Learning behaviour and cerebral protein kinase C, antioxidant status, lipid composition in senescence-accelerated mouse: influence of a phosphatidylcholine–vitamin B_{12} diet

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Our objective was to determine whether dietary supplementation with phosphatidylcholine (PC) plus vitamin B_{12} could afford beneficial effects on biochemical and biophysical events in the brain of senescence-accelerated mouse (SAM) substrain SAMP8. We measured learning behaviour, hippocampal protein kinase C (PKC) activity, cerebral antioxidant status, phospholipid composition and fatty acid composition in 6-month-old SAMP8 and in age-matched controls (SAM substrain SAMR1). In comparison with SAMR1, SAMP8 showed a significant elevation in total grading score of senescence \( P<0.05 \)† and a significant decline in acquisition \( P<0.05 \). SAMP8 had a lower hippocampal PKC activity and cerebral PKC-\( \beta \) mRNA abundance than SAMR1. SAMP8 had increased cerebral lipid peroxide levels and proportion of sphingomyelin, and a lower proportion of 20:4\( n-6 \) and 22:6\( n-3 \) in cerebral phosphatidylethanolamine than SAMR1. SAMP8 fed the PC combined with vitamin B_{12} diet had an increased PKC activity and a higher proportion of 22:6\( n-3 \) than SAMP8 fed the control diet. These results indicate the potential benefit of PC combined with vitamin B_{12} as a dietary supplement.

Phosphatidylcholine: Vitamin B_{12}: Protein kinase C: Senescence-accelerated mouse: Brain

Dysfunction of the cholinergic system in the central nervous system induces memory impairment. Many studies have demonstrated that dietary supplementation with phosphatidylcholine (PC) increases brain acetylcholine concentration and improves memory impairment in young or aged animals (Hirsch & Wurtman, 1978; Magil et al. 1981; Jope, 1982; Leathwood et al. 1982; Chung et al. 1995). In addition to choline or PC, dietary supplementation with vitamin B_{12} increased the activity of choline acetyltransferase in cat brain (Nadeau & Roberge, 1988) and improved cognitive disturbance in rodents fed a choline-deficient diet (Sasaki et al. 1992). Masuda et al. (1998) found that egg PC combined with vitamin B_{12} resulted in an increased concentration of acetylcholine in the frontal cortex and improved 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 egg PC alone or vitamin B_{12} alone was administered. The molecular mechanisms by which egg PC+vitamin B_{12} administration to animals with impaired memory can ameliorate these brain functions have not been elucidated. Possible mechanisms may include alterations in acetylcholine synthesis and release, because basal forebrain cholinergic neurons participate in memory processes (Loy et al. 1991), or they may be related to the function of choline-containing phospholipids in cell signalling events in the brain (Zeisel & Blusztajn, 1994). We reported that egg PC combined with vitamin B_{12} ameliorates second messenger-mediated injury induced by ibotenic acid by modulating the activity of protein kinase C (PKC; Hung et al. 2000).

The senescence-accelerated mouse (SAM) was established as an experimental model of accelerated ageing by Takeda et al. (1981). SAM comprises many substrains, and each substrain has relatively specific pathological phenotypes. The senescence-accelerated-prone (SAMP) SAM substrain SAMP8 shows age-dependent deterioration in learning and memory abilities, and has been used as a useful animal model for research in senile memory impairment. The senescence-accelerated-resistant (SAMR) SAM substrain

Abbreviations: PKC, protein kinase C; PC, phosphatidylcholine; PCR, polymerase chain reaction; SAM, senescence-accelerated mouse; SAM, senescence-accelerated prone SAM substrain; SAMR, senescence-accelerated-resistant SAM substrain; TBARS, thio Barbtric acid-relative substances.

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SAMR1 serves as the control strain. Compared with SAMR1, SAMP8 is characterized by a lower activity of medial septal and hippocampal choline acetyltransferase (Matsukawa et al. 1999) and lower amounts of hippocampal total and particulate PKC (Armbrecht et al. 1999). In addition, the brain level of lipid peroxides was higher in SAMP8 than in SAMR1 at both 3 and 6 months of age (Matsugo et al. 2000). It is likely, therefore, that alterations in cholinergic neurotransmitter and signal transduction or oxidative stress intervene in the senescence-related degeneration and impairments seen in SAMP8.

