N–3 fatty acid deficiency in the rat pineal gland: effects on phospholipid molecular species composition and endogenous levels of melatonin and lipoxygenase products

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Abstract N–3 essential fatty acid deficiency affects a number of biological and physiological processes. In this study, we investigated the effect of n–3 essential fatty acid status on two key pineal biochemical functions, melatonin production and lipoxygenation, using pineal glands from rats given an n–3-adequate or n–3-deficient diet. The pineal total lipid profile and phospholipid molecular species distribution altered by n–3 deficiency were evaluated in parallel. In pineal glands from n–3-deficient rats, an 87% reduction of 22:6n–3 (docosahexaenoic acid) was observed, and this decrease was accompanied by increases in 22:4n–6 (docosatetraenoic acid, 3-fold), 22:5n–6 (docosapentaenoic acid, 12-fold), and 20:4n–6 (arachidonic acid, 48%). The significant decrease of 22:6n–3 containing species in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) was also evident. These decreases in 22:6n–3 containing PL species were compensated by substantial accumulations of 22:4n–6 or 22:5n–6 and slight increases in 20:4n–6 containing PL species in PC and PE. In PS, however, the accumulation of n-6 species was not adequate to compensate for the loss of 22:6n–3 species. N–3 deficiency significantly reduced non-esterified 20:4n–6 and 22:6n–3 levels in pineals (25% and 65%, respectively). Concomitantly, the endogenous 12-HETE level decreased by 35% in deficient pineals. In contrast, n–3 deficiency led to a more than 60% increase in the daytime pineal melatonin level. In conclusion, n–3 fatty acid deficiency not only has profound effects on pineal lipid profiles but also on pineal biochemical activities. These results suggest that n–3 fatty acids may play a critical role in regulating pineal function.—Zhang, H., J. H. Hamilton, N. Salem, Jr., and H-Y. Kim. **N–3 fatty acid deficiency in the rat pineal gland: effects on phospholipid molecular species composition and endogenous levels of melatonin and lipoxygenase products. *J. Lipid Res.* 1998.** 39: **1397–1403.**

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Mammalian brain lipids contain substantial amounts of n–3 polyunsaturated fatty acids (n–3 PUFA), principally docosahexaenoic acid (22:6n–3). Increasing evidence has suggested that maintenance of a high level of 22:6n–3 is necessary for optimal neural development and function (1–4). The pineal gland also contains high levels of n–3 and n–6 PUFA. It has been reported that at least 25% of the total fatty acids identified in the rat pineal lipids are represented by arachidonic acid (20:4n–6) and 22:6n–3 (5, 6).

The mammalian pineal gland is a prominent secretory organ that synthesizes and releases a number of exocrine and endocrine substances (7). Melatonin, an indoleamine hormone, is synthesized in the pineal gland from tryptophan and secreted for circulation. In addition, the pineal gland is capable of converting PUFA into bioactive lipid mediators. Our previous work has demonstrated that the rat pineal gland expresses both 12- and 15-lipoxygenase activities, producing 12- and 15-hydroxyeicosatetraenoic acids (12- and 15-HETEs) from 20:4n–6, and 14- and 17-hydroxydocosahexaenoic acids (14- and 17-HDoHEs) from 22:6n–3, respectively (6). The rat pineal gland has also been shown to produce hepoxilin A_3 through the 12-lipoxygenase pathway (8). Furthermore, the rat pineal lipid composition is sensitive to dietary fatty acid modification (5), and dietary n–3 deficiency has been shown to affect adenosine-dependent melatonin release in cultured pineal gland (9).

A basic strategy for investigating the role of brain 22:6n–3 in neurological functions is to manipulate tissue

Abbreviations: 20:4n–6, arachidonic acid; 22:6n–3, docosahexaenoic acid; 22:4n–6, docosatetraenoic acid; 22:5n–6, docosapentaenoic acid; ESI-MS, electrospray ionization–mass spectrometry; GC/MS–NCI, gas chromatography/mass spectrometry with negative ion chemical ionization; HDoHE, hydroxydocosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SIM, selected ion monitoring.

