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Ibotenic acid-induced lesions of the medial septum increase hippocampal membrane associated protein kinase C activity and reduce acetylcholine synthesis: Prevention by a phosphatidylcholine/vitamin B_{12} diet

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Ibotenic acid infusion into the medial septum (MS) results in biochemical alterations in the hippocampus. The biochemical events involved in this neuronal lesion are poorly understood. We investigated the effect of a purified diet supplemented with egg phosphatidylcholine (PC) and vitamin B_{12} on ibotenic acid-medicated biochemical changes in the rat hippocampus and crude synaptosomal membranes. Male Wistar rats with this MS lesion were fed a purified diet (control diet) or a purified diet supplemented with 5.7 g PC and 125 μg vitamin B_{12} per 100 g (experimental diet) for 18 days. Sham-operated rats were fed the control diet. Compared with the sham-operated rats, MS-lesioned rats fed the control diet showed increased activity of membrane-bound protein kinase C (PKC), decreased activity of choline acetyltransferase, and decreased concentrations of acetylcholine in the hippocampus. The ratio of cholesterol to phospholipid in the crude synaptic membrane was lower in the lesioned rats than in the sham-operated rats, but this was not accompanied by any alteration in membrane lipid fluidity. MS-lesioned rats fed the experimental diet showed lowered PKC activity and elevated acetylcholine concentrations than did rats fed the control diet, but there were no significant effects on choline acetyltransferase activity and the lipid ratio. The ibotenic acid-mediated elevation of PKC activity was observed as early as 2 days postinjury in the control diet-fed rats but not in the experimental diet-fed rats. We propose that ibotenic acid mediates pathophysiologic actions through the activation of PKC and that PC combined with vitamin B_{12} ameliorates the second messenger-mediated injury. (J. Nutr. Biochem. 11:159–164, 2000) *© Elsevier Science Inc. 2000. All rights reserved.*

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Introduction

Ibotenic acid, which is a rigid structural analogue of glutamate, is a neuroexcitatory compound and is also a pharmacologic tool used for studies of rat models involving lesions of cholinergic neurons by stereotaxic injections into brain.[1,2] Yamasaki et al.[3] reported that rats injected with ibotenic acid into the medial septum (MS) and the nucleus basalis magnocellularis had decreased activities of choline acetyltransferase in the hippocampus and frontal cortex, respectively, followed by impairment in memory acquisition compared with sham-operated rats. Using the same animal model, Masuda et al.[4] found that egg phosphatidylcholine (PC) combined with vitamin B_{12} resulted in an

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Table 1 Composition of control or phosphatidylcholine (PC) plus vitamin B_{12} diet

Ingredients	Group	
	Control (g/100 g diet)	PC plus vitamin B_{12} (g/100 g diet)
Casein	20.0	20.0
DL-Methionine	0.3	0.3
Corn starch	44.5	44.5
Sucrose	20.0	20.0
Cellulose powder	2.0	2.0
Corn oil	8.0	2.3
Egg PC	—	5.7
Mineral mixture	4.0	4.0
Vitamin mixture	1.0	1.0
Vitamin B_{12}	—	0.000125
Choline chloride	0.1	0.2

Mineral and vitamin mixtures were according to Harper.[7]

increased concentration of acetylcholine in the frontal cortex with improve memory acquisition and retention in rats with a lesion in the nucleus basalis magnocellularis. They also reported that these beneficial effects were not reproduced when administered with egg PC alone or vitamin B_{12} alone. However, how this dietary manipulation alleviates ibotenic acid-induced brain dysfunction remains to be determined.

