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Research paper

Vitamin B_{12} deficiency results in severe oxidative stress, leading to memory retention impairment in *Caenorhabditis elegans*

Tomohiro Bito[a,1], Taihei Misaki[a], Yukinori Yabuta[a], Takahiro Ishikawa[b], Tsuyoshi Kawano[a], Fumio Watanabe[a,*]

[a] The School of Agricultural, Biological and Environmental sciences, Faculty of Agriculture, Tottori University, Tottori 680-8533, Japan
[b] Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Shimane 690-8504, Japan

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ABSTRACT

Oxidative stress is implicated in various human diseases and conditions, such as a neurodegeneration, which is the major symptom of vitamin B_{12} deficiency, although the underlying disease mechanisms associated with vitamin B_{12} deficiency are poorly understood. Vitamin B_{12} deficiency was found to significantly increase cellular H_2O_2 and NO content in *Caenorhabditis elegans* and significantly decrease low molecular antioxidant [reduced glutathione (GSH) and *L*-ascorbic acid] levels and antioxidant enzyme (superoxide dismutase and catalase) activities, indicating that vitamin B_{12} deficiency induces severe oxidative stress leading to oxidative damage of various cellular components in worms. An NaCl chemotaxis associative learning assay indicated that vitamin B_{12} deficiency did not affect learning ability but impaired memory retention ability, which decreased to approximately 58% of the control value. When worms were treated with 1 mmol/L GSH, *L*-ascorbic acid, or vitamin E for three generations during vitamin B_{12} deficiency, cellular malondialdehyde content as an index of oxidative stress decreased to the control level, but the impairment of memory retention ability was not completely reversed (up to approximately 50%). These results suggest that memory retention impairment formed during vitamin B_{12} deficiency is partially attributable to oxidative stress.

1. Introduction

After vitamin B_{12} (B_{12}) is accumulated by living cells, it is converted into two coenzymes, 5′-deoxyadenosylcobalamin and methylcobalamin, which function as the coenzymes for methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) [1] and methionine synthase (MS; EC 2.1.1.13) [2], respectively. MCM catalyzes the conversion of *L*-methylmalonyl-CoA into succinyl-CoA in the catabolic pathway of certain amino acids, odd-numbered fatty acids, cholesterol, and thymine [3]. MS catalyzes methionine synthesis from homocysteine (Hcy) with 5′-methyltetrahydrofolate. During B_{12} deficiency, the failure of B_{12}-dependent methionine biosynthesis leads to the accumulation of Hcy [4], which has prooxidant activity [4] and can activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate reactive oxygen species (ROS) [5]. These observations suggest that B_{12} deficiency disrupts cellular redox homeostasis to induce oxidative stress, which is implicated in various human diseases including atherosclerosis and neurodegenerative diseases [6]. However, there is little evidence on the relationship between the symptoms (growth retardation, metabolic disorders, megaloblastic anemia, and neurologic disorders [7]) of B_{12} deficiency and oxidative stress (or disturbances in the normal redox state).

In humans, insulin appears to regulate learning and memory in the central nervous system as well as energy metabolism in the peripheral tissues [8]. Insulin is synthesized in the brain [9], and its receptors are localized at synapses [10]. The insulin/phosphoinositide 3-kinase pathway in neural and behavioral plasticity is highly conserved in mammals [11], and it regulates various neurological processes. Inhibition of this signaling pathway in the central nervous system causes learning and memory impairment [12].

