

Folate deficiency and homocysteine induce toxicity in cultured dorsal root ganglion neurons via cytosolic calcium accumulation

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Summary

Folate deficiency induces neurotoxicity by multiple routes, including increasing cytosolic calcium and oxidative stress via increasing levels of the neurotoxin homocysteine (HC), and inducing mitochondrial and DNA damage. Because some of these neurotoxic effects overlap with those observed in motor neuron disease, we examined the impact of folate deprivation on dorsal root ganglion (DRG) neurons in culture. Folate deprivation for 2 h increased cytosolic calcium and reactive oxygen species (ROS) and impaired mitochondrial function. Treatment with nimodipine [an L voltage-sensitive calcium channel (LVSCC) antagonist], MK-801 (an NMDA channel antagonist) and thapsigargin (an inhibitor of efflux of calcium from internal stores) indicated that folate deprivation initially induced calcium influx via the LVSCC, with subsequent additional calcium derived from NMDA channels and internal stores. These compounds also reduced ROS and mitochondrial degeneration, indicating that calcium influx contributed to these phenomena. Calcium influx was prevented by co-treatment with 3-deaza-adenosine, which inhibits HC formation, indicating that HC mediated increased cytosolic calcium following folate deprivation. Nimodipine, MK-801 and thapsigargin had similar effects following direct treatment with HC as they did following folate deprivation. These findings support the idea that folate deprivation and HC treatment can compromise the health of DRG neurons by perturbing calcium homeostasis.

Key words: amyotrophic lateral sclerosis; calcium; dorsal root ganglion neurons; folate; homocysteine; motor neuron disease.

Introduction

Folate deficiency contributes to a variety of age-related neurological and psychological disorders including dementia, impaired cognition, depression, psychosis, Alzheimer's disease and Parkinson's disease (Mizrahi *et al.*, 2003; for reviews, see Mattson & Shea, 2002; Shea & Rogers, 2002a; Shea *et al.*, 2002a). Folate deficiency may also play a role in the pathogenesis of amyotrophic lateral sclerosis (ALS; reviewed in Yoshino, 1984; Shibata, 2001; Mattson & Shea, 2002; Menzies *et al.*, 2002). ALS is characterized by a progressive loss of motor neurons. Causative factors remain elusive despite decades of study (for review, see Strong, 2003). Studies from multiple laboratories implicate aberrant calcium accumulation, oxidative stress, mitochondrial dysfunction, perturbation of the axonal cytoskeleton, overactivation of one or more kinases and/or exposure to environmental neurotoxins at various stages in the disease progression. Notably, these are not mutually exclusive phenomena, because perturbations in calcium homeostasis can underlie all of them (Appel *et al.*, 2001).

Folate deficiency induces neurotoxicity by multiple routes, some of which have been shown to be relevant to ALS, including increasing cytosolic calcium and oxidative stress by increasing levels of the neurotoxin homocysteine (HC), decreasing endogenous antioxidants, depleting overall cellular methylation reactions, inducing mitochondrial and DNA damage, and depleting energy reserves and overactivation of kinases (Ho *et al.*, 2002, 2003; Kruman *et al.*, 2000, 2002; Shea & Rogers, 2002b). Increased HC accompanies folate deprivation because folate is a necessary co-factor for the enzyme (5,10-methylene tetrahydrofolate reductase) that mediates conversion of HC to methionine (Mudd *et al.*, 1965; Fiskerstrand *et al.*, 1997; Pietrzik & Bronstrup, 1998). Folate deprivation and resultant HC accumulation also potentiate the deleterious impact of certain other risk factors reported to contribute to motor neuron degeneration including excitotoxicity, glutamate and metal neurotoxicity (Gabbianelli *et al.*, 1999; Kruman *et al.*, 2000, 2002; Ho *et al.*, 2001, 2002, 2003; White *et al.*, 2001; Shea & Rogers, 2002b; Shea *et al.*, 2002b,c; Sung *et al.*, 2002).