Ibotenic acid may be involved in a process that leads to activation of metabotropic receptors by coupling to the phosphoinositide second messenger system in animal brain. Activation of this class of receptors is followed by an increase in cytosolic calcium released from intracellular stores by inositol 1,4,5-triphosphate and the activation of protein kinase C (PKC) by 1,2-diacylglycerol. These two mechanisms lead to cytotoxicity.[5] Therefore, in this study, we hypothesized that a diet containing PC and vitamin B_{12} should partially reduce the cellular toxicity related to ibotenic acid action by attenuating PKC activation. We investigated the effect of a diet containing PC and vitamin B_{12} on changes in activities of PKC and choline acetyltransferase and in concentrations of choline and acetylcholine in the rat hippocampus injured by ibotenic acid. Lipid composition and lipid fluidity of the crude synaptosomal membrane prepared from the hippocampus were also determined, because it has been suggested that synaptosomal membrane lipid composition is involved in receptor functions in neurotransmission.[6]

Methods and materials

Animals, diets, and surgery

Male Wistar rats weighing 250 to 300 g and maintained on a commercial nonpurified diet (CE-2, Japan Clea, Tokyo, Japan) were housed individually in a stainless steel mesh cage in a temperature, humidity-controlled environment (24 ± 1°C, 55 ± 10%) under a 12-hour light-dark cycle.

Composition of a purified diet (control diet) according to Harper[7] (*Table 1*) has been described elesewhere.[8,9] The purified diet was supplemented with 5.7 g/100 g of PC (PL-100LE, 92% pure PC; docosahexaenoic acid 6.1%, linoleic acid 12.2%, oleic acid 29.8%, stearic acid 13.2%, and palmitic acid 32.0%; Q.P. Co., Tokyo, Japan) and 125 μg/100 g of vitamin B_{12} (Sigma Chemical Co., St. Louis, MO USA; PC plus vitamin B_{12} diet). The amounts of PC and vitamin B_{12} in the diet were based on findings in a previous experiment.[4] The contents of vitamin B_{12} per 100 g diet were 2 μg and 127 μg for control and PC plus vitamin B_{12} diet, respectively.

The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and fixed on a stereotaxic apparatus (Narishige Co., Tokyo, Japan), as described by Yamasaki et al.[3] A Hamilton microsyringe (#7101, 1 μL, Darmstadt, Germany) was stereotaxically inserted into the MS (A:6.7, L:0, H:dura-5.3 mm) according to the atlas of Paxinos and Watson.[10] MS was injected together with ibotenic acid (Sigma Chemical Co.). Ibotenic acid was dissolved in phosphate buffered saline (PBS; pH 7.2) at a concentration of 60 nmol/μL, then infused directly into the MS in a volume of 0.7 μL at a rate of 0.1 μL/min. After the injection, the needle was left in place for 5 minutes to prevent backflow. Sham-operated rats were given an injection of PBS instead of ibotenic acid solution. After the rats had fully recovered from anesthesia, no obvious signs of the stereotaxic injection of ibotenic acid were observed. After this procedure, the rats were fed either the control diet or PC plus vitamin B_{12} diet for 18 days, after which PKC and choline acetyltransferase activities (experiment 1), crude synaptosomal membrane lipid composition and fluidity (experiment 2), and concentrations of acetylcholine and choline (experiment 3) were determined. In a separate set of experiments, the rats were fed the control diet or PC plus vitamin B_{12} diet for 2 weeks prior to the operation, and the hippocampus was dissected and used for PKC determination on days 1, 2, or 7 after the operation (experiment 4). Choline acetyltransferase was determined on day 7. These experiments were carried out under the guidelines for Animal Experiment in Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan and the Law (No. 105) and Notification (No. 6) of the Government of Japan.

Brain choline and acetylcholine concentrations

The rats were maintained on the purified diets for 18 days, after which they were sacrificed by microwave irradiation of the head (8 kW, 1.5 sec; Microwave Applicator TMW-6402A, Muromachi Co., Tokyo, Japan). The brains were rapidly removed and hippocampi were dissected. Brain choline and acetylcholine concentrations were measured according to the method of Fujimori and Yamamoto[11] using high performance liquid chromatography with electrochemical detection (BAS400A analyzer, BAS, Tokyo, Japan).

