Experimental Neurology 222 (2010) 191–203

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Methylcobalamin increases Erk1/2 and Akt activities through the methylation cycle and promotes nerve regeneration in a rat sciatic nerve injury model ☆

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ARTICLE INFO

Article history:
Received 28 August 2009
Revised 24 November 2009
Accepted 17 December 2009
Available online 4 January 2010

Keywords:
Methylcobalamin
Sciatic nerve
Cerebellar granule neuron
Dorsal root ganglion
Neurite outgrowth
Neuronal survival

ABSTRACT

Methylcobalamin is a vitamin B12 analog and is necessary for the maintenance of the nervous system. Although some previous studies have referred to the effects of methylcobalamin on neurons, the precise mechanism of this effect remains obscure. Here we show that methylcobalamin at concentrations above 100 nM promotes neurite outgrowth and neuronal survival and that these effects are mediated by the methylation cycle, a metabolic pathway involving methylation reactions. We also demonstrate that methylcobalamin increases Erk1/2 and Akt activities through the methylation cycle. In a rat sciatic nerve injury model, continuous administration of high doses of methylcobalamin improves nerve regeneration and functional recovery. Therefore, methylcobalamin may provide the basis for better treatments of nervous disorders through effective systemic or local delivery of high doses of methylcobalamin to target organs.

Introduction

Vitamin B12 (cobalamin) is important for normal functioning of the nervous system and its deficiency causes a systemic neuropathy called subacute combined degeneration of the spinal cord (Scalabrino et al., 1990). Vitamin B12 has analogs such as cyanocobalamin (CNCbl), methylcobalamin (MeCbl), hydroxocobalamin (OHCbl), and adenosylcobalamin (AdoCbl). In mammalian cells, CNCbl and OHCbl are inactive forms, AdoCbl acts as a coenzyme of methylmaronyl Co-A mutase in mitochondria, and MeCbl acts as a coenzyme of methionine synthase (MS), which is required for the formation of methionine from homocysteine in the methylation cycle that involves methylation of DNA or proteins (Banerjee and Ragsdale, 2003; Ghosh et al., 1991; Pfohl-Leszkowicz et al., 1991; Toohey, 2006). There are reports that vitamin B12, including MeCbl, has a beneficial effect on the nervous system. *In vitro*, the vitamin B complex, including vitamin B12, promotes neurite outgrowth and vitamin B12-enriched medium produces the greatest mean neurite outgrowth (Fujii et al., 1996). MeCbl protects cortical neuron and retinal cell cultures against glutamate cytotoxity (Akaike et al., 1993; Kikuchi et al., 1997). In *in vivo* studies, high doses of MeCbl improved nerve conduction and regeneration in a rat sciatic nerve injury model (Yamatsu et al., 1976a,b), streptozotocin-diabetic rats (Sonobe et al., 1988), and experimental acrylamide neuropathy (Watanabe et al., 1994). MeCbl promoted regeneration of motor nerve terminals that were degenerating in the anterior gracile muscle of the gracile axonal dystrophy mutant mouse (Yamazaki et al., 1994). Despite these previous reports about the effects of vitamin B12, including MeCbl, on neurons, the most effective analog and its mechanism of action on neurons remains to be clarified.

In the nervous system, the protein kinases Erk1/2 and Akt are activated by certain neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Segal, 2003). Activation of Erk1/2 promotes neurite outgrowth and Akt mediates branching of neurites in dorsal root ganglion (DRG) neurons (Markus et al., 2002). The survival of cerebellar granule neurons (CGN) is promoted by the activation of Erk1/2 (Bonni et al., 1999) and Akt (Bhave et al., 1999). The activation of Erk1/2 and Akt induced by the FGL peptide (a fibroblast growth factor receptor agonist) results in neurite outgrowth and neuronal survival in primary rat neurons (Neiiendam et al., 2004).

In this study, we demonstrate that MeCbl is the most effective of several vitamin B12 analogs in promoting neurite outgrowth and neuronal survival with activation of Erk1/2 and Akt and that these effects are brought about through the methylation cycle. We also show that MeCbl promotes nerve regeneration and functional recovery in a rat model of sciatic nerve injury.

☆ Methylcobalamin promotes nerve regeneration.

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doi:10.1016/j.expneurol.2009.12.017

Materials and methods

Cell culture

DRG neurons were cultured as described previously (Higuchi et al., 2003). DRG were obtained from Wistar rats at postnatal day (P9) and dissociated by incubation with 0.25% trypsin, 0.1% collagenase, and 200 U/ml DNase I for 30 min at 37 °C. The enzymatic reaction was blocked by adding Dulbecco's modified Eagle's medium (DMEM, not including vitamin B12) containing 10% fetal bovine serum, and after trituration and centrifugation, the cells were resuspended in modified Sato medium (DMEM containing 5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, 0.1 μg/ml L-thyroxine, 0.08 μg/ml triiodo-L-thyronine, and 4 mg/ml bovine serum albumin) and plated on poly-L-lysine-coated four-well chamber slides. Proliferation of non-neuronal cells was prevented by addition of 10 μM 5-fluoro-2′-deoxyuridine.

For CGN culture, the cerebellum was removed from Wistar rats at P9 as described previously (Mimura et al., 2006) and dissociated by incubation with 0.25% trypsin and 200 U/ml DNase I for 30 min at 37 °C. The enzymatic reaction was blocked by adding DMEM containing 10% fetal bovine serum, and after trituration and centrifugation, the cells were resuspended in modified Sato medium. The cells were centrifuged and resuspended in modified Sato medium again, and then plated on poly-L-lysine-coated culture dishes or four-well chamber slides.

