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Unaltered brain membranes after prolonged intake of highly oxidizable long-chain fatty acids of the $(n-3)$ series

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Feeding rats a diet enriched in $n-3$ polyunsaturated fatty acids (Menhaden oil) increased the content in eicosapentaenoic acid 20:5 $n-3$ of brain phospholipids. Conversely 22:4 $n-6$ was reduced. These changes were not associated with alterations in either vitamin E concentration or glutathione peroxidase and catalase activities in cerebrum and cerebellum. No increase in peroxidative damage was found. Interestingly the major very-long-chain fatty acids (22:6 $n-3$ and 22:5 $n-3$) were not affected.

Cell membranes of the central nervous system are especially rich in highly oxidizable polyunsaturated fatty acids, such as arachidonic and docosahexaenoic acids [21]. Given its high oxygen consumption, the central nervous system is therefore highly susceptible to oxidative damage.

Although an increased intake in vitamin E, the main lipid-soluble free radical scavenger, should help to prevent lipid peroxidation, there is some evidence that a dietary overload in polyunsaturated fatty acids can lead to an increase in lipid peroxidation in a number of tissues [8, 22, 24].

In recent years, there has been a growing interest in the use of dietary fish oils that are rich in eicosapentaenoic acid, because of the potential antithrombotic [4, 6, 11], anti-inflammatory [12] and hypolipidemic [17] effects of this long-chain polyunsaturated fatty acid. Hence, daily intakes of 20:5 $n-3$ ranging from 0.1 to 10 g have been recommended for elderly people [4, 10, 11].

In the brain, long-chain fatty acids of the $(n-3)$ series can be obtained through

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in situ elongation–desaturation of dietary α-linolenic acid [2, 5, 15]; alternatively, there is evidence that Δ^6-desaturase activity is very low in the adult brain, and therefore that end products of the liver desaturases should be directly incorporated into the brain [15].

When the amount of dietary 22:6 $n-3$ and/or its precursor 20:5 $n-3$ largely exceeds the corresponding biosynthetic capacity from α-linolenic acid, the step-by-step regulation of this biosynthetic pathway would no longer control the final amount of 20:5 $n-3$ and 22:6 $n-3$ that is incorporated into membrane phospholipids. In the brain, this control would then have to be maintained through feedback regulation, i.e. metabolic retroconversion and/or inhibition of transport of liver end products through the blood–brain barrier.

Any failure in this additional regulative capacity would lead to large increases in 22:6 $n-3$ and/or 20:5 $n-3$ within brain phospholipids, and this would increase the susceptibility of brain membranes to free radical damage unless it was accompanied by a compensatory increase in antioxidant protection.

This work was undertaken to assess the effects of a large dietary intake in Menhaden oil on the rat brain content of polyunsaturated fatty acids and antioxidant components after a 2-month-long administration.

Thirty male rats of the Sprague–Dawley strain (Iffa-Credo, l'Arbresle, France), weighing 200 g (approx. 2 months) at the beginning of the experiment, were divided into two groups of 15 animals. All the animals were fed a rat chow diet ad libitum (containing 5% lipids). In addition, rats of the first group received a daily amount of 100 μl of Menhaden oil (MAX-EPA, Scherer, Strasbourg, France) per 100 g body wt., for 60 days through gastric intubation, while rats of the second group received the same amount of olive oil, of current commercial quality (Puget, Marseille, France).

Fatty acid composition of commercial chow diet was: 16:0 (21%), 16:1 (1.5%), 18:0 (7.2%), 18:1 $n-9$ (28.6%), 18:1 $n-7$ (1.4%), 18:2 $n-6$ (37.6%), 18:3 $n-3$ (2.7%).

Menhaden oil fatty acid composition was: 14:0 (10.0%), 16:0 (21.3%), 16:1 (11.6%), 18:0 (4.0%), 18:1 $n-9$ (13.5%), 18:1 $n-7$ (4.2%), 18:2 $n-6$ (1.7%), 20:5 $n-3$ (20.8%), 22:6 $n-3$ (12.9%).

Olive oil fatty acid composition was: 16:0 (14.3%), 16:1 (1.3%), 18:0 (3.5%), 18:1 $n-9$ (65.9%), 18:1 $n-7$ (2.7%), 18:2 $n-6$ (11.6%), 18:3 $n-3$ (0.7%).

As adult animals ate approximately 20 g/day of 5% lipid standard chow, when adding 200 μl/day of either Menhaden or olive oil, the final dietary intake was approx. 5.9% lipid. Adding oil to the diet did not change the body weight.

All the rats were anesthetized by diethylether inhalation, and immediately perfused through the left heart with 50 ml of physiological saline containing 10 U/ml of heparin and 0.5% pyrogallol. The animals were then killed by decapitation and the brain and cerebellum were excised and cut in two approximately equal pieces through the median longitudinal axis. One piece was used to make an aqueous homogenate with a Teflon pestle homogenizer, at 4°C, in 50 mM Tris-HCl, 0.1 mM EDTA, 0.1% lubrol, pH 7.6 (7/1, v/w). Aliquots of low-speed supernatants of the aqueous homogenates were used for measurements of enzyme activities and total proteins. The oth-

TABLE I

FATTY ACID COMPOSITION[a] OF BRAIN LIPIDS OF 'MENHADEN' AND 'OLIVE' RAT GROUPS ($n=15$ each)

$^{*}P<0.05$, $^{**}P<0.01$.

