Effect of Dietary Vitamin E on L-[^3]H]Glutamate Binding in Rabbit Cerebellum

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Summary To demonstrate the role of vitamin E on cerebellar function, studies on rabbits fed low and high levels of dietary vitamin E were performed. The L-[^3]H]glutamate binding to cerebellar membranes of rabbits fed normal, high and low vitamin E diet showed receptor density (Bmax) values (mean±SEM) of 274±13, 637±37, and 265±60 pmol/mg protein, respectively, and dissociation equilibrium constant (Kd) values of 257±99, 233±77, and 120±15 nM, respectively. Significant difference of Bmax from control was observed in the high dietary vitamin E group and of Kd from control for the low dietary vitamin E group. These results indicate that dietary vitamin E levels have demonstrable effects on the central nervous system, especially the glutamate neurotransmitter system in rabbit cerebellum.

Key Words low vitamin E, high vitamin E, rabbit cerebellum, glutamate receptor, neurological disorder, aging

Several clinical studies on human patients have shown that low or inadequate levels of vitamin E are associated with a syndrome of neurological impairment characterized by areflexia, peripheral neuropathy, cerebellar involvement with gait and limb ataxia and decreased proprioception and vibration sense (1-5). In addition, these neurological signs could be reversed by supplementary vitamin E diet. The influence of vitamin E on nervous tissue function has been the subject of several studies (review see Ref. 6). The pathology of the nervous system of vitamin E-deficient animals revealed histologic evidence for degeneration of posterior columns, axonal dystrophy (especially in the gracile and cuneate nuclei) and distal axonopathy in the peripheral nervous system (7). Neurochemical investigations showed that the cerebellum is particularly active in the metabolism and utilization of vitamin E (8), making it more susceptible to damage from vitamin E deficiency.
than other parts of the brain. In addition, a decreased surface density of synaptic contact zones has been demonstrated in cerebellar glomeruli in old and \( \alpha \)-tocopherol-deprived young adult rats, compared to younger and normally fed animals (9).

The various structural and functional units of the cerebellum are either dedicated to the control of a special motor function or are involved in the regulation of muscle tone and the initiation and coordination of voluntary movements (10). Such functions are regulated by different types of neurotransmitter systems, mainly glutamic acid and gamma-aminobutyric acid (GABA). Indirect evidence implies that glutamic acid receptor in cerebellum may also be involved in some neuropathological syndromes, for example, movement disorder. Some malnutritional states, such as that manifested in vitamin E deficiency, were associated with gait and other motor dysfunction symptoms (8, 11–14), suggestive of cerebellum involvement.

In an attempt to demonstrate the role of vitamin E on the glutamic acid neurotransmitter system of cerebellum function, we have previously identified and characterized the excitatory amino acid (EAA) receptor subtype in rabbit cerebellum as an AMPA (DL-\( \alpha \) amino-3 hydroxy-5-methylisoxazole propionic acid) type (15). In the present study, we have compared the characteristics of L-[\(^{3}\)H]glutamate receptor in the cerebellum of rabbits fed low and high levels of dietary vitamin E.

**Materials and methods.** Materials: L-[\(^{3}\)H]glutamate (specific activity of 51.9 Ci/mmol) was purchased from New England Nuclear Corporation (Boston, MA). Vitamin E (DL-\( \alpha \)-tocopherol acetate) for dietary supplementation was obtained from Sigma Chemical Corporation (St. Louis, MO) and Hoffmann-La Roche & Co. (Switzerland).

Animals: White weanling rabbits, 1,000–2,000 g in weight, were housed and fed a synthetic diet, as described previously (16). The animals were divided into three groups, one on normal diet, one on low vitamin E and the other on high vitamin E-supplemented diet, for a period of 10 weeks. The ingredients of the vitamin E-free basal diet are shown in Table 1. The total amount of vitamin E given per day for the normal, low and high vitamin E diet groups was 3, 0.56 and 300 mg/100 g of diet respectively. Thereafter, rabbits were killed by exsanguination via cardiac puncture between 9:00–10:00 AM, and samples of cerebellum were immediately dissected out on ice-cold buffer. Tissues for the glutamate receptor binding assays were stored at \(-80^\circ\)C, until use (usually within 2 weeks).

