Protective Effects of Methylcobalamin, a Vitamin B_{12} Analog, Against Glutamate-Induced Neurotoxicity in Retinal Cell Culture

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Purpose. To examine the effects of methylcobalamin on glutamate-induced neurotoxicity in the cultured retinal neurons.

Methods. Primary cultures obtained from the fetal rat retina (gestation days 16 to 19) were used for the experiment. The neurotoxicity was assessed quantitatively using the trypan blue exclusion method.

Results. Glutamate neurotoxicity was prevented by chronic exposure to methylcobalamin and S-adenosylmethionine (SAM), which is formed in the metabolic pathway of methylcobalamin. Chronic exposure to methylcobalamin and SAM also inhibited the neurotoxicity induced by sodium nitroprusside that releases nitric oxide. By contrast, acute exposure to methylcobalamin did not protect retinal neurons against glutamate neurotoxicity.

Conclusions. Chronic administration of methylcobalamin protects cultured retinal neurons against N-methyl-D-aspartate-receptor-mediated glutamate neurotoxicity, probably by altering the membrane properties through SAM-mediated methylation. Invest Ophthalmol Vis Sci. 1997;38:848–854.

Vitamin B_{12} deficiency is well known to cause two clinical consequences (i.e., a megaloblastic anemia and a neuropathy, called subacute combined degeneration). Humans have two vitamin B_{12}-dependent enzymes (i.e., methionine synthase and methylmalonyl coenzyme mutase). Neuropathy has been found to occur because of lack of methionine synthase and not by a lack of activity by methylmalonyl coenzyme mutase.[1] Recently, the similarity in vacuolar myelopathy seen in patients with human immunodeficiency virus and patients with vitamin B_{12} deficiency[2,3] has increased interest in the scientific investigation of the mechanisms responsible for the neurologic degeneration in vitamin B_{12} deficiency.

S-adenosylmethionine (SAM) is a naturally occurring substance in many mammalian tissues, which is required in numerous transmethylation reactions involving nucleic acids, proteins, phospholipids, amines, and other neurotransmitters.[4,5] SAM is produced from condensation of methionine and adenosine triphosphate by the enzyme methionine adenosyltransferase. The daily dietary intake of methionine is not sufficient to supply the total amount required for SAM synthesis.[4] Thus, methionine has to be regenerated from homocysteine by vitamin B_{12}-dependent methionine synthase, because mammals are unable to synthesize homocysteine de novo. The transfer reaction of a methyl group from 5-methyltetrahydrofolate to homocysteine with regeneration of methionine, catalyzed by methionine synthase, requires methylcobalamin, an active coenzyme of vitamin B_{12} analogs, as a cofactor.

Recent evidence indicates glutamate as the proximate cause of neurodegeneration in several types of central nervous system insults, including hypoxia, ischemia, and hypoglycemia.[6–8] SAM has been reported to have a protective action against ischemia-induced neuronal death in the central nervous system.[9–11] Chronic exposure to methylcobalamin protected cultured cortical neurons against glutamate neurotoxicity.[12] There are some clinical reports on optic neuropathy associated with vitamin B_{12} deficiency.[13,14] Thus, the current study was undertaken to elucidate the effects of methylcobalamin and SAM against glutamate-induced neurotoxicity in cultured retinal neurons.

METHODS

Materials

Primary cultures obtained from the retinas of fetal rats (16 to 19 days' gestation) were used for the experi-

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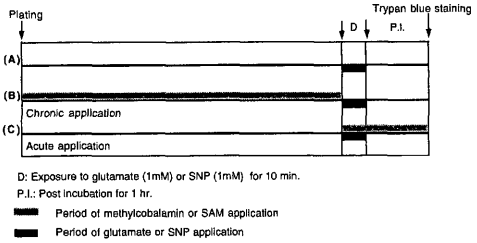

FIGURE 1. Methods of drug application. Cells were treated with 1 mM glutamate or sodium nitroprusside (SNP), followed by 1-hour postincubation in either glutamate-free or SNP-free medium (**A**). To examine chronic application of methylcobalamin or S-adenosylmethionine (SAM) (**B**), these agents were administrated immediately after plating until exposure to glutamate or SNP. To examine acute application of methylcobalamin or SAM (**C**), these agents were applied during glutamate or SNP treatment (10 minutes) and followed by postincubation (1 hour).

