Diets containing N-3 fatty acids decrease the concentrations of arachidonic acid in phospholipids, free fatty acid, and diacylglycerol fractions of submandibular salivary glands

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The purpose of this study was to determine if feeding diets supplemented with n-3 fatty acids would result in reduced levels of arachidonic acid in major phospholipids, free fatty acid (FFA), and diacylglycerol (DAG) fractions of the salivary glands. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine plus phosphatidylinositol, FFA, and DAG fractions were isolated from the submandibular salivary glands (SMSG) of rats fed nutritionally adequate, semi-purified diets enriched with n-6 or n-3 fatty acids for 6 weeks. The fatty acid composition of the major phospholipids present in the SMSG and the concentrations of arachidonic acid were measured by gas chromatography-mass spectrometry after the addition of 18:4n-3 as an internal standard and transesterification. Arachidonic acid concentrations were significantly lower in each of the lipid fractions of the SMSG of rats fed diets rich in n-3 fatty acids than those of the control group. The results suggest that the diet-induced decrease in the potentially available sources of free arachidonic acid may result in a decrease in the levels of endogenous PGE_2 and in vitro synthesis of LTC_4, which was previously observed in the SMSG of rats fed diets containing n-3 fatty acids.

Keywords: arachidonic acid; phospholipids; FFA; diacylglycerols; n-3 fatty acids; salivary glands

Introduction

Arachidonic acid can be released from membrane phospholipids in response to a number of receptor-mediated signals. The enzyme most responsible for the release of arachidonic acid is mainly phospholipase A_2[1] and to a lesser extent 1,2-diacylglycerol (DAG) lipase.[2] Diacylglycerol is a second messenger that is produced along with inositol trisphosphate as a result of a receptor-mediated hydrolysis of phosphatidyl-inositol-4,5-bisphosphate (PIP_2) by a specific phospholipase C.[3] In addition to the hydrolysis of PIP_2, another source of DAG in plasma membranes may be phosphatidic acid hydrolysis by phosphatidate phosphohydrolase.[4,5] Evidence indicates that endogenous phosphatidylcholine undergoes rapid turnover upon specific stimulation and that this turnover is, at least in part, mediated by activable phospholipase D.[6] Phosphatidic acid thus produced may be hydrolyzed to DAG and inorganic phosphate, a reaction catalyzed by phosphatidate phosphohydrolase. The contribution of this pathway to the DAG pool is especially important because the levels of phosphatidylcholine in the membranes are several-fold higher than those of PIP_2.

A previous study from our laboratory showed that dietary n-3 (ω3) fatty acids are incorporated into the submandibular salivary gland (SMSG) lipids and alter adenylate cyclase activity.[7] Recently, we reported that the feeding of diets enriched with n-3 fatty acids results in lower concentrations of arachidonic acid in total phospholipids of the rat gingiva and SMSG.[8] Reduction in arachidonic acid levels in phospholipids was asso-

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ciated with a concomitant decrease in the levels of prostaglandin E_2 (PGE_2) and the in vitro synthesis of leukotriene C_4 (LTC_4).

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) are the major phospholipids that constitute 60–75% of the total phospholipids in the rat SMSG.[9] These phospholipids could be potential sources of arachidonic acid. We have, therefore, measured the fatty acid composition along with the concentrations of arachidonic acid in these phospholipids in the SMSG of rats fed diets supplemented with n-3 or n-6 fatty acids. Because it is the non-esterified (free) arachidonic acid that acts as a substrate for the cyclooxygenase and lipoxygenase pathways, we measured the levels of arachidonic acid in the free fatty acid pool of the SMSG of rats fed diets enriched with n-3 fatty acids. Arachidonic acid levels in the DAG fraction also were measured because, in addition to phospholipids, DAG provides free arachidonic acid by DAG lipase action.