In the present study we used SAMP8 as an animal model for senile memory impairment, and compared hippocampal PKC activity, cerebral PKC-β mRNA abundance, cerebral membrane lipid composition and oxidation status with those found in SAMR1. Subsequently, we investigated whether dietary supplementation of egg PC+vitamin B12 to SAMP8 would ameliorate learning behaviour and improve in biochemical and biophysical events in this strain.

Materials and methods

Animals and diet

Mice purchased from Seiwa Experimental Animals (Fukuoka, Japan) were housed individually in standard transparent-plastic cages with free access to diet and non-ionized water, and kept in a temperature-controlled environment of 22 ± 2°C with a 12 h light–dark cycle (lights off at 20.00 hours). The mice were fed a commercial non-purified diet (NMF; Oriental Yeast, Tokyo, Japan) until 3 months of age. Subsequently, ten male SAMR1 and ten SAMP8 were fed a control diet until 6 months of age and fifteen male SAMP8 were either fed the control diet (Table 1) or a diet supplemented with PC+vitamin B12 (Table 1) until 6 months of age. The diets were based on the AIN-93G diet (Reeves et al. 1993). The PC+vitamin B12 diet contained 40 g/kg egg-yolk PC (PL-100LE, 89.2% pure PC (% total fatty acids; palmitic acid 32.0, palmitoleic acid 1.4, stearic acid 13.2, oleic acid 29.8, linoleic acid 12.2, α-linolenic acid 0.1, eicosamonoenoic acid 0.3, arachidonic acid 2.7, eicosapentaenoic acid 0.3, docosahexaenoic acid 6.1); Q.P., Tokyo, Japan) and 500 μg vitamin B12 (Sigma, St Louis, MO, USA). The amounts of PC and vitamin B12 used in the diet were based on findings from our previous experiment (Hung et al. 2000). To balance the fatty acid composition and cholesterol content, the control diet was supplemented with (g/kg): 24:08 palm oil, 4:93 arachidonic acid-rich oil (Suntory, Kyoto, Japan), 0:29 docosahexaenoic acid (Maruha, Tokyo, Japan), 1:9 cholesterol (Nacalai Tesque, Kyoto, Japan). The vitamin B12 contents of the diets were 25 and 525 μg/kg for the control and PC+B12 diets respectively.

Mice were killed by withdrawing blood from the heart after anaesthetization with diethyl ether, and the hippocampus and cerebral cortex were rapidly excised and weighed. The hippocampus was homogenized immediately for PKC determination. The cerebral cortex and the residual cerebrum were frozen at −35°C until required for analysis.

These experiments were carried out under the guidelines for Animal Experiments in the 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.

The degree of senescence in SAM

The total grading score of senescence in 6-month-old SAM was assessed using the method of Hosokawa et al. (1984). The items in the grading score system include eleven categories selected from clinical signs and gross lesions considered to be associated with the ageing process. These eleven items are as follows: (1) reactivity, (2) passivity, (3) glossiness, (4) coarseness, (5) loss of hair, (6) skin ulcer, (7) periophthalmic lesion, (8) opacity of cornea, (9) ulcer of cornea, (10) cataract, (11) lordokyphosis. The degree of senescence in each category is graded from 0 to 4. The total grading score is the sum of the degrees for the eleven items.

Passive avoidance task

A step-through passive avoidance apparatus (SFK-1; O’Hara & Co., Tokyo, Japan) evaluated learning and memory abilities 3 weeks before dissection (Jarvik & Kopp, 1967). In the acquisition trial, each mouse was placed in the light chamber. After a 60 s habituation period, the guillotine door separating the light and dark chambers was opened, and the initial latency time to enter the dark chamber recorded. Immediately after the mouse entered the dark chamber, electric foot shock (250 V, 1 mA, 50 Hz) was delivered to the floor grids for 1 s. Those mice that had an initial latency time of more than 60 s, or that did not return to the light chamber within 3 s of being shocked were excluded from further experiments. After 24 h, the retention latency time was measured in the same way as in the acquisition trial, but foot shock was not delivered and the latency time was recorded to a maximum of 500 s.