ments, and drug-induced neurotoxicity was assessed as described previously.[15-17] In brief, retinal tissues were dissociated mechanically and single-cell suspensions were plated on plastic coverslips (1.2 to 1.8 $\times$ 10^6 cells/dish). Cultures were incubated in Eagle's minimal essential medium (Eagle's salts, Nissui, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (1 to 8 days after plating), or 10% heat-inactivated horse serum (10 to 12 days after plating) containing 2 mM glutamine, 11 mM glucose (total), 24 mM sodium bicarbonate, and 10 mM HEPES. After a 6-day culture, growth of nonneuronal cells was removed by the addition of 10^{-5} M cytosine arabinoside. We used only those cultures maintained for 10 to 12 days in vitro and only isolated cells in this study. Clusters of cells were excluded from the results because cells located in the clusters could not be used for histologic experiments or intracellular Ca^{2+} recording. A previous immunocytochemical study showed that these isolated cells consisted mainly of amacrine cells.[15] All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Drug Application

In our previous study, we showed that cell viability was decreased by exposure to glutamate (1 mM) for 10 minutes followed by postincubation in glutamate-free medium for more than 1 hour.[15,17] Therefore, in the current study, cultures were exposed as follows (Fig. 1): 10 minutes to glutamate and sodium nitroprusside (SNP) followed by 1-hour after incubation in either glutamate-free or SNP-free medium (Fig. 1A). Protective effects of the drugs were assessed by chronic (Fig. 1B) and acute (Fig. 1C) drug application. To study chronic drug application, a drug was added to the incubation medium immediately after cell plating until immediately before glutamate or SNP exposure. We examined the effect of methylcobalamin (1 μM) application for 48 hours or 96 hours before glutamate exposure. Cell viability in the control culture was 88.8 $\pm$ 0.7%. Ten-minute exposure to glutamate followed by 1-hour incubation in glutamate-free medium decreased cell viability in cultures pretreated with methylcobalamin for 48 or 96 hours before glutamate exposure to 40.3 $\pm$ 1.3% or 43.5 $\pm$ 1.9%, respectively, whereas that of nontreated culture was decreased to 32.6 $\pm$ 0.8%. Although a significant difference ($P < 0.01$, by Dunnett's two-tailed test) was noted between cell viability in 48- or 96-hour pretreated cultures compared to that of nontreated cells, cell viability consistently remained more than 60% from the time the culture was added to the incubation medium immediately after cell plating until immediately before glutamate exposure. Thus, we investigated the effects of chronic application. The drug was removed from culture medium during glutamate or SNP exposure followed by postincubation. To assess the effects of acute drug application, a drug was added during glutamate or SNP exposure and was followed by postincubation.

Measurement of Neurotoxicity

The neurotoxic effects of glutamate or SNP were assessed quantitatively by trypan blue exclusion method as described previously.[15,17,18] All experiments were performed in Eagle's solution at 37°C. After the completion of drug treatment, cell cultures were stained with 1.5% trypan blue solution at room temperature for 10 minutes and then were fixed with isotonic formaldehyde solution (pH 7.0, 2°C to 4°C). The fixed cultures were rinsed with physiological saline and examined while under Hoffman modulation microscopy at $\times$400. More than 200 cells on each of 5 coverslips were counted randomly to determine the viability of the cell culture. We counted on the order of 10% to 20% of the total cells grown on each coverslip, because the overall density of the cultures was 50 to 100 cells/mm^2. Though there were slight individual differ-

TABLE 1. Effects of Chronic Applications of Methylcobalamin and S-adenosylmethionine

Treatment	*Viability (%)*	*% of Control*
Control (nontreated)	92.9 $\pm$ 0.4	100
Methylcobalamin (10^{-8} M)	92.9 $\pm$ 0.2	99.2 $\pm$ 0.2
Methylcobalamin (10^{-7} M)	94.2 $\pm$ 0.4	101.3 $\pm$ 0.4
Methylcobalamin (10^{-6} M)	95.2 $\pm$ 0.4	102.5 $\pm$ 0.4
Methylcobalamin (10^{-5} M)	96.1 $\pm$ 0.2	103.4 $\pm$ 0.2
Control (nontreated)	92.9 $\pm$ 0.4	100
S-adenosylmethionine (10^{-5} M)	90.2 $\pm$ 0.9	99.1 $\pm$ 1.0

