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-β mRNA abundance than SAMR1. SAMP8 had increased cerebral lipid peroxide levels and proportion of sphingomyelin, and a lower proportion of $20:4n$-6 and $22:6n$-3 in cerebral phosphtidylethanolamine than SAMR1. SAMP8 fed the PC combined with vitamin B$_{12}$ diet had an increased PKC activity and a higher proportion of $22:6n$-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

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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 neurostimulator 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 B_{12} 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 $22 \pm 2°C$ 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 B_{12} (Table 1) until 6 months of age. The diets were based on the AIN-93G diet (Reeves *et al.* 1993). The PC+vitamin B_{12} diet contained 40 g/kg egg-yolk PC (PL-100LE, 89·2 % pure PC

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

Ingredients	Control diet	PC+vitamin B_{12} diet
Casein	200·0	200·0
L-Cystine	3·0	3·0
Maize starch	376·3	367·5
α-Maize starch	132·0	132·0
Sucrose	100·0	100·0
Cellulose	50·0	50·0
Soyabean oil	60·0	60·0
Egg-yolk phosphatidylcholine	0·0	40·0
Palm oil	24·1	0·0
Arachidonic acid-rich oil	4·9	0·0
Ethyl docosahexaenoate	0·3	0·0
Cholesterol	1·9	0·0
Mineral mix	35·0	35·0
Vitamin mix	10·0	10·0
Vitamin B_{12}	0·0	0·0005
Choline bitartrate	2·5	2·5
tert-Butyl hydroquinone	0·014	0·014

* AIN-93G (Reeves *et al.* 1993).

(% 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 B_{12} (Sigma, St Louis, MO, USA). The amounts of PC and vitamin B_{12} 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 B_{12} contents of the diets were 25 and 525 μg/kg for the control and PC+B_{12} 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 of 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, 1·0 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.

Assay of PKC activity

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-ethylene 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-ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N'*,*N'*-tetraacetic acid, 1 mM-phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, pH 7·4). The PKC activity was analysed by measuring the incorporation of ^{32}P from [γ-^{32}P]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-^{32}P]ATP (18·5 GBq), 500 μg histone H1 type III-S/ml, 1 mM-CaCl$_2$, 80 μg phosphatidylserine/ml, 8 μg 1-oleoyl-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, ^{32}P 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 main 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-Moar *et al.* (1991). These primers were 5′-TTCAGATTTCAGCTGAAGGAA and 5′-TGTCAAATT-TGGATATAGTGTTCG. 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-MgCl$_2$ (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′-GGTCGTACCACAGGCATTGTGATG and 5-GGAGAG-CATAGCCCTCGTAGATGG (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-KH$_2$PO$_4$, 1·1 mM-EDTA, 0·6 mM-MgSO$_4$, 0·5 μg leupeptin/ml, 0·7 μg pepstatin/ml, 0·5 μg aprotinin/ml and 40 μg phenylmethylsulfonyl fluoride/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 determined as described by 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)‡

(Mean 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 B_{12} diets)

	Substrain				Diet			
	SAMR1		SAMP8		Control		PC+vitamin B_{12}	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Soluble (^{32}P pmol/min per mg protein)	29·8	1·8	26·1*	0·5	16·2	0·9	30·8††	0·1
Membrane (^{32}P pmol/min per mg protein)	28·9	3·9	18·5**	1·2	8·3	2·2	19·1†	3·6

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): * $P < 0.05$, ** $P < 0.01$.
Mean values were significantly different from those for the control diet (paired *t* test): † $P < 0.05$, †† $P < 0.01$.
‡ For details of diets and procedures, see p. 164.

(SE 0·01) g, *n* 10; $P < 0.05$). The body and brain weights of SAMP8 fed the control diet did not differ from those of SAMP8 fed the PC+vitamin B_{12} diet.

SAMP8 had a higher total grading score (4·66 (SE 0·27; *n* 10) and shorter latency time in the retention trial (317 (SE 64) s, *n* 10) than the score (3·08 (SE 0·51, *n* 10; $P < 0.05$) and latency time (447 (SE 53) s, *n* 10; $P < 0.05$) for SAMR1. The control and the PC+vitamin B_{12} diet affected neither the grading score nor the latency time (data not given).

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 B_{12} 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 B_{12} 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 acid-reactive substances; TBARS), superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and glutathione (GSH) in the cerebral cortex of senescence-accelerated (SAM)†

(Mean 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 B_{12} diets)