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Table 1. Composition of diet (g/kg)*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control diet</th>
<th>PC+vitamin B12 diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200.0</td>
<td>200.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Maize starch</td>
<td>376.3</td>
<td>367.5</td>
</tr>
<tr>
<td>α-Maize starch</td>
<td>132.0</td>
<td>132.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Egg-yolk phosphatidylycholine</td>
<td>0.0</td>
<td>40.0</td>
</tr>
<tr>
<td>Palm oil</td>
<td>24.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Arachidonic acid-rich oil</td>
<td>4.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Ethyl docosahexaenoate</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.0</td>
<td>0.005</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>tert-Butyl hydroquinone</td>
<td>0.014</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*AIN-93G (Reeves et al. 1993).
The PKC assay was carried out as described by Hung et al. (2000). The hippocampus was homogenized with 5 vol. ice-cold homogenizing buffer (0.25 M sucrose, 10 mM Hepes, 2 mM EDTA, 5 mM-ethylen glycol bis (β-aminoethyl-ether)-N,N,N’,N’-tetra acetic acid, 1 mM-phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 10 μg leupeptin/ml, pH 7.5). The extracts were centrifuged at 105,000 g for 60 min at 4°C. The supernatant fraction contained the cytosol. The precipitate was re-extracted with 1% Nonidet P-40 (Nacalai Tesque Inc., Kyoto, Japan) and incubated at 4°C for 1 h. After centrifugation, the supernatant fraction was removed. Cytosolic and particulate fractions were partially purified through a DEAE-cellulose column (DE-52; Bio-Rad, Tokyo, Japan) equilibrated with Hepes buffer (10 mM-Hepes, 20 mM-NaCl, 0.2 mM-EDTA, 0.5 mM-ethyleneglycol-bis(β-aminoethyl ether) N,N,N’,N’-tetra acetic acid, 1 mM-phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, pH 7.4). The PKC activity was analysed by measuring the incorporation of 32P from [γ-32P]ATP (Toho Biochemicals, Tokyo, Japan) into histone. The reaction mixture contained 20 mM-Hepes at pH 7.5, 1 mM-magnesium acetate, 50 μM-[γ-32P]ATP (18.5 GBq), 500 μg histone H1 type III-S/ml, 1 mM-CaCl2, 80 μg phosphatidylserine/ml, 8 μg 1-oleyl-2-acetyl-sn-glycerol/ml and the enzyme extract in a final volume of 0.1 ml. After incubation at 30°C for 3 min, the reaction was stopped by adding 100 μl TCA (40%, v/v), followed by 10 μl 50 mM-ATP and 10 μl bovine serum albumin (5 mg/ml). After centrifugation, 32P in the precipitate was measured using a liquid-scintillation counter. The protein content of enzyme extract was determined using a Bio-Rad protein assay (Bio-Rad, Tokyo, Japan).

Preparation of mRNA and polymerase-chain reaction analysis

Total RNA from the cerebrum was extracted by the method of Chomczynski & Sacchi (1987). Reverse transcription–polymerase chain reaction (PCR) was carried out according to Sokolov & Prockop (1994). Total RNA (5 μg) was transcribed into first-strand cDNA, using You-primed cDNA Single Step Kits (Pharmacia Japan, Tokyo, Japan). In the present study, we selected the β-form of PKC for mRNA determination, since PKC-β is the predominant form of PKC isoforms in the cerebral homogenates of mice, based on our preliminary experiment using monoclonal antibodies for PKC isoforms (K Shibasaki and K Imaizumi, unpublished results). The primers of PKC-β of mice were designed according to the sequence described by Freire-Morao et al. (1991). These primers were 5'-TTCCAGATTTTCAGCCTAGGAA and 5'-TGTCACAAATTGGATATAGTTCCG. PCR amplification was carried out in 10 μl reaction mixtures composed of: 1 μl cDNA solution, 2.5 units AmpliTaq DNA polymerase (Perkin-Elmer Japan, Chiba, Japan), 1 μl 10×PCR buffer containing 2.5 mM-MgCl2 (attached to the enzyme), 1.6 mM each of deoxyribonucleoside-5'-triphosphate mixture, 5 pmol each of two oligonucleotide primers. The amplification conditions were: 94°C 3 min, 95°C 15 s, 40°C 1 min, 72°C 1 min for twenty cycles, 72°C 5 min. The size of amplification products obtained by the primer pairs was 309 bp on 2% agarose gel electrophoresis. We confirmed that the concentration (0.5, 1, 2 and 3 μl/10 μl reaction solution) of cDNA from brain total RNA had a linear correlation with the densitometry examined in u.v. light after electrophoresis (r 0.998, P < 0.01).