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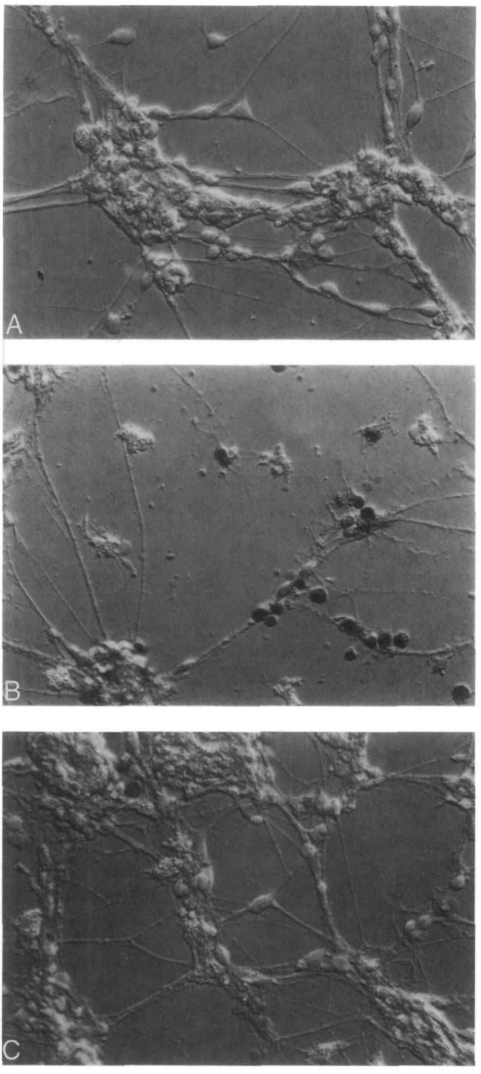

FIGURE 2. Photomicrographs showing the effect of chronic application of methylcobalamin or S-adenosylmethionine (SAM) on glutamate-induced neurotoxicity. All cultures were photographed after trypan blue staining followed by formaldehyde solution fixation using Hoffman modulation microscopy. Cells stained with trypan blue dye were regarded as nonviable. (**A**) Control (nontreated cells). Cells were stained without application of glutamate. (**B**) Cells were treated with glutamate (1 mM), followed by a 1-hour incubation with glutamate-free medium. Marked cell death occurred. (**C**) Cells were exposed chronically to methylcobalamin (1 μM) and then treated with glutamate (1 μM), followed by a 1-hour incubation with glutamate-free medium. Cell death was reduced markedly. Calibration bar = 50 μm.

ences in glutamate neurotoxicity and in the protective effects of the drugs, those individual differences did not disrupt the evaluation of drug-induced effects. Viability of culture was calculated as the percentage of the ratio of the number of unstained cells (viable cells) to the total number of cells counted (viable cells plus nonviable cells). In each experiment, five coverslips were used to obtain mean values ± standard error of the mean of cell viability. The significance of data was determined by Dunnett's two-tailed test.

Intracellular Ca^{2+} Recordings

Before recordings, the coverglasses were rinsed with HEPES-buffered salt solution, which contained (mM): sodium chloride 146, potassium chloride 4.2, magnesium chloride 0.5, calcium chloride 1.1, D-glucose 10, and HEPES 20, pH-adjusted to 7.4 with sodium hydroxide. The cells were loaded with Fura-2AM by incubation in 5 μM Fura-2AM in HEPES-buffered salt solution for 45 minutes at 37°C. After Fura-2AM loading, the cells were washed three times in HEPES-buffered salt solution and then were used in the experiments. HEPES-buffered salt solution also was used as the recording medium.