Fatty acid	Menhaden			Olive		
	TL[b]	PE[c]	PC[d]	TL	PE	PC
16:0	23.1±0.7	11.0±0.8	48.4±2.3	22.2±1.6	12.1±1.8	49.9±2.5
16:1	1.7±0.1	2.0±0.3	4.7±1.0	0.8±0.6	1.1±0.8	4.5±0.4
18:0	21.9±0.8	23.6±1.0	11.2±0.8	21.5±1.5	25.8±2.3	10.9±1.3
18:1	24.5±0.8	14.5±0.4	24.8±1.5	25.4±0.8	14.7±1.2	24.1±1.3
18:2 $n-6$	0.8±0.8	0.6±0.4	0.2±0.1*	0.9±1.2	0.9±0.3	0.8±0.3
18:3 $n-3$	<0.2	<0.2	0.2±0.1	<0.2	0.3±0.1	<0.2
20:3 $n-6$	1.3±0.9	0.5±0.2	0.3±0.1	0.8±0.2	0.4±0.1	0.3±0.2
20:4 $n-6$	9.1±0.4	20.9±1.3	6.3±0.9	9.1±0.6	20.8±2.0	6.7±0.9
20:5 $n-3$	2.5±0.5**	1.8±0.6**	0.4±0.1	0.4±0.1	0.2±0.1	0.2±0.1
22:4 $n-6$	0.7±0.4**	5.5±0.8	1.0±0.4	6.4±3.7	6.8±0.9	0.7±0.2
22:5 $n-3$	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
22:5 $n-6$	2.4±0.6	4.7±1.4	1.4±0.2	2.3±0.6	4.6±1.0	0.6±0.1
22:6 $n-3$	12.1±0.9	14.9±0.8	1.3±0.2	10.4±2.6	12.2±2.4	1.4±0.3
Saturated	45.0	34.6	59.6	43.7	37.9	60.8
Mono-unsaturated	26.2	16.5	29.5	26.2	15.8	28.6
Poly-unsaturated	28.9	48.9	11.1	30.5	46.2	10.7

[a] Results are expressed as % of total fatty acids (mean ± S.D.).

[b] Total lipids.

[c] Phosphatidylethanolamine.

[d] Phosphatidylcholine.

er piece of tissue was used to make a lipid extract [19] with a Teflon pestle homogenizer (18/1, v/w) in hexane-isopropanol (3/2, v/v) containing 0.5% butylated hydroxytoluene as an antioxidant.

The lipid extracts were used for measurement of vitamin E and lipid fluorescence [23], lipid fractionation by TLC [13] and acidic trans-methanolysis of lipid esters [20]. Analysis of the fatty acid profile of the resulting methylesters was performed by GLC on a fused-silica capillary column impregnated with carbowax 20 M (25 m length, 0.32 mm diameter); detection was performed using flame ionizing detection. Quantification was obtained with ENICA-21 Delsi integrator. Vitamin E was analyzed by a modification of a previously published procedure [9] that used reverse-phase HPLC on a 10 μm-C18 column (250 × 4 mm), with elution by 95% methanol and an absorptiometric detection at 295 nm; α-tocopheryl acetate was used as an internal standard.

Glutathione peroxidase activity was measured by a modification of the method of Paglia and Valentine [16], at 0.5 mM GSH, pH 7.6, 37°C, and using hydrogen peroxide and cumene hydroperoxide as substrates in two separate assays. Catalase activity was measured by monitoring the disappearance of 10 mM H_2O_2 at 240 nm in 50 mM Tris-HCl, 0.1 mM EDTA, 0.1% lubrol, pH 7.4 and 37°C. The presence of 0.1% lubrol allows satisfactorily linear initial rates to be obtained in brain tissue extracts (adapted from Aebi [1]).

Total protein was measured by the dye-binding assay of Bradford [3].

The fatty acid profile of brain lipids is shown in Table I. Significant differences can be observed in total lipids where the percentage of 22:4 $n-6$ was 9 times lower in the 'Menhaden' group, while the percentage of 20:5 $n-3$ was 6 times higher ($P<0.01$ in both cases).

In the phosphatidylethanolamine fraction which contains 45–50% of polyunsaturated fatty acids, the percentage of 20:5 $n-3$ is 9 times higher in the 'Menhaden' group ($P<0.01$), while the percentage of 22:6 $n-3$ was not significantly different between groups.

Finally, in the phosphatidylcholine fraction which only contains 11% of polyunsaturated fatty acids, the percentage of 20:5 $n-3$ and 22:6 $n-3$ was higher in the 'Menhaden' group, but these differences were minor when compared to those observed in the phosphatidylethanolamine fraction.