We examined the consequences of folate deprivation and exposure to HC on dorsal root ganglion neurons (DRG) in culture, and the efficacy of supplementation with several pharmacological agents.

Materials and methods

Cell culture and treatment

DRG neurons were cultured from embryonic day 12 chicks in Ham's F12 medium containing 10% fetal calf serum and

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- 2** 25 ng mL⁻¹ nerve growth factor (NGF; Shea *et al.*, 2004) and utilized within 3–5 days after culturing. To monitor the consequences of folate deprivation, cultures received DMEM lacking folate (Sigma) for 2 h. Additional cultures received 250 µM HC in the presence of folate (Ho *et al.*, 2001), and 1 µM MK-801 (an antagonist of NMDA calcium channels) 100 nM thapsigargin (which inhibits efflux of calcium from internal stores; Gray & Patel, 1995), 1 µM nimodipine [an antagonist of the L voltage-sensitive calcium channel (LVSCC); Ueda *et al.*, 1997], 50 µM 3-deaza-adenosine [DZA; an inhibitor of S-adenosyl homocysteine (SAH) hydrolase; Jeong *et al.*, 1999; Ho *et al.*, 2003] in the presence or absence of folate or in the presence of folate and HC.

Analyses of calcium influx, reactive oxygen species, mitochondrial pathology and apoptosis

To monitor cytosolic calcium, cells were rinsed with serum-free medium and received 10 µL mL⁻¹ Fluo-3 (acetoxymethyl ester; Molecular Probes) for 30 min. Living cultures were then visualized under fluorescein UV optics (Ekinci *et al.*, 1999). To monitor intracellular peroxide concentrations as an index of reactive oxygen species (ROS), cultures received 10 µL mL⁻¹ DFCD (2',7'-dichlorofluorescein diacetate; Kodak) for 20 min and were visualized under fluorescein UV optics (Ekinci *et al.*, 1999). To monitor mitochondrial membrane potential, cells cultured for 2 h in the presence or absence of folate were rinsed with fresh DMEM and incubated in 1.5 µM mL⁻¹ of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide ('JC-1', Molecular Probes) for 10 min at 37 °C (Wadia *et al.*, 1998; Ho *et al.*, 2003), washed again with medium and images acquired under fluorescence and rhodamine UV optics. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, as indicated by a fluorescence emission shift from green to red; mitochondrial depolarization can therefore be quantified by a decrease in the red/green fluorescence intensity ratio (Reers *et al.*, 1991).

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 on a PowerPC Macintosh. 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. Statistical analyses were carried out by Student's *t*-test or ANOVA. Values were considered statistically different at $P \leq 0.05$.

To quantify whether 2 h of folate deprivation or HC treatment was lethal to DRG neurons, we quantified the total number of neurons in five random microscopic fields at 10× magnification in two cultures each from three separate experiments.

Results

DRG neurons demonstrated a two-fold increase in cytosolic calcium following folate deprivation for 2 h (Fig. 1). To determine the source of this increase, additional cultures received

nimodipine, MK-801 or thapsigargin. Nimodipine prevented this increase, whereas MK-801 and thapsigargin reduced, but did not prevent, the increase in cytosolic calcium. Consistent with the earlier demonstration that folate deprivation increases ROS and induces mitochondrial degeneration in cultured cortical neurons (Ho *et al.*, 2003), DRG neurons displayed a $33 \pm 14\%$ increase in ROS and a $23 \pm 7\%$ decrease in mitochondrial membrane potential following 2 h of folate deprivation (Fig. 1). Nimodipine prevented the increase in ROS; thapsigargin also prevented this increase, whereas MK-801 attenuated, but did not prevent, the increase in ROS that accompanied folate deprivation (Fig. 1). These calcium channel antagonists had markedly differing effects on mitochondrial health during folate deprivation; the decrease in mitochondrial membrane potential was prevented by MK-801, but only attenuated by nimodipine and thapsigargin (Fig. 1).