The mRNA level of β-actin, one of the housekeeping genes, was also determined. The primers were: 5'-GGTCTGATCCACAGGCAATGTGATG and 5'-GGAAGCATTACCCCTCCTGATAGTG (Tokunaga et al. 1986). The reaction solution of the amplification was as described previously. The amplification conditions were 94°C 3 min, 95°C 15 s, 40°C 1 min, 72°C 1 min for twenty-five cycles, 72°C 5 min. The size of the products obtained by this primer pair was 294 bp.

Lipid analysis

Lipids in the cerebral cortex were extracted with chloroform and methanol according to Folch et al. (1957). The cholesterol content in the cerebral cortex was analysed using GC (Ikeda et al. 1998). Phospholipid P was determined by the method of Rouser et al. (1966). Fatty acid composition was determined using GLC, as described by Ikeda et al. (1998).

Analyses of thiobarbituric acid-reactive substances, glutathione and antioxidative enzyme activities

The cerebral cortex was homogenized in 10 mM-Hepes buffer (containing 137 mM-NaCl, 4.6 mM-KCl, 1.1 mM-KH2PO4, 1 mM-MgSO4, 0.5 mM-EDTA, 0.5 mM-phenylmethylsulfonyl fluoride, 80 μg/ml pepstatin/ml, 0.7 μg/ml pepstatin/ml, 0.5 μg/g aprotinin/ml and 40 μg/ml phosphatidylglycerol/ml; Sato et al. 1996). Homogenates of the cerebral cortex were analysed for thiobarbituric acid-reactive substances (TBARS) by the method of Ohkawa et al. (1979). Glutathione content in the cerebral cortex was analysed using the method of Hissin & Hilf (1976). The activities of glutathione peroxidase and superoxide dismutase were measured as described by Lawrence & Burk (1976) and Misra & Fridovich (1977) respectively.

Statistical analysis

Data were expressed as means with their standard errors. Students’ t test was used to determine the statistical significance. The differences were considered significant at P < 0.05.

Results

Body and brain weight, total grading score of senescence and learning ability

Final body weight was higher for SAMR1 fed the control diet (39.4 (SE 0.39) g, n 10) than seen in SAMP8 fed the control diet (31.9 (SE 0.2) g, n 10; P < 0.05). Reflecting the body weight, the whole-brain weight of the SAMR1 was higher (0.48 (SE 0.01) g, n 10) than that of SAMP8 (0.43...
Table 2. Effect of substrain of mice and diet on the activities of soluble or membrane-bound protein kinase C (PKC) activities in the hippocampus of senescence-accelerated mice (SAM)†

(Means values with their standard errors for ten mice per group for the SAMR1 and SAMP8 substrains and fifteen mice per group control and PC-vitamin B12 diets)

<table>
<thead>
<tr>
<th>Substrain</th>
<th>Soluble (*32P pmol/min per mg protein)</th>
<th>Membrane (*32P pmol/min per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMR1</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>SAMP8</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>Control</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>PC-vitamin B12</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
</tbody>
</table>

SAMR1, senescence-accelerated-resistant SAM substrain; SAMP8, senescence-accelerated-prone SAM substrain; PC, phosphatidylcholine.
Mean values were significantly different from those for the SAMR1 substrain (paired t-test): *P < 0.05, **P < 0.01.
† For details of diets and procedures, see p. 164.

Hippocampal protein kinase C activity and cerebral protein kinase C-β mRNA level

The hippocampal PKC activities of the soluble and membrane fractions from SAMP8 were lower than those from SAMR1 (Table 2). PKC activities of soluble and membrane fractions from the hippocampus of SAMP8 were increased in those fed on the PC-vitamin B12 diet in comparison with those fed on the control diet. The PKC-β mRNA level was determined in the residual region of cerebrum from which the hippocampus and cerebral cortex had been excised. The size of the amplified products of PKC-β mRNA and β-actin mRNA is shown in Fig. 1(C and D). The PKC-β mRNA level was lower for SAMP8 than for SAMR1 (Fig. 1(A and C)), but there was no evidence of dietary effects in SAMP8 (Fig. 1(B and D)).

Antioxidative status

Lipid peroxide (TBARS) of the cerebral cortex was higher for SAMP8 than for SAMR1 (Table 3), but there were no significant differences between SAMP8 and SAMR1 in the activities of superoxide dismutase and glutathione peroxidase and glutathione contents in the cerebral cortex. Ingestion of the PC-vitamin B12 diet resulted in no significant effect on these oxidative variables in the cerebral cortex of SAMP8.