The nervous system of *Caenorhabditis elegans* comprises 302 neurons, and their neural connections have been extensively studied [13]. Worms sense various chemicals by sensory neurons and exhibit

Abbreviations: Cat, catalase; DNPH, 2,4-dinitrophenylhydrazine; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; Hcy, homocysteine; HPLC, high-performance liquid chromatography; iGluR, ionotropic glutamate receptor; KPB, potassium phosphate buffer; MCM, methylmalonyl-CoA mutase; MDA, malondialdehyde; MMA, methylmalonic acid; MS, cobalamin-dependent methionine synthase; NADPH, nicotinamide adenine dinucleotide phosphate; NGM, nematode growth medium; NMDA, *N*-methyl-*L*-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; SAH, *S*-adenosylhomocysteine; SOD, superoxide dismutase

* Correspondence to: The Faculty of Agriculture, Tottori University, 4-101 Koyama-minami, Tottori 680-8553, Japan.
E-mail address: watanabe@muses.tottori-u.ac.jp (F. Watanabe).

[1] A research fellow of Japan Society for the Promotion of Science.

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several forms of plasticity. Worms that were subjected to prolonged exposure to attractive salt (NaCl) under starvation conditions display a dramatic reduction in chemotaxis to NaCl and eventually a negative chemotaxis. Exposure to NaCl in the presence of food does not reduce of chemotaxis, suggesting that worms change their behavior by integrating two stimuli, NaCl and starvation [14]. Tomioka et al. [11] demonstrated that the insulin/phosphoinositide 3-kinase pathway regulates this NaCl chemotaxis-linked learning and memory in worms.

We demonstrated that *C. elegans* is a suitable model for studying the physiological functions of B_{12} and the disordered biological processes caused by B_{12} deficiency [15]. In this study, we used this model animal to clarify the levels of oxidative stress and damage induced during B_{12} deficiency. Furthermore, we demonstrated that B_{12} deficiency resulted in severe memory impairment, which was partially attributable to oxidative stress.

2. Methods

2.1. Organisms and preparation of B_{12}-deficient C. elegans

The N2 Bristol wild-type *C. elegans* strain was maintained at 20 °C on nematode growth medium (NGM) plates using the *Escherichia coli* OP50 strain as the food source [16]. To induce dietary B_{12} deficiency, worms were grown on 1.7% (w/v) agar plates containing M9 medium (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 mmol/L $MgSO_4$, 50 μmol/L $CaCl_2$, 2 g/L glucose, 4 mg/L thiamine hydrochloride, and 5 mg/L cholesterol) in l L of water. Each plate containing M9-medium supplemented with B_{12} (100 μg/L CN-B_{12}) received one egg obtained from worms grown on NGM plates with B_{12}-deficient *E. coli* OP50 [15]. Eggs were allowed to hatch and develop into reproductively active adults. Subsequently, adults were then removed from each plate, eggs were collected and then each egg was transferred to a new control plate. After repeating this procedure at least five times, the worms were used as controls. B_{12}-deficient worms were prepared as described previously [15].

2.2. Preparation of a cell homogenate of worms

Control and B_{12}-deficient worms (0.05 g wet weight) were homogenized in 500 μL of 100 mmol/L potassium phosphate buffer (KPB) (pH 7.0) on ice using a hand homogenizer (AS ONE Corp., Osaka, Japan). The homogenates were centrifuged at 15,000×*g* for 10 min at 4 °C and these supernatants were used as crude enzymes or crude homogenates.

2.3. Enzyme activity assays

NADPH oxidase activity was determined using the luminescence assay method with lucigenin (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) as the electron acceptor [17]. The reaction was started by the addition of the crude enzyme and luminescence was monitored using a Luminescencer-Octa AB-2270 (ATTO Corp., Tokyo, Japan).

Total nitric oxide synthase (NOS) activity was assayed using an ultrasensitive colorimetric NOS assay kit (Oxford Biomedical Research, Ink., MI, USA). NOS activity was calculated based on the amount of nitrite that was converted from NO formed in the enzymatic reaction.

Total superoxide dismutase (SOD) was assayed using the SOD assay kit-WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Mn-SOD activity was determined in the reaction mixture containing 1 mmol/L KCN, which inhibits both Cu/Zn-SOD and extracellular SOD, resulting in the detection of only Mn-SOD activity [18,19].