For excitation of Fura-2 fluorescence, ultraviolet (UV) light of 340 nm or 380 nm wavelength (UV340 or UV380) was produced by a xenon lamp through 340 ± 10 or 380 ± 10 nm narrow bandpass filters and was applied to the cultures through a 40× objective lens (Fluor 40; Nikon, Tokyo, Japan). Emission fluorescence was led to a silicon intensifier camera through a bandpass filter of 510 ± 10 nm. Data were processed to calculate the ratio R = F340/F380 (F340, fluorescent images with UV340; F380, fluorescent images with UV380). A calibration curve between R and intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) was obtained by measuring Rs of Ca^{2+}-EDTAOH buffer solutions and by least-square curve fitting with the equation $[Ca^{2+}]i = K (R - R_{min})/(R_{max} - R)$.[19] All procedures from image acquisition through $[Ca^{2+}]i$ calculation were accomplished with an image processor (ARGUS 50; Hamamatsu Photonics, Hamamatsu, Japan).

The following drugs were used: monosodium L-glutamate (Nakalai tesque, Kyoto, Japan), SNP (Wako, Osaka, Japan), MK-801 (Research Biochemicals, Natick, MA), Fura-2AM (Dojin, Kumamoto, Japan), methylcobalamin, and SAM (synthesized by Eizai, Tokyo, Japan).

RESULTS

Table 1 summarizes viability in cultured retinal neurons after chronic application of methylcobalamin and SAM.

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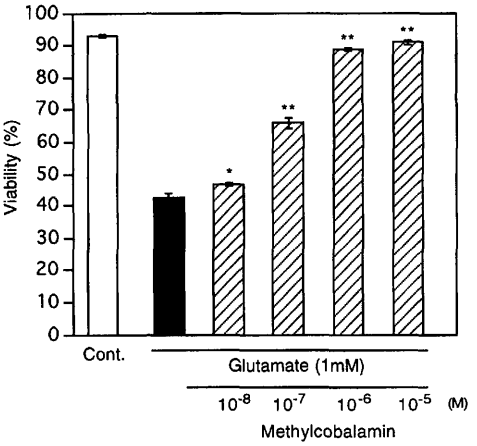

FIGURE 3. Protective action of methylcobalamin against glutamate-induced neurotoxicity. Chronic application of methylcobalamin showed protective effects against glutamate (1 mM) neurotoxicity in a dose-dependent manner at a concentration between 10^{-8} M and 10^{-5} M (* $P < 0.05$ versus the black column; ** $P < 0.01$ versus the black column). Error bars in this and the subsequent figure represent the standard errors of the means ($n = 5$).

These drugs were added respectively to the incubation medium immediately after cell plating until immediately before glutamate exposure as described in the Methods section. Neither methylcobalamin nor SAM at concentrations of 10^{-5} M induced significant changes in the viability of the cultures. Figure 2 shows an example of the effect of the chronic application of methylcobalamin on glutamate neurotoxicity. Under nontreated conditions (control), most cells were not stained by trypan blue (Fig. 2A), whereas the cells exposed briefly (10 minutes) to glutamate (1 mM) followed by 1-hour incubation in a glutamate-free medium markedly were stained by trypan blue (Fig. 2B). Chronic administration of methylcobalamin reduced cell death against glutamate neurotoxicity (Fig. 2C). Figure 3 showed quantitative assessment of the protective effect of chronic methylcobalamin administration against glutamate neurotoxicity. The protective effects of methylcobalamin was observed in a concentration-dependent manner at $10^{-8} - 10^{-5}$ M. SAM (1 μM) also showed protective action against glutamate neurotoxicity (Fig. 4). This effect was similar to those of 1 μM methylcobalamin and 10 μM MK-801, a selective NMDA blocker. Sodium nitroprusside is known to be as an NO-producing agent and 1 mM SNP exposure for 10 minutes, followed by incubation in SNP-free medium for 1 hour, also induced remarkable neurotoxicity in the cultured retinal neurons (Fig. 5). Both chronically applied methylcobalamin (1 μM) and SAM (1 μM) showed a protective effect against this neurotoxicity (Fig. 5).

As chronic application of methylcobalamin or SAM showed protective action against glutamate neurotoxicity, we examined whether the acute application of methylcobalamin or SAM was effective against glutamate neurotoxicity. As shown in Figure 6, acute application of methylcobalamin or SAM did not induce a protective action against glutamate neurotoxicity.