As 22:4 $n-6$ was decreased in total lipid from Menhaden oil-fed animals, but no significant alterations were found in neither phosphatidylethanolamine nor phosphatidylcholine, large alterations are speculated to be found in phosphatidylserine.

The main differences observed between groups in both total lipids and the phosphatidylethanolamine fraction did not change the percentage of total polyunsaturated fatty acids which remained close to 29% in the total lipids of each group.

The vitamin E content as well as the activity of glutathione peroxidase and catalase in the brain and cerebellum of 'Menhaden' and 'Olive' groups are shown in Table II. They do not indicate significant deviations between the two groups. The differences between brain and cerebellum, mainly higher values for vitamin E and for catalase activity in cerebellum, are similar in the two groups.

From the pattern of substrate specificity it can be concluded that glutathione per-

TABLE II

VITAMIN E, GLUTATHIONE PEROXIDASE AND CATALASE IN CEREBRUM AND CEREBELLUM OF 'MENHADEN' (M) AND 'OLIVE' (O) RAT GROUPS[a] ($n = 15$ each)

	Cerebrum		Cerebellum	
	(M)	(O)	(M)	(O)
Vitamin E[b]	19.1 ± 1.9	17.8 ± 3.3	12.0 ± 1.5	11.3 ± 2.0
Glutathione peroxidase[c]	30.0 ± 4.8	28.7 ± 3.2	32.7 ± 3.7	39.1 ± 3.7
Catalase[d]	94.1 ± 20.5	92.3 ± 23.9	173 ± 22	150 ± 24

[a] Results are mean ± S.D.
[b] μg/g of fresh weight.
[c] nmol NADPH oxidized/min/mg protein at pH 7.6, 37°C and 0.5 mM reduced glutathione.
[d] nmol hydrogen peroxide consumed/min/mg protein at pH 7.4 and 37°C. Student's t-test for paired comparisons.

oxidase activity is always entirely due to the selenium-dependent enzyme in brain and cerebellum.

The amounts of 20:5 $n-3$ and 22:6 $n-3$ which were ingested by rats of the 'Menhaden' group were respectively 130 and 85 mg/kg body wt./day. This would roughly correspond to a daily intake of 10 and 6.7 g respectively, for a human adult. On the contrary, undetectable amounts of 20:5 $n-3$ and 22:6 $n-3$ were present in the olive oil diet. In spite of such enormous differences in dietary intake, the polyunsaturated fatty acid content of the brain and cerebellum were very similar in the two groups of rats. While the brain content of 20:5 $n-3$ clearly increased in the 'Menhaden' group, this was apparently in 'replacement' of 22:4 $n-6$, phenomenon similar to the one that has been previously observed with a diet rich in 18:3 $n-3$ [15]. The brain content of 22:6 $n-3$, the major fatty acid of the ($n-3$) series in the brain, was not significantly different between groups. This indicates that the level of this fatty acid in brain is more tightly regulated than 20:5 $n-3$. Although the activity of Δ^6-desaturase is usually considered as the limiting step in the biosynthesis of 22:6 $n-3$ in the brain, this study suggests that another type of regulation (Δ^4-desaturase) operates in a situation of dietary overload in 20:5 $n-3$ and 22:6 $n-3$ to maintain the level of 22:6 $n-3$ within its normal range. Another possibility is involvement of transacylases. Total polyunsaturated fatty acids are unchanged in the brain of such rats and there seems to be no need for an adaptation of antioxidant systems such as vitamin E, selenium-glutathione peroxidase or catalase to prevent oxidative damage to the cerebrum and cerebellum, although 20:5 $n-3$ was increased.

In contrast to the minor cerebral variations that we found in this study, a large nutritional intake in long-chain $n-3$ fatty acids has been shown to induce adaptative changes in the phospholipid distribution of human erythrocytes [18] and 5'-nucleotidase activity in rat liver [7]. Moreover it has been shown that liver microsomes of rats fed fish oil contained high level of lipid peroxides and high epoxide-hydrolase [14].

We did not find any increase in lipidofluorescence (262 ± 61 relative fluorescence per mg phosphorus) (370 exc./450 em.) or malonaldehyde (MDA) concentrations (41.2 ± 4.3 nmol MDA/g fresh weight; index of membrane oxidative damage) or in Na,K-ATPase activity (95.5 ± 10.3 nmol/mg protein/min), in the brain of rats which had ingested Menhaden oil (data not shown).

Our results suggest that the tight regulation of the content of polyunsaturated fatty acids in the brain prevents any oxidative damage occurring as a result of a dietary overload of 20:5 $n-3$ and 22:6 $n-3$, as increasing dietary content of very-long-chain polyunsaturated fatty acids does not alter brain vitamin E, glutathione peroxidase and catalase. Such large quantities of 20:5 ($n-3$), i.e. 165 mg/kg/day and of 22:6 $n-3$, i.e. 104 mg/kg/day in the diet, produced similar alterations in the respective fatty acid level expressed as percentage of total lipid (2.4 and 17%) but the difference was only significant for 20:5 $n-3$. Thus in both cases, this could mean that the polyunsaturated fatty acid rich diet may have bred the same poor effect for both fatty acids in brain.

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