Cholesterol: phospholipid and phospholipid composition of the cerebral cortex

There were no effects of substrain on the cholesterol:phospholipid (Table 4), which is used as an index of membrane fluidity (Kessler & Yehuda, 1985). The relative amount of sphingomyelin in total phospholipid of SAMP8 was higher

Table 3. Effect of substrain of mice and diet on lipid peroxide (thiobarbituric acid-reactive substances; TBARS), superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and glutathione (GSH) in the cerebral cortex of senescence-accelerated (SAM)†

(Means values with their standard errors for ten mice per group for SAMR1 and SAMP8 substrains and fifteen mice per group for control and PC-vitamin B12 diets)

<table>
<thead>
<tr>
<th>Substrain</th>
<th>Control</th>
<th>PC-vitamin B12</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMR1</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>SAMP8</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>TBARS (mmol MDA/g tissue)</td>
<td>42.1 3.6</td>
<td>75.9* 3.9</td>
</tr>
<tr>
<td>SOD (U/g tissue)†</td>
<td>309 27</td>
<td>395 68</td>
</tr>
<tr>
<td>GSHPx (U/g tissue)§</td>
<td>3.05 0.18</td>
<td>3.11 0.23</td>
</tr>
<tr>
<td>GSH (µg/g tissue)</td>
<td>244 12</td>
<td>242 10</td>
</tr>
</tbody>
</table>

SAMR1, senescence-accelerated-resistant SAM substrain; SAMP8, senescence-accelerated-prone SAM substrain; PC, phosphatidylcholine; MDA, malondialdehyde.
Mean value was significantly different from that for the SAMR1 substrain (paired t-test); *P < 0.05.
† For details of diets and procedures, see p. 164.
‡ IU SOD = 50% inhibition of the rate of reduction of cytochrome C.
§ IU GSHPx = µmol NADPH oxidized/min.
than that of SAMR1, but there was no difference between substrains for any of the other phospholipid subclasses. There was no dietary effect on lipid concentrations or phospholipid subclass composition for SAMP8.

Fatty acid composition of phosphatidylethanolamine in the cerebral cortex

Phosphatidylethanolamine in the cerebral cortex from SAMR1 contained a higher proportion of arachidonic acid (20:4 \(n\)-6) and docosahexaenoic acid (22:6 \(n\)-3) than that from SAMP8, while the proportion of palmitic and oleic acids was lower in SAMR1 (Table 5). The PC+vitamin B\(_{12}\) diet resulted in a significant increase in docosahexaenoic acid and a decrease in palmitic acid (\(P < 0.01\) in each case), when compared with the control diet.

### Table 4. Effect of substrain of mice and diet on cholesterol:phospholipid (C:PL) and phospholipid subclass composition in the cerebral cortex of senescence-accelerated mice (SAM)†

<table>
<thead>
<tr>
<th>Substrain</th>
<th>Diet</th>
<th>SAMR1 Mean</th>
<th>SAMR1 SE</th>
<th>SAMP8 Mean</th>
<th>SAMP8 SE</th>
<th>Control Mean</th>
<th>Control SE</th>
<th>PC+vitamin B(_{12}) Mean</th>
<th>PC+vitamin B(_{12}) SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>C:PL</td>
<td></td>
<td>0.61</td>
<td>0.01</td>
<td>0.65</td>
<td>0.02</td>
<td>0.68</td>
<td>0.05</td>
<td>0.72</td>
<td>0.03</td>
</tr>
<tr>
<td>PC (mol/100 mol)</td>
<td></td>
<td>36.9</td>
<td>1.1</td>
<td>35.3</td>
<td>1.5</td>
<td>34.9</td>
<td>1.6</td>
<td>35.7</td>
<td>1.5</td>
</tr>
<tr>
<td>PE (mol/100 mol)</td>
<td></td>
<td>31.9</td>
<td>0.1</td>
<td>34.2</td>
<td>1.3</td>
<td>33.3</td>
<td>1.3</td>
<td>35.0</td>
<td>1.4</td>
</tr>
<tr>
<td>PS (mol/100 mol)</td>
<td></td>
<td>17.9</td>
<td>0.9</td>
<td>20.4</td>
<td>0.9</td>
<td>19.6</td>
<td>0.9</td>
<td>21.2</td>
<td>0.9</td>
</tr>
<tr>
<td>SPH (mol/100 mol)</td>
<td></td>
<td>5.12</td>
<td>0.15</td>
<td>7.60*</td>
<td>0.60</td>
<td>7.89</td>
<td>0.66</td>
<td>7.31</td>
<td>0.52</td>
</tr>
</tbody>
</table>

SAMR1, senescence-accelerated-resistant SAM substrain; SAMP8, senescence-accelerated-prone SAM substrain; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SPH, sphingomyelin.