Materials and methods

Male weanling Sprague-Dawley rats (Holtzman Co., Madison, WI USA) were fed semipurified diets containing 5% corn oil (group 1), 4% ethylester concentrate (EEC) of n-3 fatty acids + 1% corn oil (group 2), and 5% EEC of n-3 fatty acids (group 3) ad libitum for 6 weeks. The basal diet was similar to that of the American Institute of Nutrition (AIN-76A) diet.[10,11] All the dietary ingredients, including corn oil, were purchased from TEKLAD (Madison, WI USA). The EEC of n-3 fatty acids was supplied by the Charleston Laboratory Southeast Fisheries Science Center, National Marine Fisheries Service, U.S. Department of Commerce under the Fish Oil Test Material Program of National Institutes of Health. The fatty acid composition of the corn oil and EEC n-3 has been previously reported.[7,8] Corn oil contained (as percent of total fatty acids): 16:0, 11.3; 18:0, 2.1; 18:1, 27.1; 18:2 n-6, 58.4, and 18:3, 0.9. The EEC of n-3 fatty acids had 78.5% of the total fatty acids as n-3, with 20:5 (43.9%) and 22:6 (24.0%) as the major fatty acids. It also contained 3.4% of the total fatty acids as n-6, with 20:4 constituting 2.3% of the total. The purpose of adding 1% corn oil to the 4% EEC n-3 diet (group 2) was to exclude the possibility of inadvertently inducing essential fatty acid deficiency. Although EEC of n-3 fatty acids contained only 0.1% of 18:2n-6, the addition of 1% corn oil supplied adequate amounts (1.5% of total calories) of 18:2n-6 to satisfy its nutritional requirements for the growing rats, which is considered to be 1–2% of the total calories.[12]

Rats were killed by decapitation and the SMSG were dissected out, weighed, rinsed with ice-cold physiological saline, and the total lipids were extracted.[13] Aliquots of the total lipid extracts were subjected to silicic acid column chromatography on Biosil-A columns to separate the neutral lipids and the phospholipid fractions. Aliquots from each of the two fractions were subjected to thin layer chromatography on 0.25 mm silica gel plates that were developed with hexane/diethylether/acetic acid (80:20:1, vol/vol) for neutral lipids, and with chloroform/methanol/acetic acid/water (25:15:4:1.75 vol/vol) for phospholipids. The standards of oleic acid, 1,3-dioleoylglycerol, cholesterol, and trioleoylglycerol were used as markers for neutral lipids. PC, PE, PI, and PS were used as markers for phospholipid separation. Plates were exposed briefly (10–15 seconds) to iodine vapors to visualize the various spots. The desired lipid spots (FFA and DAG from the neutral lipids and PC, PE, and PI + PS from phospholipids) were scraped off the plates and extracted twice with chloroform/methanol/H_2O (1:2:0.8 vol/vol). PI was not separated from PS with this solvent system. After the addition of an equal volume of chloroform and water, the two phases were separated. The chloroform phase was used for measuring the concentrations of arachidonic acid. After the addition of an internal standard (1–5 μg octadecatetraenoic acid, 18:4n-3) the chloroform phase was dried under nitrogen, 2 mL of 14% boron trifluoride-methanol was added, and the fatty acids were transesterified.[14] Octadecatetraenoic acid (18:4n-3) was chosen as an internal standard instead of 15:0 or 17:0, which are normally used,[9,15] because of its structural similarity to arachidonic acid (20:4n-6). Also, this fatty acid was not found in the lipid extracts of tissues or plasma of rats fed diets rich in n-3 fatty acids (S.Q. Alam, unpublished observation). The fatty acid methyl esters (FAME) were extracted twice with hexane, the solvent was evaporated under nitrogen, and 50–100 μL of isooctane (capillary GC/GC–MS grade, American Burdick and Jackson, Muskegan, MI USA) were added. Aliquots (1–2 μL) were injected into the gas chromatographic column for gas chromatographic-mass spectrometric (GC-MS) analyses of FAME as previously described.[8]