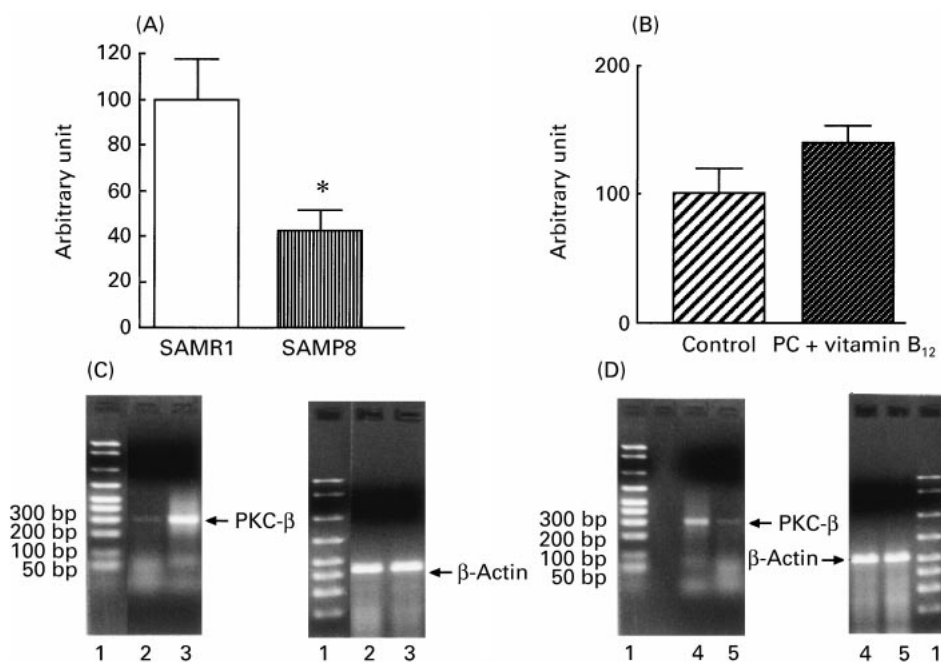|	Substrain				Diet			
	SAMR1		SAMP8		Control		PC+vitamin B_{12}	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
TBARS (mmol MDA/g tissue)	42·1	3·6	75·9*	3·9	88·1	11·3	884	12·1
SOD (U/g tissue)‡	309	27	395	68	446	108	485	23
GSHPx (U/g tissue)§	3·05	0·18	3·11	0·23	2·67	0·60	3·12	0·30
GSH (μg/g tissue)	244	12	242	10	249	8	234	22

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 procedure, see p. 164.
‡ IU SOD = 50% inhibition of the rate of reduction of cytochrome C.
§ IU GSHPx = 1 μmol NADPH oxidized/min.

Fig. 1. Effect of substrain of mice or diet on cerebral protein kinase C (PKC)-β mRNA level in senescence-accelerated mice (SAM): (A), Mice of the senescence-accelerated-resistant SAM substrain (SAMR1) and senescence-accelerated-prone SAM substrain (SAMP8) fed a control diet (n 10); (B), SAMP8 fed a control diet or a phosphatidylcholine (PC)+vitamin B$_{12}$ diet. (C), Representative patterns of the amplified products of PKC-β and β-actin mRNA for SAMR1 (sample 3) and SAMP8 (sample 2), separated on agarose gel electrophoresis. (D), Representative patterns of the amplified products of PKC-β and β-actin mRNA for SAMP8 fed a control diet (sample 4) or a PC+vitamin B$_{12}$ diet (sample 5), separated on agarose gel electrophoresis. In C and D sample 1 represents a molecular-weight marker of DNA. Values are means with their standard errors represented by vertical bars. Mean value was significantly different from that of SAMR1: * $P < 0.05$.

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 : 4n-6) and docosahexaenoic acid (22 : 6n-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.

Discussion

Effect of substrain

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

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)†

(Mean 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 B$_{12}$ diets)

	Substrain				Diet			
	SAMR1		SAMP8		Control		PC+vitamin B$_{12}$	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
C:PL	0·61	0·01	0·65	0·02	0·68	0·05	0·72	0·03
PC (mol/100 mol)	36·0	1·1	35·3	1·5	34·9	1·6	35·7	1·5
PE (mol/100 mol)	31·9	0·1	34·2	1·3	33·3	1·3	35·0	1·4
PS (mol/100 mol)	17·9	0·9	20·4	0·9	19·6	0·9	21·2	0·9
SPH (mol/100 mol)	5·12	0·15	7·60*	0·60	7·89	0·66	7·31	0·52

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.

Table 5. Effect of substrain of mice and diet on major fatty acid composition (mol/100 mol total fatty acids) of phosphatidylethanolamine in the cerebral cortex of senescence-accelerated mice (SAM)†

(Mean 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 B_{12} diets)

| | Substrain | | | | Diet | | | |
| | SAMR1 | | SAMP8 | | Control | | PC+vitamin B_{12} | |
Fatty acid	Mean	SE	Mean	SE	Mean	SE	Mean	SE
16:0	10·9	0·5	15·5*	1·5	16·1	2·4	11·5††	0·4
18:0	26·3	2·3	30·3	0·3	30·3	0·3	29·2	1·1
18:1	16·9	0·4	19·8*	1·6	19·8	1·6	18·7	0·6
20:4n-6	14·1	0·2	11·7*	1·2	11·7	1·2	13·3	0·4
22:6n-3	20·6	0·8	16·0*	1·6	16·9	1·6	20·7††	0·6

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

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 ($P \leq 0.05$) 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 O_2 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 physico-chemical 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 B$_{12}$ 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 B$_{12}$ 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 B$_{12}$ 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 phosphoinositide 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 B$_{12}$ 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 B$_{12}$ deficiency (Nourhashemi *et al.* 2000), and oral administration of vitamin B$_{12}$ 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 B$_{12}$ diet. In fact, Jones *et al.* (1997) reported that activation by the M1 cholinergic agent arecoline stimulates entry of [^{3}H]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 B$_{12}$ diet would lead to an elevation of the hippocampal PKC activity.

In the present study, SAMP8 fed the PC+vitamin B$_{12}$ 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 B$_{12}$ (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 B$_{12}$ results in hippocampal PKC activation and an increase in docosahexaenoic acid in cerebral regions. These potential benefits of a PC+vitamin B$_{12}$ diet are induced by activation of the cholinergic system.

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