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levels of 22:6n–3 and evaluate appropriate functional endpoints (4, 10, 11). It has been shown previously that an artificial rearing method with a low n–3 fat diet can produce rats with brain 22:6n–3 reduced by 50% in the first generation and more than 90% in the second generation (12). In the present study, we investigated the effect of this severe n–3 deficiency on pineal lipid profiles including the phospholipid molecular species composition. Furthermore, we determined the effects of n–3 deficiency on two important pineal biochemical functions by measuring endogenous levels of lipoxygenase metabolites and melatonin.

MATERIALS AND METHODS

Chemicals

Pentafluoropropionic acid anhydride, pentafluorobenzyl bromide, and diisopropylethylamine were purchased from Pierce (Rockford, IL); and bistrimethylsilyltrifluoroacetamide (BSTFA) and BF_3/methanol were obtained from Alltech (Deerfield, IL). Authentic hydroxyeicosatetraenoic acids (HETEs), 5(S)-, 12(S)-, and 15(S)-HETEs, as well as deuterium-labeled standards such as d_8-HETEs and d_8-20:4n–6 were purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Various phospholipid standards were obtained from Avanti Polar Lipids (Alabaster, AL). Melatonin was from Sigma Chemical Company (St. Louis, MO) and d_4-melatonin was a generous gift from Dr. S. P. Markey, National Institute of Mental Health, Bethesda, MD. Solvents for liquid–liquid extraction, solid phase extraction (SPE), HPLC, LC/MS, and GC/MS were Baxter B&J Brand™ high purity solvents (Muskegon, MI).

Animals and pineal collection

Procedures for the artificial rearing of rats were described previously in detail (12). Briefly, offspring from pregnant Sprague-Dawley rats (Germantown, NY) were artificially reared on an n–3 deficient or n–3 adequate diet via gastrostomy tube from day 5 to 18–19 of life (12). The rats were then housed in pairs under a normal light/dark cycle of 7:00 am–7:00 pm light and 7:00 pm–7:00 am dark, and maintained on the same diets in solid form, and mated; their offspring were considered the second generation and were used in the present study at 1 year of age. The diets contained 5 wt% fat in the form of saturated fats (distilled monoglycerides) and unsaturated fats (ethyl esters of purified linoleate, linolenate, and docosahexaenoate). For the n–3 adequate diet, 12% of the fat was in the form of linoleic acid while 1% each was provided by α-linolenic and docosahexaenoic acids. For the n–3-deficient diet, there was 26% linoleic acid and only trace amounts of α-linolenic acid (0.06%).

Rats were killed between 10 am–1 pm. Pineal glands were collected immediately after decapitation and placed in 1 ml of methanol containing 50 μg/ml butylated hydroxytoluene on dry ice or stored at −80 °C. Four pineal glands from each group were used in the subsequent analysis. Pineal lipids, melatonin, and lipoxygenase metabolites were extracted according to the method of Bligh and Dyer (13). Deuterium-labeled internal standards, including 100 pg of d_4–melatonin, 10 ng of d_8–20:4n–6, 1 ng each of d_8–12- and 15-HETEs, and 100 pmol each of d_{35}–18:0/20:4n–6-PC and -PE and 18:0/22:6n–3-PS, were added prior to homogenization. Total pineal extracts were reconstituted in 1 ml chloroform for further analysis.

Analysis of pineal lipid composition

Analysis of total fatty acid was performed using four separate pineal glands from each group in the presence of 3 μg tricosanoic acid (23:0) as an internal standard. Fatty acid composition of pineal lipids was determined by GC analysis after transmethylation as previously described (6). Briefly, aliquots of pineal lipid extracts were transmethylated using BF_3/methanol (14% wt/vol) at 100°C for 2 h under a nitrogen atmosphere (14). Fatty acid methyl esters were extracted with hexane and then analyzed using an HP 5890 gas chromatograph equipped with a flame ionization detector and a DB-FFAP capillary column (30 m × 0.25 mm i.d.; film thickness, 0.25 μm; J&W Scientific, Folsom, CA). Individual fatty acid methyl esters were identified by comparing retention times with known standards (Nu-Chek Prep, Elysian, MN). The content of each individual fatty acid was expressed as a weight percentage of total fatty acids.

Analysis of non-esterified fatty acids

Bazan et al. (15) reported that non-esterified fatty acids in rat brain increased substantially during the first 2–10 min after decapitation. However, when tissues were excised within 1 min after killing, the levels of brain non-esterified fatty acids were essentially identical when measured after either decapitation or focused microwave irradiation (16). In the present study, rats were decapitated and pineal glands were removed and frozen in methanol within approximately 30–40 sec. This rapid process has produced consistent and reproducible results.