Immunocytochemistry

The four-well chamber or glass slides were fixed in 4% paraformaldehyde (PFA) for 30 min, blocked for 1 h, and incubated overnight at 4 °C with primary antibody, followed by incubation for 1 h at room temperature with secondary antibody as described previously (Temporin et al., 2008a,b), and nuclei were labeled with DAPI (Wako Pure Chemical Industries, Osaka, Japan). For the terminal transferase dUTP nick-end-labeling (TUNEL) assay, the slides were stained with the DeadEnd™ Fluorometric TUNEL System (Promega Corporation, Madison, WI) after incubation with secondary antibody. The primary antibody was anti-neural class III β-tubulin (TuJ1) mouse monoclonal antibody (1:1000; Covance, Berkeley, CA), and the secondary antibodies were Alexa 488-labeled goat anti-mouse IgG antibody (1:1000; Molecular Probes, Eugene, OR) or Alexa 568-labeled goat anti-mouse IgG antibody (1:1000; Molecular Probes).

Neurite outgrowth assay

Neurons were cultured with CNCbl (10 μM; Sigma-Aldrich, St. Louis, MO), MeCbl (1 nM–100 μM; Sigma-Aldrich), OHCbl (10 μM; Sigma-Aldrich), AdoCbl (10 μM; Sigma-Aldrich), S-adenosylmethionine (SAM, 10 μM; Sigma-Aldrich), 4-nitro-2,1,3-benzothiadiazole (Nbtd, 10 μM; Sigma-Aldrich), U0126 (10 μM; Cell Signaling Technology, Beverly, MA), LY294002 (50 μM; Cell Signaling Technology), or caspase inhibitor Z-VAD-FMK (Z-VAD, 20 μM; Promega Corporation) for 72 h and then immunostained with anti-TuJ1 antibody. Total neurite length per neuron and the axonal length (the length of the longest neurite per TuJ1-positive neuron) were measured using an image analyzer (Lumina Vision; Mitani Co., Fukui, Japan) as described previously (Tanaka et al., 2002). Only neurites longer than 20 μm (about the diameter of a soma) and not in contact with other cells were measured. The mean total neurite length and axonal length were calculated from at least 30 neurons in each experiment.

Neuronal survival assay

Neurons were cultured with CNCbl (10 μM), MeCbl (1 nM–100 μM), AdoCbl (10 μM), OHCbl (10 μM), SAM (10 μM), Nbtd (1 μM), U0126 (10 μM), or LY294002 (50 μM) for 72 h. Kainic acid (100 μM; Calbiochem, San Diego, CA) was added to the medium of CGN cultures 48 h after starting the culture, and thereafter, the neurons were cultured for 24 h. The neurons were visualized by staining with anti-TuJ1 antibody and apoptotic cells were visualized in the TUNEL assay. Normal and apoptotic cells were counted in ten randomized 200× fields using an image analyzer (Lumina Vision). The apoptotic rates were calculated by dividing the number of apoptotic cells by the total number of cells. More than 100 cells were counted in each experiment.

Western blotting

CGN were cultured with BDNF (100 ng/ml; Sigma-Aldrich) or MeCbl (10 μM). At time points for 5 min to 72 h, cultured cells were homogenized with Kaplan buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 1% NP40, and protease inhibitor cocktail) and clarified by centrifugation. They were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk for 1 h, the membranes were incubated with anti-p44/42 MAPK rabbit polyclonal antibody (1:1000; Cell Signaling Technology), anti-phospho-p44/42 MAPK rabbit monoclonal antibody (1:1000; Cell Signaling Technology), anti-Akt rabbit polyclonal antibody (1:1000; Cell Signaling Technology), or anti-phospho-PKB (phospho-Akt) (pSer473) rabbit polyclonal antibody (1:1000; Sigma-Aldrich) at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:1000; GE Healthcare, Little Chalfont, UK) and ECL reagents (GE Healthcare). The densities of Erk1/2, phospho-Erk1/2, Akt, and phospho-Akt were measured using Scion Image software (Scion Corporation, Frederick, MD). To calculate the normalized density, the density of phospho-Erk1/2 or phospho-Akt was divided by the density of Erk1/2 or Akt, respectively, in the same membrane.

Surgical procedures

All animal experiments were approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

Eight-week-old female Wistar rats (180–220 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The left sciatic nerve was exposed at mid-thigh level and cut with microscissors. The dissection site was sutured end to end with 10-0 nylon, and the skin was then sutured with 3-0 nylon. Continuous administration of phosphate-buffered saline (PBS) or MeCbl (1 mg/kg/day) in PBS for 12 weeks was performed using an osmotic minipump (Model 2ML4; Alzet, Cupertino, CA), which was placed subcutaneously in the right side of the back. All surgeries were performed by the same surgeon.

Serum concentration of vitamin B12

Blood samples (0.5 ml) were collected from the jugular vein or the right ventricle of rats under sodium pentobarbital anesthesia 3 days after administration. Samples were centrifuged for 20 min at 800 *g* and the supernatant was collected. Serum concentrations of vitamin B12 were measured from four rats from the MeCbl and PBS group each, by chemiluminescent immunoassay performed at Bio Medical Laboratories, Tokyo, Japan.

Motor and sensory functional analysis

To evaluate sensory function, the toe pinch test was performed every week, in which the reaction to pinching the third, fourth, and fifth toes with fine forceps was graded based on the withdrawal reflex, escape behavior, and vocalization (0, normal, brisk withdrawal reflex, escape behavior, and strong vocalization; 1, mildly impaired; 2,