Glutamate-induced Ca^{2+} influx was measured to determine whether chronic application of methylcobalamin or SAM affected the Ca^{2+} influx, which was the first event of glutamate neurotoxicity. Retinal cultures were loaded with 5 μM Fura-2AM for 45 minutes, and fluorescence was examined by fluorescence microscopy. Intracellular Ca^{2+} level was increased immediately after a brief exposure to glutamate (50 μM, 10 seconds). Figure 7 shows examples of intracellular Ca^{2+} responses of cultured retinal neurons after a brief exposure to glutamate (50 μM, 10 seconds). Each culture dish (Fig. 7A, nontreated; Fig. 7B, chronic application of methylcobalamin; Fig. 7C, chronic application of SAM) was loaded with Fura-2AM and baseline intracellular Ca^{2+} level was acquired (Fig. 7, Before). Ten seconds after glutamate exposure, intracellular Ca^{2+} level markedly was increased (Fig. 7, $t = 10$). Then, the intracellular Ca^{2+} concentration was reduced gradually and was almost at baseline after 4 minutes followed by glutamate application (Fig. 7, $t = 240$). Table 2 summarizes the maximal intracellular Ca^{2+} concentra-

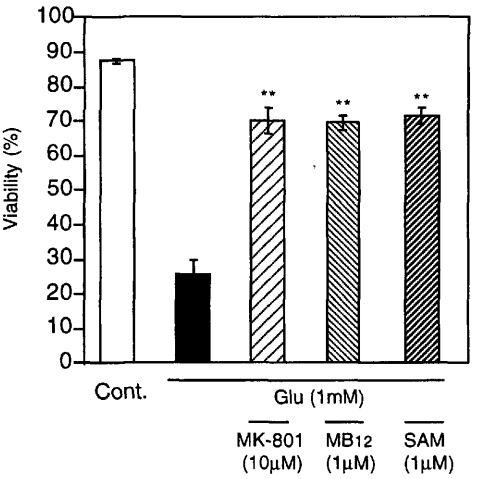

FIGURE 4. Effects of S-adenosylmethionine (SAM) on glutamate neurotoxicity. Chronic application of SAM (1 μM) showed a protective effect against glutamate neurotoxicity similar to those of methylcobalamin (MB12, chronic application) and MK-801, a selective NMDA channel blocker. The value 10 μM MK-801 was applied simultaneously with glutamate for 10 minutes and postincubation medium was both glutamate- and MK-801-free. In this treatment, MK-801 inhibited glutamate-induced cell death (**$P < 0.01$ versus the black column).

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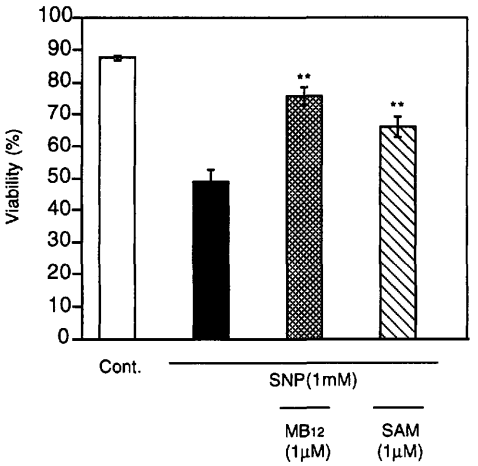

FIGURE 5. Protective effects of chronic application of methylcobalamin (MB12) and S-adenosylmethionine (SAM) against sodium nitroprusside (SNP)-induced neurotoxicity. A value of 1 mM SNP exposure for 10 minutes, followed by incubation in SNP-free medium for 1 hour, induced remarkable cell death in this culture. This SNP-induced neurotoxicity was prevented by chronic application of methylcobalamin (1 μM) or SAM (1 μM) ($^{**}P < 0.01$ versus the black column by Dunnett's two-tailed test).

tions induced by brief application of glutamate (50 μM, 10 seconds). Chronic application of neither methylcobalamin nor SAM affected the maximal intracellular Ca^{2+} concentration induced by glutamate in comparison to that of the control.