One major mechanism by which folate deprivation can promote calcium influx is by increased generation of HC (Pietrzik & Bronstrup, 1998). The SAH hydrolase inhibitor, DZA, prevents HC accumulation and resultant calcium influx following folate deprivation in cultured neurons (Ho *et al.*, 2003). Consistent with these prior studies, treatment of DRG neurons with 50 µM DZA prevented the increase in cytosolic calcium that normally accompanied folate deprivation (Fig. 1), suggesting that increased cytosolic calcium following folate deprivation of DRG neurons was mediated by increased HC generation. To test this possibility further, we treated DRG neurons with 250 µM HC. HC increased cytosolic calcium, ROS and mitochondrial membrane depolarization. The calcium antagonists demonstrated similar but not identical effects on HC treatment as were observed following folate deprivation. Nimodipine prevented the HC increase in cytosolic calcium and ROS, whereas MK-801 attenuated these increases. Thapsigargin attenuated the increase in cytosolic calcium, but completely prevented the increase in ROS. Only MK-801 prevented the decrease in mitochondrial membrane potential following HC treatment; neither nimodipine nor thapsigargin was effective (Fig. 1).

Neither folate deprivation nor HC treatment were lethal for this short interval (2 h); statistically identical numbers of neurons per microscopic field were observed following 2 h in the presence (4.4 ± 0.6) or absence (3.9 ± 0.9) of folate, or in the presence of HC (5.2 ± 2.4 ; $P = 0.53$; ANOVA). However, cultured neurons degenerate within 24 h following this extent of calcium influx unless accompanying ROS are quenched by antioxidants (Ekinci *et al.*, 1999), providing an experimental window for therapeutic attempts in relation to ALS.

Discussion

The findings of the present study indicate that even short-term folate deprivation can increase cytosolic calcium and ROS, and impair mitochondrial function. Reduction in ROS and mitochondrial degeneration by calcium channel antagonists demonstrated that increased cytosolic calcium contributed to these phenomena. Prevention of calcium accumulation by treatment with