Assays of choline acetyltransferase and PKC activities

The brains were rapidly removed after cervical dislocation of the animals and the hippocampi were immediately dissected. Half the amount of tissue was homogenized in 20 volumes of ice-cold 50 mM phosphate buffer (pH 7.4) and the activity of choline acetyltransferase was measured by the method of Mantione et al.[12] as described elsewhere.[4] [1-^{14}C]Acetyl-coenzyme A (55 mCi/mmol, Amersham, Tokyo, Japan) was used as a substrate.

The remaining tissue was homogenized in 40 volumes of ice-cold buffer [50 mM Tris, 2 mM 2Na-ethylendaimine-tetraacetic acid (EDTA), 2 mM dithiothreitol, 100 mM leupeptin, pH 7.5]and the homogenates were centrifuged for 60 minutes at 100,000 × g in a Beckman TL-100 ultracentrifuge to obtain cytosolic and membrane fractions. Membrane pellets were resolubilized in homogenization buffer. PKC was then partially purified by a modified procedure.[13] The activity of PKC was measured in vitro by incorporation of [^{32}P] from [γ-^{32}P]-adenosine triphos-

Table 2 Protein kinase C activity, lipid composition, membrane fluidity, choline acetyltransferase activity, and concentrations of acetylcholine and choline of hippocampus in rats fed the control diet or the phosphatidylcholine (PC) plus vitamin B_{12} diet for 18 days

	Sham	MS-lesioned	
	Control	Control	PC plus vitamin B_{12}
Experiment 1 (n = 6 for each group)			
Protein kinase C (pmol/min/mg protein)			
Cytosol fraction	1,233 ± 130	860 ± 31	1050 ± 63
Membrane fraction	1,438 ± 13[a]	2,485 ± 245[b]	1,620 ± 236[a]
Choline acetyltransferase (pmol/min/mg protein)	241 ± 6[a]	139 ± 6[b]	158 ± 3[b]
Experiment 2 (n = 7 for each group)			
Lipid composition (nmol/mg protein)			
Cholesterol	210 ± 34[a]	121 ± 18[b]	95.1 ± 4.2[b]
Phospholipids	536 ± 133	790 ± 76	806 ± 92
Cholesterol/Phospholipids	0.392 ± 0.052[a]	0.153 ± 0.032[b]	0.118 ± 0.088[b]
Membrane fluidity (P)	0.272 ± 0.015	0.246 ± 0.009	0.268 ± 0.013
Experiment 3 (n = 6 for each group)			
Concentration (mmol/g tissue)			
Acetylcholine	28.3 ± 0.5[a]	12.9 ± 1.1[b]	18.0 ± 2.0[c]
Choline	26.2 ± 2.9[a]	43.9 ± 1.8[b]	20.7 ± 1.3[a]

Values represents mean ± SE. [abc]Values without a common superscript letters are significantly different at $P < 0.05$.

phate (ATP; Toho Biochemical Co., Tokyo, Japan) into histone III-S (Sigma Chemical Co.), as described previously.[14]

Preparation of crude synaptosomal membranes and determinations of lipid and membrane lipid fluidity

The hippocampus was placed in 20% w/v of cold 0.32 M sucrose solution and was homogenized by a Teflon glass homogenizer. The homogenate (diluted to 10%) that served as the crude synaptosomal membrane preparation was ultracentrifuged according to Cotman and Matthews.[15] Briefly, a crude nuclear fraction was obtained by centrifugation at 1,100 × g for 5 minutes. The supernatant was transferred and centrifuged at 17,000 × g for 10 minutes in 50 Ti rotor (Hitachi Koki, Ibaraki, Japan). The crude mitochondrial fraction was resuspended in 10% sucrose solution. The suspension was applied to a two-step discontinuous Ficoll (13% and 7.5%, Pharmacia Japan, Tokyo, Japan)-sucrose gradient. After centrifugation, a synaptosomal fraction was obtained at the interface of the 7.5 to 13% Ficoll-sucrose layer. The synaptosomal band was removed, diluted with 10% sucrose, and pelleted at 30,000 rpm for 30 minutes. The synaptosomal pellet was resuspended in a small volume of 10% sucrose. This preparation was designated as crude synaptosomal membrane and used for lipid and membrane lipid fluidity determinations.[6]

The degree of fluidity in crude synaptosomal membrane lipids was determined by steady-state fluorescence polarization of the apolar probe 1,6-diphenyl-1,3,5-hexatriene (special grade, Tokyo Kasei, Co., Tokyo, Japan) to probe the membrane lipid deep core at one constant temperature of 25°C as described.[14] The degree of fluorescence polarization (P) was calculated.