A Hewlett Packard (Palo Alto, CA USA) gas chromatograph (Model 5890) with a mass selective detector (Model 5970) was used. FAME were analyzed on a fused silica capillary column (DB-23, 20 m × 0.172 mm, i.d., J & W Scientific, Folsom, CA USA). The samples were injected in a splitless mode. The column temperature was programmed as follows: T_i = 70° C (3 min) then increased to 200° C at 10° C/min, then held for 1 min at this temperature and further increased to 230° C at 2° C/min. Samples were also injected in a split mode. The column temperature was programmed from a 3 min hold at 140° C–220° C at 5° C/min. The detector and injector temperatures were 280° C and 250° C, respectively. Electron impact spectra were obtained at 70 electron volt. FAME were quantitated by using total ion currents (TIC). The peaks for fatty acid methyl esters of 18:4n-3 and 20:4n-6 were identified by their retention times and electron impact mass fragmentation patterns (m/z of 290, 261, 221, and 194 for 18:4 and 318, 217, and 203 for 20:4). The quantitation was based on the relative proportions of areas (20:4/18:4) using TIC mode.

The data on body weight gains, arachidonic acid concentrations, and the fatty acid composition were statistically analyzed using analysis of variance (ANOVA), and the differences among the three dietary groups were calculated using Newman-Keul's test.[16]

Results

There was no significant difference in the final body weight gains of rats among the three dietary groups (group 1, 335 ± 12 g; group 2, 331 ± 14 g; and group 3, 343 ± 18 g). The values are mean ± SEM (six rats/group).

The concentrations of arachidonic acid present in the various lipid fractions are shown in *Table 1*. The type of dietary fat had a marked effect on the arachidonic acid levels. For each of the lipid fractions that was analyzed, the feeding of a diet enriched with n-3 fatty acids (4% EEC + 1% corn oil group or 5% EEC

group) resulted in a significant decrease in arachidonic acid levels as compared with those in the control group fed a diet containing 5% corn oil. The arachidonic acid pool contained in the FFA and DAG fractions of the SMSG was several-fold lower than that in PC, PE, or PI + PS fractions.

The fatty acid composition of PC, PE, and PI + PS fractions isolated from the SMSG of rats fed the various diets is shown in *Tables, 2, 3, and 4,* respectively. In each of the phospholipid fractions, the levels of 18:2n-6 (linoleic acid) and 20:4n-6 (arachidonic acid) were lower in SMSG of rats fed diets containing n-3 fatty acids than those fed 5% corn oil. Dietary n-3 fatty acids (20:5, 22:5, and 22:6) were readily incorporated into the gland phospholipids. These three fatty acids constituted 20–22% of the total fatty acids in PC and PI + PS fractions and 25–29% of the total fatty acids in PE fraction of SMSG of rats fed diets containing EEC of n-3 fatty acids. These fatty acids were virtually absent from the SMSG phospholipids of rats fed the corn oil diet. The feeding of 1% corn oil with 4% EEC did not alter the incorporation of n-3 fatty acids into the various phospholipids.

Discussion

Previous studies from our laboratory have shown that dietary n-3 fatty acids are incorporated into the SMSG phospholipids, alter membrane fluidity and adenylate cyclase activity,[7] and can influence the transmembrane signaling system in the SMSG.[17] In a recent study, we found that feeding diets rich in n-3 fatty acids results in a decrease in arachidonic acid levels in total phospholipids of gingiva and SMSG, with a concomitant decrease in the synthesis of PGE_2 and LTC_4.[8] The focus of the current investigation was to determine if the observed decrease in arachidonic acid concentrations was more pronounced in some phospholipids than others and also if it is reflected in the DAG and FFA fractions of the SMSG lipids. The results of this study show that the arachidonic acid levels were decreased in all the phospholipids (PC, PE, and PI + PS) as well as in FFA and DAG fractions of the salivary gland. Lower arachidonic acid levels in the SMSG of rats fed n-3–rich diets is consistent with previous observations in other tissues.[18–22] The lower concentration of arachidonic acid in the FFA fraction and the reduced availability of arachidonic acid from DAG and phospholipids may account for the decrease in the in vivo levels of PGE_2 and in vitro production of LTC_4 that we previously reported in the SMSG of rats fed diets containing n-3 fatty acids.[8]

In this study, we measured the arachidonic acid pool in the total DAG. It is known that 1,2-sn-DAG is involved in the protein kinase C (PKC) activation. However, there are multiple forms of PKC.[23,24] It has