Non-esterified fatty acids were separated from bulk pineal lipids using Bond Elut–aminopropyl columns (Varian Sample Preparation Products, Harbor City, CA) (17). The free fatty acids were then converted into PFB-esters and determined using GC/MS-NCI. The instrument used was an HP 5989A mass spectrometer coupled to an HP 5890 gas chromatograph. An SPB-1 fused silica capillary column (30 m × 0.32 mm, 0.25 μm film thickness; Supelco, Bellefonte, PA) was used with an oven temperature programmed from 150°C to 220°C at 10°C/min., 220°C to 260°C at 3°C/min., and 260°C to 300°C at 30 °C/min. The injector and transfer line temperatures were maintained at 280°C and 250°C, respectively. The mass spectrometer was operated at: emission current, 300 μA; electron energy, 105 ev; source temperature, 200°C; and quadrupole temperature, 100°C. Using the selected ion monitoring (SIM) mode, linear calibration curves were generated for both 20:4n–6 and 22:6n–3 using d_8–20:4n–6 as an internal standard.

Analysis of pineal phospholipid molecular species

Molecular species of the pineal phospholipids were determined using an HP 5989B mass spectrometer coupled with an HP 1050 HPLC system and a reversed-phase HPLC column (150 × 20 mm, 5 μm; Phenomenex, Torrance, CA). The separation was accomplished using a linear solvent gradient as previously described (18) with a flow rate of 0.3 ml/min. Approximately 5 μl of pineal lipids (representing 1/5 of the total lipid extract from one pineal gland) was injected onto the HPLC column and then analyzed by electrospray ionization-mass spectrometry (ESI-MS) (19). The levels of individual PL species were determined against the added deuterium-labeled internal standards.

Analysis of endogenous pineal lipoxygenase metabolites

Lipoxygenase metabolites were separated from pineal lipids by solid phase extraction using C18 SPE columns (Varian Sample Preparation Products, Harbor City, CA) (20). These partially purified metabolites were then derivatized to PFB-esters by reacting with 1% diisopropylethylamine and 0.35% pentafluorobenzyl bromide in acetonitrile (6). After platinum oxide (PtO_2)-catalyzed hydrogenation (21), the hydroxy groups of these hydrogenated PFB-

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esters were converted to TMS-ethers with BSTFA. Finally, the fully derivatized lipoxygenase metabolites were analyzed by GC/MS-NCI with an HP 5989A GC/MS system (6). The mass spectrometer was operated in the SIM mode to record ions of *m/z* 399 (HETEs), *m/z* 427 (HDoHEs), and *m/z* 407 (d_8-HETEs).

Analysis of endogenous melatonin

Aliquots of pineal extracts (typically 1/5 or 1/3 of the total extract of one pineal gland) were evaporated with nitrogen and melatonin was derivatized with 20 μl pentafluoropropionic acid anhydride and 20 μl ethyl acetate in 2 ml screw-capped vials at 60 °C for 15 min (22). The reaction mixture was then evaporated with nitrogen and partitioned with 0.5 ml acetonitrile and 1 ml hexane after vigorous vortexing (23). After the removal of the hexane layer, the acetonitrile layer was dried, the melatonin derivative was redissolved in 6 μl ethyl acetate, and 2–4 μl was then analyzed by GC/MS-NCI. The partitioning step greatly enhanced chromatographic performance and reduced background (23, 24). Quantitative determination of melatonin was conducted using the SIM mode for ions at *m/z* 320 (melatonin) and *m/z* 323 (d_4-melatonin) (23).

Statistical analysis

The data are presented as mean ± SD. The statistical significance of differences between the means of individual animal groups were determined using unpaired Student's *t*-test. Correlation coefficient (*r*) between two sets of data was obtained using Pearson product-moment correlation test.