DISCUSSION

In the current study, chronic administration of methylcobalamin showed the protective action against glutamate-induced neurotoxicity. Glutamate neurotoxicity also was prevented by SAM, which is formed as an intermediate metabolite of methylcobalamin in the metabolic route of vitamin B_{12}.[4] These results indicate that the effects of methylcobalamin are caused by the formation of SAM. In contrast to chronic administration where marked protection against glutamate neurotoxicity was noted, acute application of methylcobalamin or SAM was not effective in restoring cell viability after glutamate exposure. In acute experiments, methylcobalamin or SAM was administered during both 10-minute glutamate application and 1-hour postincubation periods. In chronic experiments, methylcobalamin and SAM were added to the incubation medium after cell plating until immediately before the exposure of cells to glutamate. However, the drug was removed from the glutamate-containing medium as well as from glutamate-free medium for the subsequent 1-hour incubation period. Thus, protective actions seen

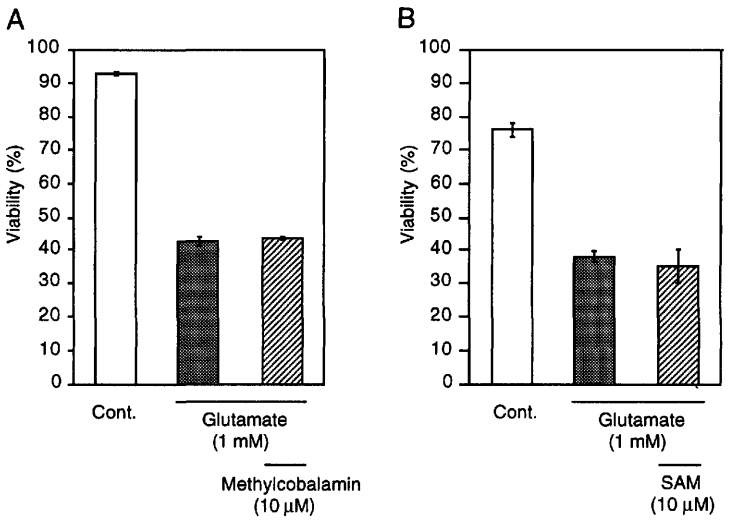

FIGURE 6. The effects of acute application of methylcobalamin or S-adenosylmethionine (SAM). When methylcobalamin (10 μM) or SAM (10 μM) was administered during glutamate exposure (10 minutes) followed by postincubation (1 hour), neither methylcobalamin nor SAM had any significant influence on glutamate neurotoxicity.

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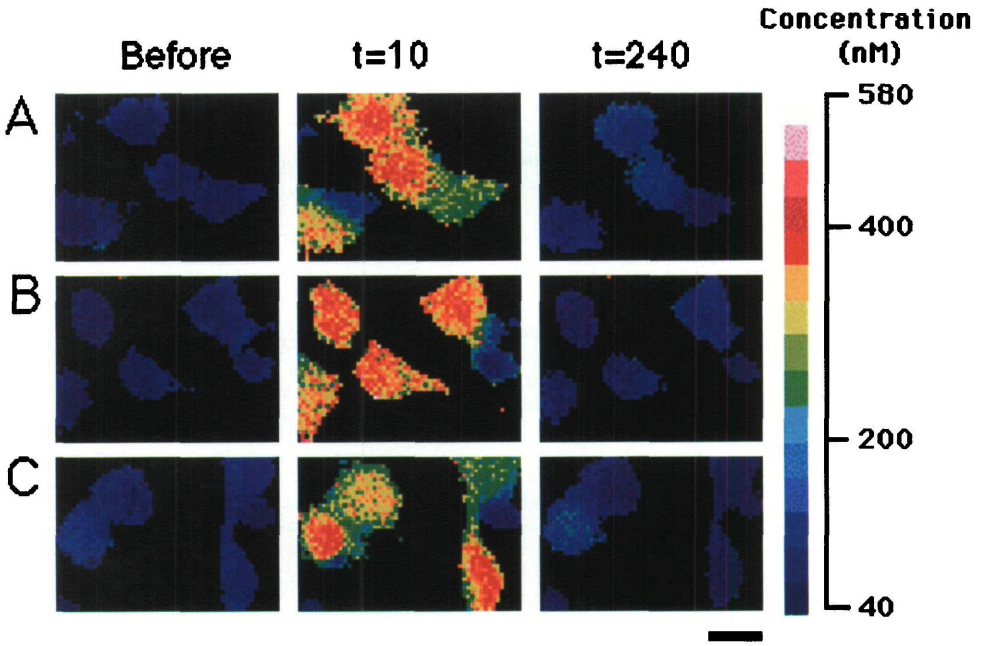