Catalase (Cat) activity was determined using an NWLSS™ catalase activity assay kit (Northwest Life Science Specialties, WA, USA). Cat activity was calculated on the basis of the amount of H_2O_2 remaining in the enzymatic reaction mixture, which was monitored by measuring the absorbance at 240 nm using a spectrophotometer (UV-2550, Shimadzu Corp., Kyoto, Japan).

Glutathione peroxidase (GPx) activity was assayed using a GPx assay kit (Northwest Life Science Specialties). The crude enzyme was diluted 10 fold using 100 mmol/L KPB (pH 7.0) before use. After preincubation of the reaction mixture for 1 min, changes in the absorbance at 340 nm were monitored for 5 min using a Shimadzu spectrophotometer (UV-2550).

NOS, SOD, Cat, and GPx activities were assayed according to the respective manufacturer's instructions.

2.4. Assays of oxidative stress markers

H_2O_2 concentrations were determined using a H_2O_2 assay kit (BioVision, Inc., CA, USA). OxiRed probe reacts with H_2O_2 to produce a product with color (570 nm) in the presence of horseradish peroxidase. The reaction product was determined by measuring the absorbance at 570 nm using a Sunrise Rainbow RC-R microplate reader (Tecan Austria GmbH, Salzburg, Austria).

NO was assayed using an NO (NO_2/NO_3) assay kit (NO_2/NO_3 colorimetric assay kit-C II, Dojindo Laboratories, Kumamoto, Japan). The crude homogenate described previously was heat-treated to remove proteins before use. NO content was calculated based on the amount of NO_2/NO_3 by measuring the absorbance at 540 nm using a microplate reader (Tecan).

Malondialdehyde (MDA) was determined using a TBARS assay kit (ZeptoMetrix Crop., NY, USA). MDA-thiobarbituric acid adducts formed from the reaction of MDA in the sample were determined by measuring the absorbance at 540 nm using a microplate reader (Tecan).

The carbonyl proteins of control and B_{12}-deficient worms were determined using a modified 2,4-dinitrophenylhydrazine (DNPH) method [20]. Carbonyl content was determined by measuring the absorbance at 380 nm and calculated using a molar absorption coefficient of 21,000 $(mol/L)^{-1}$ cm^{-1}.

All oxidative stress markers were assayed according to the respective manufacturer's instructions or the cited reference.

2.5. Fluorescent staining of H_2O_2 in the living worms

To detect cellular H_2O_2 levels in control and B_{12}-deficient worms, the fluorescent probe BES-H_2O_2-Ac (Wako Pure Chemical Industry, Ltd., Tokyo, Japan) was used. BES-H_2O_2-Ac was dissolved in a small amount of dimethyl sulfoxide, diluted to a final concentration of 200 μmol/L using sterilized M9 medium, and used as a staining solution. Control and B_{12}-deficient worms (approximately 12 worms each) were treated with 150 μL of the staining solution for 1 h under aseptic conditions. To remove the remaining staining solution on the worm body surface, each worm was transferred to a B_{12}-deficient medium without *E. coli* and incubated at 20 °C for 1 h. The washed worms were treated with 20 μL of 1 mmol/L sodium azide solution on a slide glass and observed using an SZX-RFL-2 fluorescence microscope (Olympus, Tokyo, Japan) (λex: 485 nm, λem: 530 nm).

2.6. Assays of antioxidants

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined using a GSH/GSSG assay kit (OXIS International Inc., OR, USA). 5,5′-Dithiobis (2-nitrobenzoic acid) was monitored by measuring at absorbance at 412 nm using a Shimadzu UV-2550 spectrophotometer. GSH and GSSG content were assayed according to the manufacturer's instruction.

L-Ascorbic acid was assayed according to the DNPH method [21]. In brief, ascorbic acid was oxidized to dehydroascorbic acid, derivatized to its osazone, and then assayed using a Shimadzu high-performance liquid chromatography (HPLC) system (SPD-6AV UV–Vis spectrophotometric detector, LP-6A liquid delivery pump, and CTO-6V