RESULTS

Pineal fatty acid composition

The fatty acid composition of total pineal lipids from n–3-adequate and n–3-deficient rats is summarized in **Table 1**. As previously observed with brain lipids (12), 22:6n–3 decreased markedly in the n–3-deficient pineal gland, from 12.5 ± 1.3 to 1.6 ± 0.3% (an 87% reduction). The decrease in pineal 22:6n–3 was accompanied by substantial increases in the accumulation of both 22:4n–6 (3-fold; from 1.1 ± 0.4 to 3.4 ± 0.4%) and 22:5n–6 (12-fold; from 0.5 ± 0.1 to 6.1 ± 0.9%). In addition, 20:4n–6 content increased by more than 48% (from 12.7 ± 1.1 to 18.9 ± 1.3%) in the n–3-deficient rats. However, total unsaturated fatty acids remained relatively unchanged (approximately 58% of total fatty acids). The mean 20:4n–6/22:6n–3 ratio in the pineal glands of the n–3-adequate and n–3-deficient groups was approximately 1 and 12, respectively.

TABLE 1. Fatty acid composition of total pineal lipids

Fatty Acids	n–3 Adequate	n–3 Deficient
Non-essential fatty acids		
14:0	1.1 ± 0.1	1.0 ± 0.2
16:0	20.7 ± 1.1	20.4 ± 1.7
16:1n–9	3.8 ± 0.9	3.1 ± 0.8
18:0	19.1 ± 1.2	21.0 ± 0.9
18:1n–9	12.2 ± 1.2	9.4 ± 1.1[a]
18:1n–7	6.7 ± 0.5	5.3 ± 0.6[a]
20:0	0.2 ± 0.0	0.1 ± 0.1
20:1n–9	0.6 ± 0.0	0.4 ± 0.0
22:0	0.1 ± 0.0	0.1 ± 0.1
22:1n–9	0.1 ± 0.0	0.1 ± 0.1
24:0	0.2 ± 0.1	0.1 ± 0.1
24:1n–9	0.8 ± 0.5	1.1 ± 0.2
N–6 polyunsaturates		
18:2n–6	5.4 ± 0.5	5.7 ± 0.4
20:2n–6	0.3 ± 0.0	0.3 ± 0.1
20:3n–6	1.2 ± 0.3	1.5 ± 0.2
20:4n–6	12.7 ± 1.1	18.9 ± 1.3[b]
22:2n–6	0.2 ± 0.1	0.1 ± 0.1
22:4n–6	1.1 ± 0.4	3.4 ± 0.4[b]
22:5n–6	0.5 ± 0.1	6.1 ± 0.9[b]
Total n–6	21.4 ± 1.8	36.0 ± 2.7[b]
N–3 polyunsaturates		
18:3n–3	0.1 ± 0.1	0.1 ± 0.1
20:5n–3	0.1 ± 0.0	0.1 ± 0.1
22:5n–3	0.6 ± 0.1	0.1 ± 0.1[b]
22:6n–3	12.5 ± 1.3	1.6 ± 0.3[b]
Total n–3	13.3 ± 1.3	1.9 ± 0.4[b]

Data are mean ± SD from four different animals and expressed as a percentage of total fatty acids.
[a] $P < 0.05$ and [b] $P < 0.001$ (unpaired Student's *t*-test).

Pineal phospholipid molecular species

Using d_{35}–18:0/20:4n-6-PC, d_{35}–18:0/20:4n-6-PE, and d_{35}–18:0/22:6n-3-PS as internal standards, many PL species of the rat pineal lipids were identified and quantified (18, 19). While PC could be detected at approximately 1 pmol/pineal, the detection limits for PE and PI (5 pmol/pineal) and PS (20 pmol/pineal) were considerably higher. Thus, PL species that were present at a level near these detection limits could not be reliably quantified. From the LC/MS analysis, we found that the majority of pineal PL species contain 16:0, 16:1, 18:0, or 18:1 at the *sn*-1 position. Accordingly, the quantitative analysis was performed for these species in each phospholipid class. In **Table 2**, absolute and relative distributions of PC, PE, PS, and PI classes are shown. The polyunsaturated portion of each phospholipid class is also indicated (Table 2). The major phospholipid class in the rat pineal gland was PC, which represented more than 70% of the total PL (Table 2). The next abundant phospholipid class was PE (20%) and the proportion of PS or PI was below 5% of the total phospholipids. In PS, all the species identified were PUFA-containing species. N–3 deficiency did not alter the distribution of PC, PE, and PI or the total phospholipid content significantly; however, a significant decrease (by 28%) in both the total amount and mole percentage of PS was observed. A slight increase in total PUFA PC was also observed in the pineal glands from the n–3-deficient animals.