Preparation of cerebellar membranes: Cerebellar membrane was prepared by a modification of the techniques of Coyle (17) and Govitrapong and Kittiwatanapaïsan (15). The frozen cerebellum was homogenized in 20 volumes of ice-cold 0.32 M sucrose in 5 mM Tris acetate buffer, pH 7.4. The homogenate was centrifuged at 900 \( \times \) g for 10 min in a refrigerated centrifuge (Hitachi). The pellet was discarded and supernatant was centrifuged at 48,000 \( \times \) g for 20 min. The crude cerebellar membrane preparation was resuspended by an ultra turrax T25 homogenizer, setting at 135,000 rpm for two 10-s periods, in 50 volumes of 50 mM

Table 1. Composition of basal diet and amount of vitamin E given.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Sucrose</td>
<td>39.2</td>
</tr>
<tr>
<td>Casein</td>
<td>22.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>22.9</td>
</tr>
<tr>
<td>Tocopherol-stripped lard</td>
<td>3.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>4.2</td>
</tr>
<tr>
<td>Hawk-Oser salt mixture(^1)</td>
<td>3.0</td>
</tr>
<tr>
<td>Inositol</td>
<td>0.1</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Water-soluble vitamin mixture(^2)</td>
<td>0.4</td>
</tr>
<tr>
<td>Fat-soluble vitamin mixture(^3)</td>
<td>0.15</td>
</tr>
<tr>
<td>Cellulose powder (alfacel)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Total tocopherol content given per day (mg/100 g diet)

- Normal diet group: 3.0 mg
- Low vitamin E diet group: 0.56 mg
- High vitamin E diet group: 300.0 mg

\(^1\) Each 100 g of the basal mixture contained (in g): calcium carbonate, 0.0686; calcium citrate, 0.3083; calcium biphosphate, 0.1128; magnesium carbonate, 0.0352; magnesium sulfate, 0.0383; potassium chloride, 0.0771; cupric sulfate, 0.48; ferric ammonium citrate, 94.33; manganese sulfate, 1.24; ammonium alum, 0.57; potassium iodide, 0.25; and sodium fluoride, 3.13.  
\(^2\) Each 100 g contained nicotinamide, 5.2 g; calcium pantothenate, 260 mg; riboflavin, 130 mg; folic acid, 130 mg; biotin, 1.3 mg; pyridoxine-HCl, 130 mg; thiamine-HCl, 130 mg; vitamin B\(_{12}\), 1.17 mg; and sucrose, 93.3 g.  
\(^3\) Each 100 g contained vitamin A palmitate 1.7 g; vitamin D\(_2\), 350 mg; vitamin K (menadione), 16.25 mg; and sucrose, 97.8 g.

Tris acetate buffer, pH 7.4, followed by centrifugation at 48,000 × g for 20 min. This washing procedure was repeated three times to remove the endogenous glutamate. The cerebellar membrane was kept at −80°C until use (usually within 2 weeks).

Glutamate binding assays: \(\text{L-[^{3}H]}\)glutamate binding assays were carried out according to the modified techniques of Govitrapong and Kittiwatanapaisan\(^{15}\) and Coyle\(^{17}\). The frozen cerebellar membrane was dispersed into ice-cold 50 mM Tris acetate buffer, pH 7.4 by the ultra turrax T25 homogenizer. The membrane suspension was diluted to a concentration of approximately 20 µg protein/ml; 0.9 ml of this suspension was added to the reaction tube, and the reaction mixtures were preincubated for 5 min in either the presence or absence of 50 µl unlabeled L-glutamate with 100 µM in the final concentration. The reaction was initiated by adding 50 µl of \(\text{L-[^{3}H]}\)glutamate to achieve a final concentration of 100 nM (2 nM of \(\text{L-[^{3}H]}\)glutamate, specific activity 51.9 Ci/mmol, and 98 nM unlabeled L-glutamate) and the incubation was continued for 45 min at 37°C. The reaction
was terminated by filtering the reaction mixture through a Whatman GF/C filter paper, followed by two rinses with 3 ml of ice-cold 50 mM Tris acetate buffer, pH 7.4. The sample was placed directly into 5 ml scintillation fluid containing 35% Triton X-100. The radioactivity was counted by a liquid scintillation counter. The counting efficiency for tritium was 44%. Nonspecific binding was determined by including 100 μM L-glutamate in the reaction mixture. Specific binding was defined as the binding obtained by subtracting the nonspecific binding from the total binding. Specific L-[3H]glutamate binding was 60–70% of the total binding.

**Determination of protein:** The concentration of protein was determined by the Lowry method (18) using bovine serum albumin as a standard.

**Analysis of data:** Saturation curves were initially analyzed by the method of Scatchard, and finally analyzed by using the nonlinear least-squared regression analysis program, known as LIGAND (19). The dissociation equilibrium constant (K_D) and the maximum number of binding sites (B_max) were obtained. All experiments were conducted in triplicate for each individual condition a minimum of six times. Significance was determined by multivariate analysis of variance, followed by the Tukey method for differences in means.

**Results:** The saturation studies of L-[3H]glutamate binding to rabbit cerebellum were conducted at 37°C by incubating the membrane suspension with L-[3H]glutamate at concentrations ranging from 10 to 1,000 nM. \( K_D \) and \( B_{\text{max}} \) values were obtained from the analysis of data by Scatchard plot. The lines of best fit were derived by the analysis of data using LIGAND (19) computer program. A typical result of the saturation data from an animal on a normal, high or low vitamin E diet is shown in Fig. 1.