Mean value was significantly different from that for the SAMR1 strain (paired t-test): * \(P < 0.05\).

† For details of diets and procedure, see p. 164.

### Discussion

#### Effect of substrate

SAM mice were developed by selective sister–brother mating from the AKR/J strain with an inherited form of senescence (Takeda et al. 1981). SAM comprise SAMP and...
SAMR substrains; the latter shows normal ageing characteristics. Among the former, it has been found that the substrain SAMP8 shows significant impairments in various learning tasks when compared with the substrain SAMR1 (Takeda et al. 1981). Hosokawa et al. (1984) reported no difference in the evaluation of the degree of senescence between SAMP and SAMR at a young age, but that a difference became obvious after the age of 6 months. In agreement with these reports, 6-month-old SAMP8 had a higher total degree of senescence and a shorter latency time in the retention time trial than did the corresponding SAMR1.

In addition to these age-dependent morphological and behavioural differences SAMP8 had a lower PKC activity in the soluble and membrane fractions than did SAMR1. These results are in agreement with the findings of Armbrecht et al. (1999), who found an age-dependent decline in PKC-γ protein in the hippocampus and an altered distribution in the soluble and particulate fractions in SAMP8. Shearman et al. (1991) reported that PKC isoforms are distributed differently between pre- and post-synaptic neurons in the hippocampus; PKC-α is localized presynaptically, PKC-β is localized both pre- and post-synaptically and PKC-γ only is localized in post-synaptic neurons. Since we have not determined individual PKC isoforms, it is not clear whether the decreased PKC activity in the hippocampus of SAMP8 can be attributed to PKC-γ or to other isoforms. Moreover, our results show that SAMP8 has a significantly lower PKC-β mRNA level than SAMR1 (P ≤ 0.05) in the residual region of the cerebrum from which the hippocampus and cerebral cortex were excised. Thus, it is likely that a decreased gene expression of PKC isoforms contributes to the lowered PKC activity in the hippocampus of SAMP8. Several studies have shown the involvement of PKC in both the induction and the maintenance phases of long-term potentiation (Bliss & Collingridge, 1993; Colley & Routtenberg, 1993; Sacktor et al. 1993). Battaini et al. (1990) also observed that PKC activity is reduced in both the soluble and the particulate fractions of the cortical structures of aged Sprague-Dawley rats, as compared with findings in young rats. Taken together, the gene expression-mediated PKC activity in the hippocampus of SAMP8 explains the shorter latency time for SAMP8 in the retention trial.

Antioxidative status has been implicated in the physiological decline associated with ageing, since the brain utilizes much O₂ and contains relatively high concentrations of peroxidizable fatty acids (Harman, 1981). Although the present study shows no significant difference in the activities of superoxide dismutase and glutathione peroxidase, and glutathione content in the cerebral cortex, SAMP8 has a higher lipid peroxide concentration in the cerebral cortex than SAMR1. In addition, SAMP8 has lower proportions of 20:4n-6 and 22:6n-3 in the cortical phosphatidylethanolamine, accompanied by an elevation of saturated fatty acids. Thus, increased oxidative stress in the brain of SAMP8 could lead to a reduction in these polyunsaturated fatty acids. Reduction of polyunsaturated fatty acids in synaptosomal membranes could also be responsible for the altered PKC activity in the hippocampus of SAMP8, because polyunsaturated fatty acids are related to PKC activation (Epand & Lester, 1990).

Oxidative stress could also indirectly be responsible for an increased proportion of sphingomyelin in the cerebral cortex of SAMP8, since sphingomyelin contains mainly saturated and monounsaturated fatty acids. Delion et al. (1997) also reported an age-induced increase in sphingomyelin in the rat hippocampus, whereas PC and phosphatidylethanolamine decreased. The elevation of sphingomyelin in some brain regions is of critical importance in accelerated ageing, since it was reported that increasing membrane sphingomyelin levels in rat haemochromocytoma cells led to a dramatic increase in vulnerability to oxidative stress and to a significant loss of Ca homeostasis (Denisova et al. 1997).