The major long-chain PUFA PC present in the pineal glands from the n–3-adequate rats were 16:0/20:4n–6, 16:0/22:6n–3, 18:0/20:4n–6, and 18:0/22:6n–3 species (**Table 3**). Species with the ether linkage at the *sn*-1 position, namely alkenylacyl and alkylacyl subclasses, represented a small fraction in total PC, identified as 16:0/20:4n–6 and 18:0/20:4n–6 species. In the n–3-deficient rats, 16:0/22:6n–3-PC and 18:0/22:6n–3-PC decreased by more than 80%, from 6.0 ± 0.5 to 1.0 ± 0.1% and 6.2 ± 0.5 to 1.1 ± 0.03%, respectively. These decreases in 22:6n–3-containing PC species were accompanied by dramatic increases in the accumulation of 18:0/22:5n–6-PC, 16:0/22:5n–6-PC and 18:0/22:4n–6-PC. Most PC species containing 20:4n–6 did not change significantly as a result of n–3 deficiency, except 16:0/20:4n–6 increased from 10.9 ± 1.7 to 12.2 ± 0.5%. Other species such as 16:0/16:1, 16:0/

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TABLE 2. Distribution of individual PL class in the rat pineal lipids

	n-3 Adequate		n-3 Deficient	
	pmol/pineal	%	pmol/pineal	%
Total PC	5276 ± 568	72.7 ± 2.1	5552 ± 801	73.3 ± 2.7
PUFA-PC[a]	2701 ± 250	37.3 ± 2.3	3230 ± 529	42.6 ± 2.0[b]
Total PE	1436 ± 135	19.9 ± 1.6	1482 ± 139	19.7 ± 2.1
PUFA-PE	1425 ± 135	19.7 ± 1.6	1461 ± 139	19.5 ± 2.2
Total PS	297 ± 36	3.9 ± 0.3	213 ± 19[c]	2.8 ± 0.5[c]
PUFA-PS	297 ± 36	3.9 ± 0.3	213 ± 19[c]	2.8 ± 0.5[c]
Total PI	253 ± 33	3.5 ± 0.6	299 ± 34	4.1 ± 0.3
PUFA-PI	222 ± 40	3.1 ± 0.4	268 ± 38	3.6 ± 0.3
Total PL	7249 ± 690		7557 ± 878	

Data are mean ± SD obtained from four different animals.
[a]PUFA indicates that the fatty acids at the *sn*-2 position are C20 and C22 polyunsaturated fatty acids.
[b]$P < 0.05$ and [c]$P < 0.01$ (unpaired Student's *t*-test).

TABLE 3. Mole percentage of indvidual PC species in total pineal PC

	n-3 Adequate	n-3 Deficient
16/16:1	8.8 ± 0.5	7.2 ± 1.2
16:0/18:1[a]	7.2 ± 0.6	6.9 ± 0.8
16:1/18:1 or 16/18:2n-6	6.0 ± 0.6	6.3 ± 0.8
16:1/182n-6	0.5 ± 0.1	0.4 ± 0.0
18:0/18:1	12.1 ± 2.9	9.8 ± 1.4
18:1/18:1 or 18/18:2n-6	12.7 ± 1.4	10.4 ± 1.5
18:1/18:2n-6	2.2 ± 0.1	1.7 ± 0.4
16:0/20:4n-6		
diacyl-	10.9 ± 1.7	12.2 ± 0.5
alkenylacyl-	0.7 ± 0.1	1.0 ± 0.2
alkylacyl-	0.6 ± 0.0	0.7 ± 0.1
16:0/22:4n-6	nd	0.6 ± 0.1[c]
16:0/22:5n-6	nd	4.0 ± 0.3[c]
16:0/22:6n-3	6.0 ± 0.5	1.0 ± 0.1[c]
16:1/20:4n-6	1.6 ± 0.1	2.2 ± 0.1[b]
16:1/22:4n-6	nd	0.1 ± 0.0
16:1/22:5n-6	nd	2.3 ± 0.2[c]
16:1/22:6n-3	0.9 ± 0.2[c]	nd
18:0/20:4n-6		
diacyl-	17.9 ± 1.4	17.2 ± 1.7
alkenylacyl-	0.2 ± 0.0	0.2 ± 0.0
alkylacyl-	0.8 ± 0.1	0.6 ± 0.1
18:0/22:4n-6	0.6 ± 0.0	3.1 ± 0.6[b]
18:0/22:5n-6	nd	6.3 ± 0.1[c]
18:0/22:6n-3	6.2 ± 0.5	1.1 ± 0.0[c]
18:1/20:4n-6	3.8 ± 0.2	3.7 ± 0.4
18:1/22:4n-6	nd	0.2 ± 0.0[b]
18:1/22:5n-6	nd	0.3 ± 0.1[b]
18:1/22:6n-3	0.9 ± 0.1[c]	nd