Table 1 Arachidonic acid concentrations in major phospholipids, diacylglycerol, and free fatty acids of SMSG of rats fed diets containing ethyl ester concentrate of n-3 fatty acids

Diet fed	PC	PE	PI + PS	DAG	FFA
	mg/g	mg/g	mg/g	μg/g	μg/g
5% Corn oil	0.86 ± 0.11[a]	0.99 ± 0.12[a]	0.41 ± 0.10[a]	19.1 ± 1.1[a]	16.2 ± 0.9[a]
4% EEC n-3 + 1% Corn oil	0.16 ± 0.02[b]	0.42 ± 0.05[b]	0.12 ± 0.01[b]	7.6 ± 0.9[b]	6.0 ± 0.8[b]
5% EEC n-3	0.15 ± 0.01[b]	0.38 ± 0.07[b]	0.13 ± 0.01[b]	7.5 ± 1.3[b]	7.8 ± 1.6[b]

Arachidonic acid concentrations are expressed per g wet weight of the gland. Values are mean ± SEM of 3–5 rats/group. Values with different superscripts in the same column are significantly different from each other ($P < 0.05$) using analysis of variance, Newman-Keul's test.
EEC n-3 = ethyl ester concentrate of n-3 fatty acids.

Table 2 Fatty acid composition of phosphatidylcholine in SMSG of rats fed diets containing ethyl ester concentrate of n-3 fatty acids

Fatty acid	5% Corn oil	4% EEC n-3 + 1% corn oil	5% EEC n-3
16:0	35.3 ± 0.4[a]	34.6 ± 4.0[a]	31.8 ± 3.2[a]
16:1n-7	1.1 ± 0.1[a]	2.1 ± 0.2[b]	2.1 ± 0.2[b]
16:2	—	1.3	2.1 ± 0.9
18:0	10.2 ± 0.2[a]	7.4 ± 0.3[a]	13.7 ± 5.7[a]
18:1n-9	6.4 ± 0.6[a]	6.0 ± 0.5[a]	5.1 ± 0.3[a]
18:1n-7	5.0 ± 0.3[a]	3.9 ± 0.4[a]	4.5 ± 0.7[a]
18:2n-6	11.1 ± 0.2[a]	7.4 ± 0.4[b]	2.3 ± 0.4[c]
18:3n-3	—[a]	2.7 ± 0.2[b]	1.4 ± 0.3[c]
20:3n-6	5.1 ± 0.9[a]	—[b]	—[b]
20:4n-6	20.9 ± 2.1[a]	5.4 ± 0.2[b]	4.8 ± 0.4[b]
20:5n-3	—[a]	14.7 ± 1.2[b]	17.6 ± 2.4[b]
22:5n-3	—[a]	1.7 ± 0.1[b]	1.2 ± 0.3[c]
22:6n-3	—[a]	3.3 ± 0.9[b]	2.7 ± 0.3[b]

Values are area percent (mean ± SEM of three rats/group). Values with different superscripts in the same row are significantly different from each other ($P < 0.05$) using analysis of variance, Newman-Keul's test.

Table 3 Fatty acid composition of phosphatidylethanolamine in SMSG of rats fed diets containing ethyl ester concentrate of n-3 fatty acids