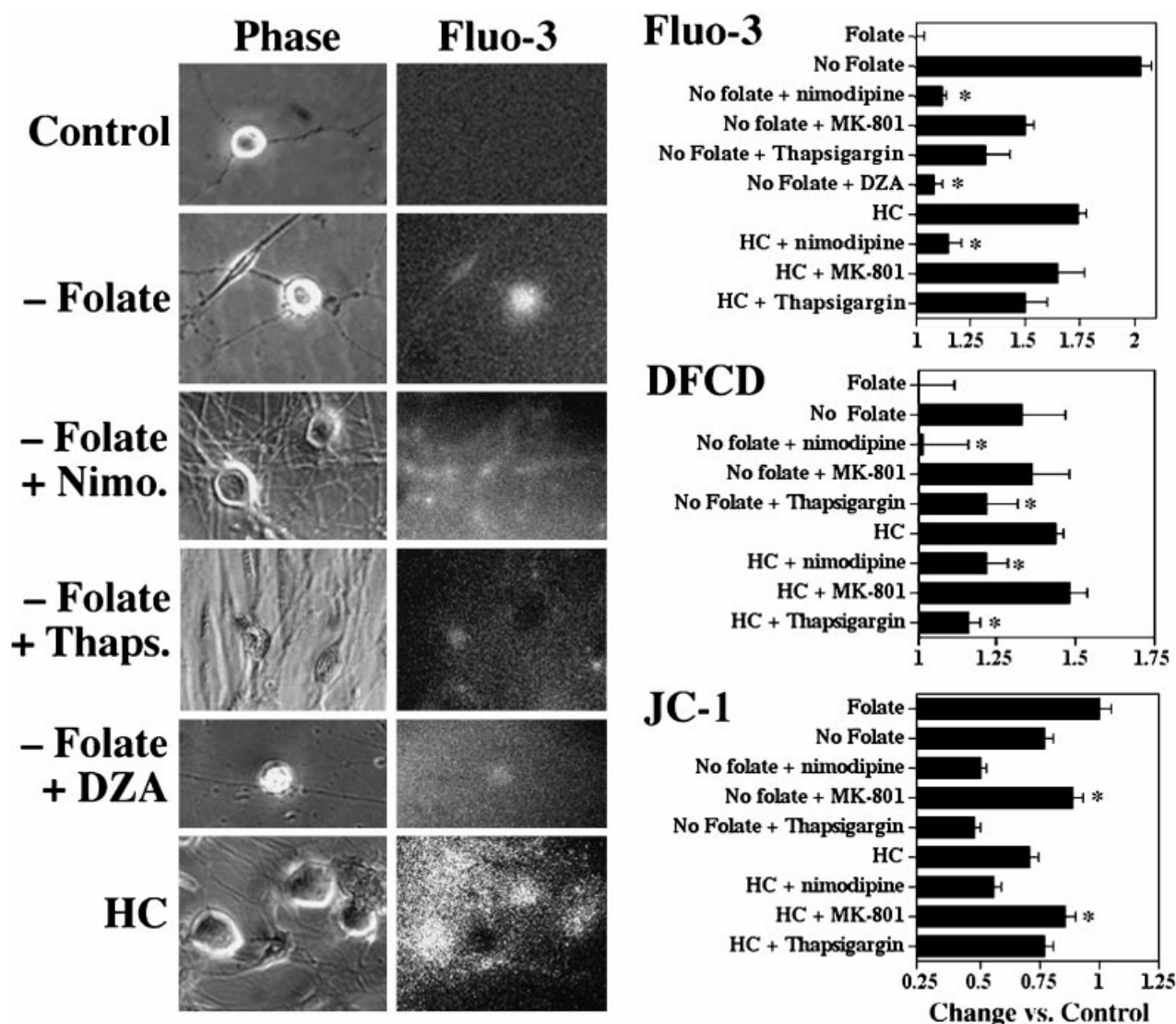


Fig. 1 Folate deprivation and HC treatment increase cytosolic calcium and ROS, and reduce the mitochondrial membrane potential: effect of calcium channel antagonists and DZA. The panels present phase-contrast and fluorescent images of representative DRG neurons cultured in the presence (control) or absence of folate for 2 h, with or without treatment with nimodipine, thapsigargin, DZA or HC in the presence of folate as indicated, after which cytosolic calcium was visualized with Fluo-3 as described in Materials and methods. The accompanying graphs present densitometric analyses of multiple neurons in duplicate cultures from 2 to 8 experiments. Neurons were incubated for 2 h in the presence or absence of folate with or without HC, DZA, nimodipine, MK-801 or thapsigargin as indicated, then probed for cytosolic calcium (Fluo-3), ROS (DFCD) or mitochondrial membrane potential (JC-1) as described in Materials and methods; HC was administered in the presence of folate. Values are presented as the relative change (mean \pm SEM) vs. values obtained for cultures receiving folate with no additional additives, which were defined as controls with values of 1. Values statistically identical to controls (ANOVA) are labelled with an asterisk. Note that values for individual cultures treated with JC-1 represent the ratio of red vs. green fluorescence; a decrease in this ratio indicates increased loss of membrane potential.

DZA, which inhibits HC formation (Ho *et al.*, 2003), coupled with the prior demonstration that HC activates calcium channels, also induces ROS and mitochondrial degeneration in cultured neurons (Kruman *et al.*, 2000; Ho *et al.*, 2001, 2002; White *et al.*, 2001). These data collectively suggest that increased HC formation is a major mechanism by which short-term folate deprivation can induce neurodegeneration in cultured DRG neurons. Treatment with the related compound homocysteic acid also induced calcium accumulation within cytosol of spinal neurons (Adalbert *et al.*, 2002).