The receptor density \( B_{\text{max}} \) and dissociation equilibrium constant \( K_D \) of rabbit cerebellar membranes in the normal, high and low vitamin E diet groups are shown in Table 2. The data are expressed as the mean ± SEM. The \( B_{\text{max}} \) value of the high vitamin E diet group (637 ± 37 pmol/mg protein) was significantly increased \( (p<0.0005) \) when compared to the normal diet group (274 ± 13 pmol/mg protein) whereas the \( K_D \) value did not change. The \( K_D \) value of the low vitamin E diet group was slightly decreased \( (p<0.01) \) when compared to the normal group, whereas the \( B_{\text{max}} \) value did not change.

**Discussion.** There has so far been no direct evidence concerning the mechanism of neurological symptoms caused by vitamin E deficiency. However, there have been some reports indicating that the cerebellum may be the preferred target for such involvement. The cerebellum may be more susceptible to damage from vitamin E deficiency than other parts of the brain, because this area has lower concentration of vitamin E (20) and also the region is particularly active in metabolizing vitamin E compared with other parts of the brain (8). Rats fed a vitamin E-deficient diet for 9 months showed a decrease of choline acetyltransferase activity in the cerebellum (8, 21).

Our data indicated that the glutamic acid neurotransmitter system in the cerebellum was altered by dietary vitamin E condition. The significant increase in
Fig. 1. Saturation curves of L-[3H]glutamate binding to rabbit cerebellar membrane of normal (A), high (B), and low (C) vitamin E diet groups. The binding was carried out at concentration ranging from 10 to 1,000 nM of L-[3H]glutamate. The points, which were obtained from triplicated determinations, represent the specific binding of glutamate. The inset represents the Scatchard plot, with the line indicating the specific binding. The data were analyzed by using computer program, LIGAND. The results of the experiments shown here provide the $K_D$ values of 110.80, 165.80, and 130.70 nM and $B_{max}$ values of 121.60, 721.90, and 130.70 pmol/mg protein for the normal (A), high (B), and low (C) vitamin E diet group respectively.

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Table 2. Cerebellum receptor density ($B_{\text{max}}$) and dissociation equilibrium constant ($K_D$) of rabbits fed normal, high and low vitamin E diets.

<table>
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<th>$B_{\text{max}}$ (pmol/mg protein)</th>
<th>$K_D$ (nm)</th>
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<tbody>
<tr>
<td>Normal diet</td>
<td>274 ± 13</td>
<td>257 ± 99</td>
</tr>
<tr>
<td>High vitamin E diet</td>
<td>637 ± 37*</td>
<td>233 ± 77</td>
</tr>
<tr>
<td>Low vitamin E diet</td>
<td>265 ± 60</td>
<td>120 ± 15$^b$</td>
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The $B_{\text{max}}$ and $K_D$ of each group were calculated from the saturation experiments. Data are presented as the mean ± SEM of 6 rabbits per group. Significant difference from the normal diet group with $^a p < 0.0005$ and $^b p < 0.01$.

The number of glutamate receptors, found in the high vitamin E diet group, may be due to vitamin E acting as an inducer for the production of glutamate receptor binding sites in rabbit cerebellum. Alternately, it may unmask the spare receptors or activate the modulatory protein (22), existing in the AMPA glutamate receptor sites, thus resulting in a greater number of receptors available for L-[3H]glutamate to bind. The binding data seems compatible with our preliminary histological result showing highly stained and densely packed cells in the granular cell layer. Recently, megavitamin E supplement studies have been reconsidered. It has been reported that there is no apparent toxic side effect of high doses of vitamin E supplement in rabbits (23). This has led to numerous groups of investigators administering pharmacological doses of vitamin E to patients with abetalipoproteinemia (4, 24), cholestasis (4) and congenital biliary atresia and cystic fibrosis (25), resulting in either improvement of their neurological function or at least halting further deterioration of their nervous systems (26). Additionally, vitamin E as a therapeutic agent in the treatment of Parkinson’s disease has also been clinically tried (27).

The maximal L-glutamate binding number in the low vitamin E diet group did not significantly alter, whereas the dissociation equilibrium constant was slightly decreased. The change in the affinity of L-glutamate binding may be due to the phenomenon of receptor up-regulation, in order to compensate for alterations around the synapses. This would seem to support the observation of the increased sympathetic nervous activity in vitamin E-deficient animals (28).

The mechanism by which vitamin E exerts its effect on cerebellar function remains to be elucidated. It is conceivable that vitamin E affects membrane structure (29), which in turn influences membrane-bound receptors in the molecular layer of the cerebellum, the area which contains AMPA receptors on cerebellar granule cells (29, 30).

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