FIGURE 7. The temporal profile of calcium images of retinal cultures at the indicated time (seconds) after exposure to 50 μM glutamate for 10 seconds. (**A**) Nontreated (control), (**B**) chronic application of methylcobalamin (1 μM), (**C**) chronic application of S-adenosylmethionine (1 μM). Pseudocolor representation of fluorescence intensities, using arbitrary fluorescence intensity units indicated on the linear color scale (calibration bar = 20 μm).

only after chronic application of methylcobalamin or SAM make direct interaction of methylcobalamin to glutamate receptors less likely to take place. This notion also is supported by findings with Ca^{2+} influx measurements. Considering that chronic application of methylcobalamin or SAM might alter the expression of NMDA receptors (i.e., might reduce receptor expression in the cultured retinal neurons), we investigated whether chronic administration of the drug would affect the Ca^{2+} influx through NMDA receptors, the first event of glutamate-induced neurotoxicity. Neither methylcobalamin nor SAM had significant effects on glutamate-induced Ca^{2+} influx (Table 2). Thus, chronically applied methylcobalamin was less likely to reduce NMDA receptor expression. However, because findings were based on a gross measure of similarity in Ca^{2+} influx in response to glutamate between nontreated and pretreated cultured neurons, we cannot dismiss completely the effect of chronic treatment of methylcobalamin on the expression of NMDA receptors in the cultured retinal neurons.

TABLE 2. Maximal Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_{imax}$) Induced by Brief Application of Glutamate*

Treatment	$[Ca^{2+}]_{imax}$ *(nM)*
Control (nontreated)	415.4 ± 44.6
Methylcobalamin (1 μM)†	398.0 ± 15.8
SAM (1 μM)†	398.6 ± 30.5

SAM = S-adenosylmethionine.
There were no significant differences among the three groups.
* 50 μM, 10 seconds, n = 5.
† Methylcobalamin and SAM were treated chronically.

Chronic application of methylcobalamin or SAM prevented not only glutamate-induced neurotoxicity but SNP-induced neurotoxicity as well in a dose-dependent manner. Sodium nitroprusside is a classic NO donor and clinically is used to produce NO. The results indicate that retinal cultures treated chronically with methylcobalamin or SAM became tolerant to NO-induced neurotoxicity. Our previous study showed that in cultured retinal neurons, NO alone had no toxic effects on retinal neurons but became toxic in the presence of superoxide anion ($O_2^{\bullet -}$).[16] The peroxynitrite anion formed as a result of the reaction between NO and $O_2^{\bullet -}$ decomposes to a hydroxy racial ($^{\bullet}OH$)[20] that reacts at great speed with mem-

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brane lipids. The retinal neurons contain large amounts of polyunsaturated fatty acids within membranes, which particularly are vulnerable to free radical attack. The double bonds of polyunsaturated fatty acid side chains in membrane allow easy removal of hydrogen atoms by ·OH. Thus, the action of a single molecule of ·OH can initiate a chain reaction that generates numerous toxic reactants that rigidify membranes by cross-linking, disrupt membrane integrity, and damage membrane proteins.[21] S-adenosylmethionine has been known to increase the fluidity of neuronal membranes by increasing the phosphatidylcholine content of the lipid core of the membrane.[22] Recently, enzymatic methylations mediated by SAM also have been shown in the rat retina, which include phospholipid N-methyltransferases (I and II) and fatty acid carboxymethylase.[23] These membrane phospholipid N-methylation and fatty acid carboxymethylation are known to alter membrane fluidity and thereby alter membrane function and activities of membrane-bound enzymes.[5] Thus, SAM-mediated methylation induces some alterations in the membrane properties of cultured retinal neurons maintained in the methylcobalamin-containing medium, which reinforce the cell body, making it more resistant to radical toxicity.

In conclusion, chronic administration of methylcobalamin protects cultured retinal neurons against NMDA-receptor-mediated glutamate neurotoxicity. It is suggested that altered membrane properties induced by SAM-mediated methylation is the predominant route of the neuroprotective effect of methylcobalamin. However, the current study does not exclude completely the possibility that the effect was related to generation of new glutamate-resistant cell types rather than to a change in glutamate sensitivity in the original cells. Further studies are necessary to determine the mechanism related to the neuroprotective action of methylcobalamin.