Data are mean ± SD obtained from four different animals.
[a]Indicates that 18:1 containing species represent the combination of both n-7 and n-9 fatty acids.
[b]$P < 0.01$; [c]$P < 0.001$ (unpaired Student's *t*-test).

18:1, 16:1/18:1 (or 16:0/18:2), 18:0/18:1, 18:1/18:1 (or 18:0/18:2), and 18:1/20:4n-6 were also prominent PC species identified and their molar distribution was not affected significantly by n-3 deficiency.

In **Table 4**, the mole percentage distributions of PE subclasses including diacyl, alkenylacyl, and alkylacyl species are shown. Diacyl-PE was the major PE subclass, although alkenylacyl- and alkylacyl-PE represented significant portions in some PE species. As in the case of PC, the 22:6n-3-containing PE species were also substantially decreased in all PE subclasses due to n-3 deficiency. The decreases in these 22:6n-3 species were again associated with significant accumulation of 16:0/22:4n-6-, 16:0/22:5n-6-, 18:0/22:4n-6-, and 18:0/22:5n-6-PE species. While most of other 20:4n-6-containing species showed little or no change, there was a significant increase of 16:0/20:4n-6 and 18:1/20:4n-6 in diacyl- and alkenylacyl-PE in the n-3-deficient animals. This increase together with the increase of 16:0/20:4n-6-PC (Table 3) might reflect the observed elevation of the 20:4n-6 content in total lipid composition (Table 1).

In PS, 18:0/20:4n-6, 18:0/22:6n-3, and 18:0/22:5n-6 were the only species identified. While 18:0/22:6n-3 decreased significantly from 221 ± 26 pmol/pineal in the n-3-adequate rats to 39 ± 6 pmol/pineal in the n-3-deficient animals, the accumulation of 18:0/22:5n-6 was also observed (from a non-detectable level to 66 ± 9 pmol/pineal). Although statistically insignificant, an increase of 18:0/20:4n-6-PS was observed in the pineal glands of the n-3-deficient rats (from 77 ± 17 to 109 ± 15 pmol/pineal). Surprisingly, the loss of 18:0/22:6n-3-PS was not fully compensated by the increase in 18:0/22:5n-6- and 18:0/20:4n-6-PS, and thus the level of total PUFA-PS was substantially depressed (Table 2).

In PI, 18:0/20:4n-6 species was the major species detected, representing 69.3 ± 3.7% of the total PI. In addition, 16:0/20:4n-6 (11.8 ± 2.7%), 18:1/20:4n-6 (5.5 ± 2.7%), and 16:0/18:1 (12.8 ± 1.7%) species were also identified. As indicated in Table 2, n-3 deficiency did not affect the levels of these species significantly.

Non-esterified 20:4n-6 and 22:6n-3 levels

The endogenous non-esterified 20:4n-6 and 22:6n-3 levels in the pineal glands of the n-3-adequate and n-3-deficient rats are shown in **Table 5**. The levels of non-esterified 20:4n-6 and 22:6n-3 in the pineal glands from the n-3-deficient rats decreased by 25% and 65%, respectively. The ratios of 20:4n-6/22:6n-3 in the non-esterified fatty acid fractions were approximately 4 in the n-3-adequate and 9 in the n-3-deficient rats, which were significantly different from the 20:4n-6/22:6n-3 ratios observed in total pineal fatty acids (Table 1).

Pineal lipoxygenase metabolites

The major lipoxygenase metabolites observed were 12- and 15-HETEs derived from 20:4n-6, and 14- and 17-HDoHEs derived from 22:6n-3. Endogenously, 14- and 17-HDoHEs were produced at very low levels (<20 pg/pineal) and could not be reliably quantified. Therefore, 12- and 15-HETEs were monitored as indicators of endogenous pineal lipoxygenase product accumulation.