Comparison of the relative increase in cytosolic calcium following treatment with various calcium channel antagonists allows speculation regarding the sequence of recruitment of individual channels, as well as the severity of calcium influx derived from each source (Mujumdar *et al.*, 2000; Ho *et al.*, 2002; Kim *et al.*, 2002). Prevention of increased cytosolic calcium by nimodipine, but only a reduction by treatment with MK-801 and thapsigargin, suggests that folate deficiency initially increases cytosolic calcium via the LVSCC, with subsequent additional influx of calcium via NMDA channels and efflux from

internal sources. Increased ROS was also prevented by thapsigargin, indicating that calcium derived from internal sources also contributed to ROS generation. Conversely, impaired mitochondrial function was prevented by treatment with MK-801, but not by either nimodipine or thapsigargin, suggesting that influx of calcium from NMDA channels was a crucial determinant of mitochondrial health. These findings for ROS and mitochondrial depolarization indicate that downstream effects, rather than the initial entry of calcium via the LVSCC, may mediate neuropathology. Analogous results were obtained following treatment of cortical neurons with amyloid-beta (Ekinici *et al.*, 1999), in which the initial influx of calcium was apparently benign provided downstream increases in ROS were quenched. Nimodipine, MK-801 and thapsigargin had similar effects on accumulation of cytosolic calcium, ROS and mitochondrial health following direct treatment with HC-increased cytosolic calcium; however, whereas MK-801 prevented mitochondrial damage following HC treatment, nimodipine and thapsigargin partially prevented this, suggesting that secondary influx via the LVSCC and efflux from internal stores contributed to mitochondrial degeneration following HC treatment. These findings are in contrast with that of another study in smooth muscle cells, in which HC first induced release of calcium from internal stores, followed by influx from the external environment (Mujumdar *et al.*, 2000); these differences may reflect the diverse physiology of neurons and muscle cells. Similarly, MK-801 prevented cytosolic calcium accumulation in cultured cortical neurons, whereas nimodipine was ineffective, suggesting that HC induced calcium influx into cortical neurons via NMDA channels (Ho *et al.*, 2002), while the present findings indicate that the LVSCC is the primary source of calcium influx following HC treatment of DRG neurons; these diverse results are also likely to reflect distinct differences between cortical and DRG neurons.

Deficiencies in folate metabolism may contribute to the progression of ALS by multiple mechanisms (Yoshino, 1984; Mattson & Shea, 2002). Certain mutations in Cu/Zn superoxide dismutase (SOD1) that promote a gain of toxic function and increased ROS are strongly associated with ALS (for reviews, see Julien, 1998; Strong, 2003). Accumulation of intracellular calcium is one of the primary mechanisms of motor neuronal degeneration associated with SOD1 mutations (for review, see Shibata, 2001). Calcium apparently accumulates from sequential sources (e.g. Van Den Bosch *et al.*, 2000; Kim *et al.*, 2002). Studies comparing influx of extracellular calcium vs. efflux of intracellular calcium from internal stores have suggested that a major source of the increased cytosolic calcium is derived from extracellular sources (Kim *et al.*, 2002). This process induces oxidative stress generation, resulting in motor neuronal death through the guanylyl cyclase–cGMP-dependent cascade. Perturbations in calcium homeostasis resulting from dietary deficiencies in folate, and/or genetic deficiencies in folate metabolism (Mattson & Shea, 2002), may potentiate the consequences of mutations in SOD1 that alter calcium and ROS levels. Increased free cytosolic copper, resulting from impaired binding of copper by mutant SOD1,

has been reported to increase oxidative stress in human neuroblastoma cells expressing mutant SOD1 (Gabbianelli *et al.*, 1999) and transgenic mice exhibiting aberrant copper metabolism (Sung *et al.*, 2002). HC potentiates copper neurotoxicity (White *et al.*, 2001), suggesting an additional mechanism by which aberrations in folate metabolism could contribute to ALS. Alterations in the mitochondrial electron transport chain have been reported in ALS (e.g. Menzies *et al.*, 2002) and represent an early pathological event in transgenic mice expressing mutant human SOD1 (Kong & Xu, 1998; Jung *et al.*, 2001, 2003); the findings of the present study leave open the possibility that folate deficiency could potentiate mitochondrial trauma. Folate deficiency may also potentiate the consequences of antibodies to calcium channels (including the LVSCC), found in sera from ALS patients, that increase cytosolic calcium in motor neurons in culture and in mice (Engelhardt *et al.*, 1995; Muchnik *et al.*, 2002).