All these observations indicate a close relationship between learning behaviour and biochemical or physicochemical properties in some regions of the brain of SAMP8. Thus, analysing the effect of nutritional intervention on these
properties in the brain of SAMP8 could indicate how to prevent age-dependent deterioration of cerebral functions.

**Effect of diet**

The PC+vitamin B₁₂ diet did not affect growth variables, total grading score of senescence and memory retention, and there were no dietary effects on cerebral lipid peroxide levels and the membrane phospholipid subclass composition. Interestingly, the PC+vitamin B₁₂ diet resulted in increased activity of hippocampal PKC in both the cytosol and membrane fractions in SAMP8. Elevation of the enzyme activity was not accompanied by changes in cerebral PKC-β mRNA level, thereby indicating that the PC+vitamin B₁₂ diet affects PKC activity at the post-transcriptional step rather than at PKC gene expression. However, it is also possible that the diet influences the abundance of PKC isoform mRNA other than that of the β-form.

Transmitters that alter the hippocampal PKC activity are likely to be acetylcholine and glutamate (Vanderzee et al. 1997), because acetylcholine depolarizes pyramidal neurons, up regulates N-methyl-D-aspartate channels and activates the phosphoinosistide cascade. Nishizaki et al. (1999) reported that arachidonic acid sustains enhanced activity of the acetylcholine receptor by interacting with a PKC pathway, thereby increasing glutamate release from presynaptic terminals and leading to a ‘long-term potentiation-like’ facilitation of hippocampal synaptic transmission. Although we did not measure the concentration of cerebral acetylcholine, the PC+vitamin B₁₂ diet influenced the cerebral cholinergic system in SAMP8. Many researchers have found that dietary supplementation with PC increases brain acetylcholine concentrations in aged animals (Hirsch & Wurtman, 1978; Magil et al. 1981; Jope, 1982; Leathwood et al. 1982; Matsumoto et al. 1994; Chung et al. 1995). Furthermore, Alzheimer-type dementia is associated with vitamin B₁₂ deficiency (Nourhashemi et al. 2000), and oral administration of vitamin B₁₂ improves inferior learning performance in an animal model of vascular dementia (Kimura et al. 1998) or increased the acetylcholine concentration in the brain of rats that have ibotenic acid-induced lesions (Masuda et al. 1998; Hung et al. 2000).

Activation of the cholinergic system rather than improvement of the antioxidative status involves a significantly increased proportion of 22:6n-3 of phosphatidylethanolamine in the cerebral cortex in SAMP8 fed the PC+vitamin B₁₂ diet. In fact, Jones et al. (1997) reported that activation by the M1 cholinergic agent arecoline stimulates entry of [³H]docosahexaenoic acid into phospholipid of the synaptosomal and microsomal membrane fractions in rats. One possible consequence of this change in phosphatidylethanolamine bilayers may be that it facilitates the binding of PKC and rearrangement to an active form, because phosphatidylethanolamine with 18:1 and 22:6 causes an increase in the rate of histone phosphorylation by PKC, whereas PC with 18:1 and 22:6 had no effect on this enzyme (Giorgione et al. 1995). Thus, activation of the cholinergic system by the PC+vitamin B₁₂ diet would lead to an elevation of the hippocampal PKC activity.

In the present study, SAMP8 fed the PC+vitamin B₁₂ diet showed no significant improvement in learning behaviour. This result is in contrast to results showing an improvement of learning behaviour in rats with ibotenic acid-induced lesions that received a PC+vitamin B₁₂ (Masuda et al. 1998; Hung et al. 2000). This discrepancy may result from the nature of the brain lesions, since ibotenic acid-induced brain lesions can be reversible (Wree et al. 1993), but the impairment of the neural system in aged SAMP8 is not reversible.

In summary, our results show that a dietary supplement of PC+vitamin B₁₂ results in hippocampal PKC activation and an increase in docosahexaenoic acid in cerebral regions. These potential benefits of a PC+vitamin B₁₂ diet are induced by activation of the cholinergic system.

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**References**


Tokunaga K, Taniguchi H, Yoda M, Shimizu M & Sakiyama S...