Crude synaptosomal membranes were extracted with chloroform and methanol according to the method of Folch et al.[16] The phospholipid-phosphorus was determined using the Malachite green method.[17] Cholesterol was determined enzymatically[18] and fatty acid composition was determined by gas liquid chromatography, as previously described.[14] The membrane protein was determined by protein-dye binding method.[19]

Statistics

Fisher's protected least squares difference test was used to determine the statistical significance by the software of superANOVA (Abaous Concepts, Berkeley, CA USA).

Results

Table 2 summarizes biochemical data of the hippocampus in MS-lesioned and sham-control rats. In experiment 1, MS-lesioned rats on the control diet had a higher activity of membrane-bound PKC than did the sham control rats. MS-lesioned rats on the PC plus vitamin B_{12} diet had as low membrane-bound PKC activity as did sham-control rats and lower activity than MS-lesioned rats on the control diet. There were no significant differences in PKC activities in the cytosol fractions among three groups. MS-lesioned rats on the control or PC plus vitamin B_{12} diets had a lower activity of choline acetyltransferase than did sham-control ones. In this experiment, food intake was similar among three groups on day 18 after the operation (260 ± 7, 270 ± 6 and 275 ± 7 g/18 days for sham-control, MS-lesioned rats with control diet and MS-lesioned rats with PC plus vitamin B_{12} diet, respectively; values are mean ± SE for 6 rats per group). Water intake and behavior seemed regular among all the groups. Both MS-lesioned groups had higher body weights gain than did the sham-control group [30 ± 1, 48 ± 4, and 44 ± 5 g/day for sham-control, MS-lesioned rats with control diet, and MS-lesioned rats with PC plus vitamin B_{12} diet, respectively ($P < 0.05$ for all groups)].

In experiment 2, MS-lesioned rats on the control or PC plus vitamin B_{12} diet had a lower concentration of cholesterol in the crude synaptosomal membrane of hippocampus than did sham-control rats. The concentration of phospholipids in the crude membrane was similar among the three groups. Accordingly, the ratios of cholesterol to phospholipids were lower in MS-lesioned groups than in the sham-control group. Fluidity of the crude membrane was not influenced by the ibotenic acid injection. Fatty acid composition of the membrane lipids was not significantly altered by the operation or by the diet, except for a slightly higher proportion of oleic acid in the MS-lesioned rats compared with sham-operated rats (proportion of oleic acid in wt%: 13.9 ± 0.6, 15.8 ± 0.6, and 16.3 ± 0.4 for sham-control rats, MS-lesioned rats with control diet, and

Table 3 Protein kinase C and choline acetyltransferase activities of hippocampus in the rats fed the control diet or the phosphatidylcholine (PC) plus vitamin B_{12} diet for 1, 2, or 7 days

	Sham	MS-lesioned	
	Control	Control	PC plus vitamin B_{12}
Experiment 4			
Protein kinase C (*p*mol/min/mg protein)			
Cytosol			
Day 1	1,255 ± 66	1,100 ± 38	1,241 ± 63
Day 2	1,167 ± 46	1,286 ± 85	1,120 ± 95
Day 7	1,464 ± 318	1,554 ± 633	1,045 ± 177
Membrane			
Day 1	1,539 ± 41	1,444 ± 43	1,437 ± 54
Day 2	1,603 ± 8[a]	1,965 ± 89[b]	1,510 ± 110[a]
Day 7	1,684 ± 197[a]	3,271 ± 172[b]	2,165 ± 116[a]
Choline acetyltransferase (*p*mol/min/mg protein)			
Day 7	320 ± 5[a]	267 ± 5[b]	263 ± 12[b]

Values represent mean ± SE for six rats. [ab]Values without a common superscript letters are significantly different at $P < 0.05$.