The levels of endogenous pineal 12- and 15-HETEs are shown in Table 5. In the pineal glands from the n-3-deficient rats, the endogenous 12-HETE level decreased by 35%, while the 15-HETE level remained relatively unchanged. This decrease in the 12-HETE level correlated with the reduction in the non-esterified 20:4n-6 level in the n-3 deficient animals ($r = 0.93$, $P < 0.01$) (Table 5).

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TABLE 4. Mole percentage of individual PE species in total pineal PE

	n–3 Adequate	n–3 Deficient
18:0/18:1[a]	0.9 ± 0.1	1.3 ± 0.5
16:0/20:4n–6		
diacyl-	1.7 ± 0.2	2.7 ± 0.2[d]
alkenylacyl-	2.4 ± 0.5	4.9 ± 0.3[e]
alkylacyl-	5.6 ± 1.0	5.3 ± 1.2
16:0/22:4n–6		
diacyl-	nd	2.7 ± 0.6[e]
alkenylacyl-	nd	4.5 ± 0.5[b, e]
alkylacyl-	nd	6.2 ± 1.0[e]
16:0/22:5n–6		
diacyl-	nd	3.1 ± 0.4[e]
alkenylacyl-	nd	1.8 ± 0.3[e]
alkylacyl-	nd	[b]
16:0/22:6n–3		
diacyl-	6.4 ± 0.5	1.4 ± 0.3[e]
alkenylacyl-	2.9 ± 0.4[e]	nd
alkylacyl-	12.5 ± 1.8[e]	nd
18:0/20:4n–6		
diacyl-	19.2 ± 2.7	22.4 ± 1.4
alkenylacyl-	6.4 ± 0.5	7.6 ± 1.3
alkylacyl-	3.2 ± 0.4	3.3 ± 0.7
18:0/22:4n–6		
diacyl-	nd	3.6 ± 0.1[e]
alkenylacyl-	nd	3.9 ± 0.8[c, e]
alkylacyl-	nd	1.9 ± 0.1[e]
18:0/22:5n–6		
diacyl-	nd	8.1 ± 0.8[e]
alkenylacyl-	nd	1.3 ± 0.2[e]
alkylacyl-	nd	[c]
18:0/22:6n–3		
diacyl-	12.3 ± 1.3	2.4 ± 0.4[e]
alkenylacyl-	2.9 ± 0.3[e]	nd
alkylacyl-	7.3 ± 0.3[e]	nd
18:1/20:4n–6		
diacyl-	1.9 ± 0.0	3.1 ± 0.3[e]
alkenylacyl-	0.9 ± 0.1	4.4 ± 0.5[e]
alkylacyl-	2.3 ± 0.4	2.4 ± 0.1
18:1/22:6n–3		
diacyl-	4.7 ± 0.5	nd
alkenylacyl-	0.9 ± 0.2	nd
alkylacyl-	5.6 ± 0.7	nd

Data are mean ± SD obtained from four different animals.
[a] Indicates that 18:1-containing species represent the combination of both n–7 and n–9 fatty acids.
[b] and [c] Indicate that those species were possibly co-eluted, and they share the same molecular mass, respectively, and therefore could not be distinguished.
[d] $P < 0.01$; [e] $P < 0.001$ (unpaired Student's *t*-test).

TABLE 5. Endogenous levels of non-esterified 20:4n–6 and 22:6n–3, 12- and 15-HETEs, and melatonin in the rat pineal glands

	n–3 Adequate	n–3 Deficient
20:4n–6 (ng/pineal)	7.0 ± 0.7	5.2 ± 0.3[a]
22:6n–3 (ng/pineal)	1.7 ± 0.2	0.6 ± 0.03[b]
12-HETE (pg/pineal)	448 ± 29	289 ± 19[b]
15-HETE (pg/pineal)	147 ± 20	129 ± 22
Melatonin (pg/pineal)	52 ± 3	85 ± 14[b]

Data are mean ± SD from four different animals.
[a] $P < 0.01$; [b] $P < 0.001$ (unpaired Student's *t*-test).

Pineal melatonin accumulation

The daytime (the light phase) pineal melatonin accumulation was also affected by n–3 deficiency (Table 5). In the pineal glands from the n–3-deficient rats, the endogenous melatonin level increased more than 60% when compared to that of the n–3-adequate animals. The pineal melatonin level was negatively correlated with the levels of non-esterified 20:4n–6 ($r = -0.98$, $P < 0.001$) , 22:6n–3 ($r = -0.99$, $P < 0.001$), and 12-HETE ($r = -0.89$, $P < 0.01$) in these animals.