Key Words

glutamate, methylcobalamin, retinal cell culture, S-adenosylmethionine, vitamin B_{12}

References

1. McKeever M, Molloy A, Weir DG, et al. An abnormal methylation ratio induces hypomethylation in vitro in the brain of pig and man, but not in rat. *Clin Sci.* 1995;88:73–79.
2. Gray F, Gheradi R, Scaravilli F. The neuropathology of the acquired immune deficiency syndrome (AIDS): A review. *Brain.* 1988;111:245–266.
3. Petito CK, Navia BA, Cho ES. Vacuolar myelopathy resembling subacute combined degeneration in patients with the acquired immunodeficiency syndrome. *New Engl J Med.* 1985;312:874–879.
4. Bottiglieri T, Hyland K, Reynolds EH. The clinical potential of ademethionine (S-adenosylmethionine) in neurological disorders. *Drugs.* 1994;48:137–152.
5. Baldessarini RJ. Neuropharmacology of S-adenosyl-L-methionine. *Am J Med.* 1987;83:95–103.
6. David P, Lusky M, Teichberg VI. Involvement of excitatory neurotransmitters in the damage produced in chick embryo retinas by anoxia and extracellular high potassium. *Exp Eye Res.* 1988;46:657–662.
7. Mosinger JL, Olney JW. Photothrombosis-induced ischemic neuronal degeneration in the rat retina. *Exp Neurol.* 1989;105:110–113.
8. Yoon YH, Marmor MF. Dextromethorphan protects retina against ischemic injury in vivo. *Arch Ophthalmol.* 1989;107:409–411.
9. Yamamoto Y, Shibata S, Hara C, et al. Methylcobalamin attenuates the hypoxia/hypoglycemia- or glutamate-induced reduction in hippocampal fiber spikes in vitro. *Eur J Pharmacol.* 1995;281:335–340.
10. Matsui Y, Kubo Y, Iwata N. S-adenosyl-L-methionine prevents ischemic neuronal death. *Eur J Pharmacol.* 1987;144:211–216.
11. Trovarelli G, de Medio GE, Porcellati S, et al. *Neurochem Res.* 1983;8:1597–1609.
12. Akaike A, Tamura Y, Sato Y, el al. Protective effects of a vitamin B12 analog, methylcobalamin, against glutamate cytotoxicity in cultured cortical neurons. *Eur J Pharmacol.* 1993;241:1–6.
13. Areekul S, Roongpisuthipong C, Churdchu K, et al. Optic neuropathy in a patient with vitamin B12 deficiency: A case report. *J Med Assoc Thai.* 1992;75:715–718.
14. Stambolian D, Behrens M. Optic neuropathy associated with vitamin B12 deficiency. *Am J Ophthalmol.* 1977;83:465–468.
15. Kashii S, Takahashi M, Mandai M, et al. Protective action of dopamine against glutamate neurotoxicity in the retina. *Invest Ophthalmol Vis Sci.* 1994;35:685–695.
16. Kashii S, Mandai M, Kikuchi M, et al. Dual action of nitric oxide in N-methyl-D-aspartate receptor-mediated neurotoxicity in the cultured retinal neurons. *Brain Res.* 1996;711:93–101.
17. Kikuchi M, Kashii S, Honda Y, et al. Protective action of zinc against glutamate neurotoxicity in cultured retinal neurons. *Invest Ophthalmol Vis Sci.* 1995;36:2048–2053.
18. Choi D, Maulucci-Gedde M, Kriegstein A. Glutamate neurotoxicity in cortical cell culture. *J Neurosci.* 1987;7:357–368.
19. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem.* 1985;260:3440–3450.
20. Beckman JS, Beckman TW, Chen J, et al. Apparent hydroxylradical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA.* 1990;87:1620–1624.
21. Coyle JT, Puttfarcken P. Oxidative stress, glutamate, and neurodegenerative disorders. *Science.* 1993;269:689–695.
22. Muccioli G, Scordamaglia A, Bertacco S, et al. Effect of S-adenosyl-L-methionine on brain muscarinic receptors of aged rats. *Eur J Pharmacol.* 1992;227:293–299.
23. Sastry BVR, Vidaver PS, Janson VE, et al. S-adenosyl-L-methionine-mediated enzymatic methylations in the rat retinal membranes. *J Ocular Pharmacol.* 1994;10:253–263.

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