MS-lesioned rats with PC plus vitamin B_{12} diet, respectively; values are mean ± SE for 6 rats per group; $P < 0.05$ for all groups).

In experiment 3, the concentrations of acetylcholine in MS-lesioned rats were lower than in the sham-control rats. In MS-lesioned groups, PC plus vitamin B_{12} diet-fed rats had a higher concentration of acetylcholine than did control diet-fed rats. The concentration of choline in the hippocampus was the highest in the MS-lesioned rats on the control diet and there was no difference between the sham-control rats and MS-lesioned ones with PC plus vitamin B_{12} diet.

In experiment 4, MS-lesioned rats on the control diet had a higher activity of membrane-bound PKC on days 2 and 7 than did sham-control rats or MS-lesioned ones on the PC plus vitamin B_{12} diet (*Table 3*). There was no significant difference in PKC activity between the latter two groups. The activity of choline acetyltransferase in the hippocampus on day 7 was lower in both MS-lesioned groups than in sham-control group.

Discussion

Our data show that ibotenic acid infusion into the rat MS led to an increase of membrane-bound PKC activity in hippocampus from days 2 to 18 postinjury. No difference in the cytosolic PKC activity occurred during the same period after injury. These results suggest an active translocation of cytosolic PKC. Ibotenic acid-induced functional alteration was also demonstrated by Wree et al.,[20] who found widespread reductions of glucose utilization in all the layers and sectors of the hippocampus in the ipsilateral lesioned hemisphere; the reductions occurred as early as 3 days after lesioning and persisted up to 3 months. Several studies have shown that the ibotenic acid-infused cerebral lesion can lead to an elevation of PKC in the brain or the neuronal cells. Horsburgh et al.[21] found a significant increase of [^{3}H]phorbol 12,13-dibutyrate binding to PKC in the superficial layers of entorhinal cortex of rat brain on day 21 after ibotenate lesioning. Scholz[22] reported an ibotenate-mediated stimulation of phosphorylation of three types of PKC substrate in cultured pyramidal neurons. Collectively, these data support the notion that MS injury can induce activation of PKC by coupling to the second messenger system,[5] and this activation may play a role in the pathophysiology of MS lesions, such as the decreased choline acetyltransferase activity and acetylcholine concentration observed in the present study, or the impairment of memory acquisition reported by Yamasaki et al.[3]

In intact animals, however, dietary PC plus vitamin B_{12} exerted an effect on the hippocampal PKC activity that was different from that seen in the ibotenic acid-lesioned rats: The same diet-fed mice instead showed an increased PKC activity in the soluble fraction in the hippocampus (unpublished observation). In any event, dietary PC plus vitamin B_{12} influenced the PKC activity in the hippocampus.

Our MS-lesioned rats had a lower cholesterol concentration in the hippocampal crude synaptosomal membrane than the sham-control rats, but the effects on phospholipid concentration and the lipid fluidity were nil. Wree et al.[20] reported that the ultrastructure was characterized by abundant organelles and an indented nucleus containing a rod in the dentate gyrus molecular layer of rats 14 days after ibotenic acid infusion, whereas in the surrounding neuropils there was a degeneration of dendrites, glycogen-containing astrocytes, and astrocytic processes but myelinated axons remained intact. Therefore, selective loss of the membrane cholesterol in the lesioned rats might relate to the specific nature of ibotenate, which destroys neuronal cell bodies with little influence on neighboring axons.