Fatty acid	5% Corn oil	4% EEC n-3 + 1% Corn oil	5% EEC n-3
16:0	9.3 ± 0.3[a]	10.3 ± 0.3[a]	9.1 ± 0.3[a]
16:1n-7	1.1 ± 0.1[a]	2.3 ± 0.3[b]	2.3 ± 0.3[b]
18:0	17.2 ± 1.2[a]	15.7 ± 0.8[a]	16.1 ± 0.5[a]
18:1n-9	11.9 ± 0.6[a]	13.1 ± 1.6[a]	10.1 ± 0.9[a]
18:1n-7	3.6 ± 0.3[a]	3.3 ± 0.4[a]	3.5 ± 0.5[a]
18:2n-6	8.7 ± 1.5[b]	6.7 ± 1.6[a,b]	2.7 ± 0.3[a]
18:3n-3	2.1 ± 0.5[a]	1.5 ± 0.1[a]	1.9 ± 0.3[a]
20:3n-6	2.4 ± 0.4[a]	1.0 ± 0.2[a]	3.2 ± 1.3[a]
20:4n-6	25.9 ± 2.5[a]	9.8 ± 1.0[b]	8.8 ± 0.8[b]
20:5n-3	—[a]	14.5 ± 0.5[b]	16.4 ± 1.4[b]
22:4n-6	2.9 ± 0.1[a]	—[b]	—[b]
22:5n-3	—[a]	3.3 ± 0.1[b]	3.5 ± 0.2[b]
22:6n-3	—[a]	7.6 ± 0.5[b]	8.9 ± 1.2[b]

Values are area percent (mean ± SEM of three rats/group). Values with different superscripts in the same row are significantly different from each other ($P < 0.05$) using analysis of variance, Newman-Keul's test.

Table 4 Fatty acid composition of phosphatidylinositol plus phosphatidylserine in SMSG of rats fed diets containing ethyl ester concentrate of n-3 fatty acids

Fatty acid	5% Corn oil	4% EEC n-3 + 1% Corn oil	5% EEC n-3
16:0	10.2 ± 0.5[a]	12.8 ± 1.5[a]	10.1 ± 0.6[a]
16:1n-7	1.8 ± 0.4	1.2	1.1 ± 0.3
18:0	34.4 ± 0.5[a]	36.4 ± 0.3[a]	36.0 ± 2.8[a]
18:1n-9	6.8 ± 0.9[a]	6.1 ± 0.8[a]	5.8 ± 0.6[a]
18:1n-7	5.5 ± 0.3[a]	5.6 ± 0.3[a]	4.0 ± 0.1[b]
18:2n-7	6.4 ± 0.3[a]	5.0 ± 0.6[b]	2.8 ± 0.4[c]
18:3n-3	2.7 ± 0.3	2.9	3.4 ± 1.0
20:3n-6	6.0 ± 0.3[a]	—[b]	—[b]
20:4n-6	17.8 ± 1.5[a]	7.1 ± 0.1[b]	7.5 ± 0.2[b]
20:5n-3	—[a]	8.1 ± 0.5[b]	7.7 ± 0.5[b]
22:4n-6	2.1 ± 0.3[a]	—[b]	—[b]
22:5n-3	—[a]	5.4 ± 0.3[b]	6.4 ± 0.6[b]
22:6n-3	—[a]	6.3 ± 0.3[b]	6.5 ± 0.6[b]

Values are area percent (mean ± SEM of three rats/group). Values with different superscripts in the same row are significantly different from each other ($P < 0.05$) using analysis of variance, Newman-Keul's test.

been suggested that different molecular species of DAG may exert differential effects on the various forms of PKC.[25]

There are a number of studies on the beneficial effects to health of diets rich in n-3 fatty acids. One of the important questions is the optimum level of intake of n-3 fatty acids at which their beneficial effects are exerted. There is some evidence that the consumption of as little as 30 g of fish per day can reduce the incidence of heart disease in humans.[26] There is very little information in the literature regarding the structure-function relationship in tissues as affected by progressive increases of dietary n-3 fatty acids. In one study,[27] a dose-dependent suppression of arachidonic acid concentration in tissue lipids and the formation of eicosanoids was observed when rats were fed graded amounts of n-3 fatty acids in the presence of a constant amount of linoleic acid. However, when the n-3:n-6 ratio of fatty acids in the diet was kept constant, this dose-dependent response of n-3 fatty acids was not observed.[28] In our studies, we used essentially one level of n-3 fatty acids, i.e., 4–5% in the present study and 9–10% in a previous one.[8] Our objective in these studies was to determine if a maximal incorporation of n-3 fatty acids in tissue lipids would result in significant reductions in arachidonic acid levels and eicosanoid synthesis in the salivary glands. Future studies are planned to test the effects of different levels of dietary intake of n-3 fatty acids on gland function.

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