These findings in culture support the use of nimodipine for treatment of ALS (Miller *et al.*, 1996; Kriz *et al.*, 2003). The findings of the present study also suggest that supplementation with the pharmacological inhibitor DZA, to inhibit HC accumulation and resultant cytosolic calcium accumulation, may be useful under conditions of folate deficiency (Sung *et al.*, 2002; Shea *et al.*, 2004). DZA also has anti-inflammatory effects (Krenitsky *et al.*, 1986; Medzihradsky *et al.*, 1992), which may provide an additional mechanism by which DZA reduces neurodegeneration (e.g. Launer, 2003; Naccari, 2003; Sun *et al.*, 2003). It is unclear at present whether effective administration of DZA may be compromised by the blood–brain barrier. However, DZA is a nucleoside analogue (similar nucleoside analogues such as AZT have been shown to enter the nervous system and are in current use for HIV therapies; e.g. Masereeuw *et al.*, 1994; Mayers *et al.*, 1995), and prevents the increased oxidative stress and cognitive impairment that otherwise accompanies folate deprivation in transgenic mice (Shea *et al.*, 2004).

The present findings in cultured DRG neurons support the idea that folate deprivation and HC treatment can contribute to the progression of ALS by multiple mechanisms (Yoshino, 1984; Mattson & Shea, 2002; Sung *et al.*, 2002). Homocysteine and homocystinuria should perhaps therefore be considered as potentially contributing factors in the development and progression of ALS. Prior studies have demonstrated that dietary folate deprivation and HC treatment potentiated the neurotoxic impact of genetic predisposition towards Alzheimer's disease in transgenic mice (Kruman *et al.*, 2002; Shea & Rogers, 2002a). In this regard, a growing body of evidence supports the idea that the interaction of dietary deficiencies and genetic predisposition exerts profound influence on age-related neurodegeneration (Mattson, 2003). Several studies support the efficacy of nutritional supplements on the progression of ALS and other motor neuron disorders, and suggest that commonalities may be observed among effective nutritional approaches for multiple age-related neurological disorders, including ALS, Alzheimer's disease and Parkinson's disease (reviewed in Cameron & Rosenfeld, 2002). It would be of interest therefore to test the consequences of

folate deprivation and HC treatment, coupled with calcium channel blockers and nutritional supplements (e.g. vitamin B6, which also regulates HC metabolism, and vitamin E, deficiency of which augments the deleterious impact of folate deficiency on neurodegeneration; Mattson & Shea, 2002), on mouse models of motor neuron disease (Sung *et al.*, 2002).

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Change to capitals	≡ under matter to be changed	≡
Change to small capitals	= under matter to be changed	=
Change to bold type	~ under matter to be changed	~
Change to bold italic	≡ under matter to be changed	≡
Change to lower case	Encircle matter to be changed	⊖
Change italic to upright type	(As above)	⤴
Insert 'superior' character	/ through character or ⤴ where required	γ under character e.g. γ
Insert 'inferior' character	(As above)	⤵ over character e.g. ⤵
Insert full stop	(As above)	⦿
Insert comma	(As above)	,
Insert single quotation marks	(As above)	γ and/or γ
Insert double quotation marks	(As above)	γ and/or γ
Insert hyphen	(As above)	Ⓜ
Start new paragraph	⤴	⤴
No new paragraph	~	~
Transpose	⤴	⤴
Close up	linking ⦿ letters	⦿
Insert space between letters	⤴ between letters affected	#
Insert space between words	⤴ between words affected	#
Reduce space between letters	↑ between letters affected	↑
Reduce space between words	↑ between words affected	↑