, which inhibits HC formation from SAH; this finding indicates that overall antioxidant and/or methylation capacity is an important aspect of controlling HC generation during folate deficiency. Whereas the efficacy of DZA is likely to be restricted to preventing an inappropriate increase in HC that may only accompany folate deprivation, NAC and SAM may perhaps provide more general protection. A deficiency of SAM accompanies normal aging and AD (Bottiglieri et al., 1994; Trolin et al., 1994; Morrison et al., 1996; Kennedy et al., 2004), and may underlie the gradual hypomethylation of DNA that accompanies aging (Seshadri et al., 2002); supplementation with SAM (Loehrer et al., 1997) may alleviate this deficiency. The endogenous antioxidant, glutathione (GSH) is reduced in AD (Schultz et al., 2000; Liu et al., 2004); and supplementation with NAC may maintain appropriate GSH production.

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We only examined the efficacy of combined treatments with these agents on ROS generation in cultured neuroblastoma. Prior studies demonstrate that DZA, SAM, and NAC provide neuroprotection against different aspects of folate deprivation or HC treatment (Ho et al., 2001, 2002, 2003). It would perhaps be useful to examine whether or not combined treatment provided superior neuroprotection against additional aspects of folate deprivation. However, it should be noted that cultured neuronal cells were examined here only to determine whether or not DZA, SAM, and NAC exerted effects on neuronal cells themselves, in order to gain insight regarding whether or not these agents provided neuroprotection directly, or instead, indirectly via affecting brain vasculature (Candito et al., 1997; Shea et al., 2002a; Cacebelos et al., 2004).

Of interest would be to carry out longer tests (e.g., 9 mo; Veinbergs et al., 2000) as well regarding maintain mice on the deficient diet for varying periods prior to supplementation with these agents, in order to determine whether or not these agents could reverse oxidative damage and/or cognitive impairment. Also of interest would be to test multiple concentrations of these agents.

An important facet of the observed potentiation of the neurotoxic consequences of folate deprivation by ApoE deficiency is that genetic predispositions to AD, and/or other age-related neurodegenerative conditions, may remain latent pending age-related decline in nutrition (Shea and Rogers, 2002a). In this regard, ApoE deficiency can potentiate environmental, nutritional, and other genetic AD risk factors (Matsubara-Tsutsui et al., 2002; Shea and Rogers, 2002a, b; Marui et al., 2003; Pastor et al., 2003; DeMattos, 2004; Lahiri et al., 2004). Folate deprivation may also indirectly potentiate other neurodegenerative risk factors; for example, SAM depletion increases expression of another gene, presenilin-1, the overexpression of which is associated with AD (Scarpa et al., 2003; Popescu and Ankacrona, 2004). HC, which increases as a consequence of folate deprivation, potentiates Aβ neurotoxicity (Ho et al., 2001). Accordingly, folate deprivation may directly or indirectly potentiate multiple additional AD risk factors. In addition to dietary inadequacy, functional folate deficiency, and impaired function of the methionine cycle can result from polymorphisms of methionine cycle enzymes that exhibit diminished function. For example,

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polymorphisms of MTHFR that exhibit reduced activity induce mild hyperhomocysteinemia that is further augmented by diminished dietary folate, and is associated with arterial disease and venous thrombosis (Frosst et al., 1995; Arrula et al., 1997; Shields et al., 1999; Botto and Yang, 2000; Friso et al., 2002). Diminished MTHFR also reduces production of tetrahydrofolate and adenosylmethionine (required for DNA synthesis and methylation, respectively; Stern et al., 2000). MS deficiency results in hyperhomocysteinemia, homocysteinuria, and diminished cellular methionine (Ma et al., 1999; Watkins and Rosenblatt, 1989). Cystathionine _-synthase (CBS) converts HC to cysteine (Mudd et al., 1995) and promotes glutathione (GSH) synthesis. Polymorphisms of cysthathionine _-synthase (CBS) that exhibit diminished activity increasing HC export (leading to increased excitotoxicity; Ho et al., 2001; Kruman et al., 2000) and simultaneously decreasing GSH synthesis (Djurhuus et al., 1989) compensate oxidative buffering capacity by preventing GSH synthesis. Deficiency in CBS leads to homocysteinuria, a rare autosomal recessive disease of sulfur amino acid metabolism, and hyperhomocysteinemia, which results in multiple organ/system damage severe vascular disease and mental retardation (Mudd et al., 1995). Notably, a 36% increase in one particular MTHFR polymorphism has recently been reported among young people; such polymorphisms were present in 4.63% of individuals >24 yr of age, yet in 6.31% of those <24 yr of age (Reyes-Engel et al., 2002). The investigators considered that increased maternal dietary folate (confirmed in their samples) has allowed an increase in fetal viability despite latent deficiencies in MTHFR. These data indicate an increase in critical genetic deficiencies in folate metabolism within the population that may manifest only with age-related nutritional decline. The findings of the present study suggest that treatment with a combination of agents, perhaps including DZA, SAM, and NAC may be useful as part of a therapeutic approach to delay the onset or progression of AD.

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