We have demonstrated for the first time that MS-lesioned rats on a PC plus vitamin B_{12} diet had a lower activity of membrane-bound PKC in the hippocampus than did MS-lesioned rats on a control diet, and there was an inverse relationship between the membrane-bound PKC activity and acetylcholine concentration in the PC plus vitamin B_{12} diet-fed rats. These results indicate that a diet containing PC plus vitamin B_{12} might play a role in lessening damage to cholinergic neurons in the hippocampus by preventing neurotoxic amino acids from activating metabotropic receptors being coupled to PKC activation,[5] thereby leading to a reduction in neuronal loss or an increase of the activity of spared cholinergic neurons in

ibotenic acid-injured rats. An inverse relationship between suppressing PKC activity and reducing damage to cholinergic neurons has been demonstrated by Nabeshima et al.[23] They showed that an intraperitoneal injection of staurosporine, which is an inhibitor of PKC, attenuated the impaired performance in basal forebrain-lesioned rats and partially reversed the decrease of choline acetyltransferase activity in the front-parietal cortex. In the present study, MS-lesioned rats fed the PC plus vitamin B_{12} diet, in comparison with sham-controlled rats, had a low choline acetyltransferase activity but a lowered choline concentration in the hippocampus than did MS-lesioned rats on the control diet. These results suggest that rats on PC plus vitamin B_{12} diet, compared with those on the control diet, increased utilization of choline for acetylcholine synthesis in the hippocampus by activating the cholinergic system rather than activating the enzyme activity.

According to the concept of a precursor control of neurotransmitter release,[24] dietary supplements of choline and lipid-bound forms of choline may be one therapeutic approach to improve symptoms of cholinergic deficiency. This was confirmed by Chung et al.[25] who showed that administration of an egg PC resulted in an increased acetylcholine concentration in the frontal cortex and hippocampus in mice with dementia. More recently, Masuda et al.[4] found that intragastric administration of either egg PC or vitamin B_{12} alone did not increase acetylcholine concentration in the frontal cortex or improve memory acquisition in rats given ibotenate infusion into the nucleus basalis magnocellularis. Instead they found that the administration of the egg PC combined with vitamin B_{12} led to an increase in acetylcholine concentration and improvement in memory acquisition.[4] Therefore, it is likely that PC combined with vitamin B_{12} is required to alleviate the action of exocitotoxic amino acids.

Our preliminary study using monoclonal antibodies for PKC isoforms showed that the β-isoform was the major subspecies in the hippocampal homogenates (unpublished observation). Therefore, it is likely that the activation of PKC after MS lesioning might be due to the PKC-β. The possibility that the prolonged activation of PKC after MS lesioning may involve the membrane-inserted constitutive PKC is not inconsistent with its complex with phospholipids.[26] Diacylglycerol production resulting from polyphosphoinositide and PC hydrolysis is believed to cause further diacylglycerol production from PC, a process that is sustained and leads to persistent PKC activation.[27] In the present study, PC plus vitamin B_{12} diet might alleviate the increased diacylglycerol production, because the concentration of choline in the hippocampus was lower in the MS-lesioned rats on PC plus vitamin B_{12} than in the MS-lesioned rats on control diet (experiment 3 in *Table 2*).

Several lines of evidences showed that vitamin B_{12} is involved in metabolism of choline in the brains of experimental animals. Nadeau and Roberge[28] showed that vitamin B_{12} enhanced the activity of choline acetyltransferase in the cat brain. Sasaki et al.[29] showed that 10 mg/kg diet of vitamin B_{12} increased cortical acetylcholine concentrations in rats fed a choline-deficient diet. Relevance of vitamin B_{12} in neuropathology was also suggested by clinical studies.[30–32] Together these animal and clinical studies suggest the important role of vitamin B_{12} in neurologic disorders.

In the present study, when compared with the control diet, PC plus vitamin B_{12} diet did not improve the cholesterol/phospholipid ratio, the fluidity in crude synaptosomal membranes, or the fatty acid composition in MS-lesioned rats. Therefore, it is not likely that PC plus vitamin B_{12} diet alleviates the neurotoxic action of ibotenic acid by improving the membrane structure.

In summary, our results suggest that dietary PC plus vitamin B_{12} may aid in overcoming pathophysiologic conditions related to enhanced PKC activation provoked by overactivity of exocitotoxic amino acids. The molecular mechanism related to the attenuation of PKC activation by PC combined with vitamin B_{12} is the subject of ongoing studies.

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