DISCUSSION

It has been shown previously that in artificially reared rats, n–3 essential fatty acid deficiency has resulted in significant losses in brain n–3 PUFA, particularly 22:6n–3 (12). This severe loss of brain 22:6n–3 was compensated by increases in n–6 PUFA, especially 22:5n–6, 22:4n–6, and 20:4n–6, so that the total PUFA content was maintained (12, 25–27). Using the second generation of these artificially reared rats, we determined pineal fatty acid composition and phospholipid molecular species distribution in the present study, with a focus on the effect of n–3 deficiency on two key biochemical functions of the rat pineal gland, lipoxygenation and melatonin production.

As observed in brain fatty acid profiles (12), n–3 deficiency also led to a significant reduction in the 22:6n–3 content of pineal lipids and this loss was compensated by the increases in 22:5n–6, 22:4n–6, and 20:4n–6 (Table 1). These results indicate that the brain and the pineal gland may share a similar mechanism in maintaining total long-chain PUFA. The content of n–6 long-chain PUFA (n–6 LCPUFA) in tissues is influenced by linoleic acid in the diet, as linoleic acid can be chain elongated and desaturated to form a series of n–6 LCPUFA (28, 29). Therefore, the presence of a higher level of linoleic acid in the n–3-deficient diet in comparison to the control diet is considered an important factor for high levels of n–6 LCPUFA observed in pineal glands of the n–3-deficient rats. However, a high level of linoleic acid in the diet did not lead to a significant elevation of linoleic acid in the pineal lipids, suggesting that an active metabolism of linoleic acid to n–6 LCPUFA by the n–3-deficient animals may have occurred to compensate for the loss of DHA.

Using HPLC/ESI-MS, we determined for the first time the profiles of PL molecular species in rat pineal lipids, particularly those containing long-chain PUFA. As our data indicated, the depletion of pineal 22:6n–3 due to n–3 deficiency was reflected in the PL classes of PC, PE, and PS, in which the levels of 16:0/22:6n–3 and 18:0/22:6n–3 species were significantly reduced. However, this severe loss of 22:6n–3 containing species was generally compensated by the accumulation of PL species that contained 22:5n–6 and 22:4n–6, suggesting the importance of maintaining the overall long-chain PUFA content for proper pineal structure and function (12, 25, 26). Although the majority of long-chain PUFA resided in PC (Table 2), the accumulation of long-chain n–6 PUFA due to n–3 deficiency was broadly observed among PC and PE molecular species (Tables 3 and 4). In PS, however, the loss of 18:0/22:6n–3 species was not fully compensated by 18:0/22:5n–6 accumulation, indicating that the mechanism underly-

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ing the redistribution of n–6 LCPUFA in the rat pineal gland may exhibit a preference toward specific PL classes. The decrease in total PS due to n-3 deficiency observed in the present study was consistent with our previous findings in brain microsomal lipids (27), and this decrease may have a significant effect on membrane function.

N–3 essential fatty acid deficiency has been linked to a number of biological dysfunctions (30–33). In the present study, we observed that the endogenous pineal 12-HETE level was significantly decreased in the n–3-deficient rats, while the 15-HETE level was also reduced but could not be statistically substantiated. These data suggest that n–3 deficiency influenced the metabolism of 20:4n–6 via the lipoxygenase pathway in the rat pineal gland. The reduction in 12-HETE correlated closely with the lowered non-esterified 20:4n–6 level in these animals. As 20:4n–6 is the precursor of 12-HETE, it seems likely that the reduced non-esterified 20:4n–6 level during n–3 deficiency led to the decreased 12-HETE production. It is interesting to note that the lowered non-esterified 20:4n–6 level was associated with a higher 20:4n–6 content in total pineal lipids (Tables 1 and 5). One possible explanation is that the severe loss of 22:6n–3 in cell membranes may lead to the modification of lipase or acyltransferase activities, so that a higher level of membrane 20:4n–6 can be preserved. A higher membrane 20:4n–6 content may be required in order to maintain the membrane LCPUFA status. This could be achieved through an enhanced esterification of 20:4n-6 or through the inhibition of lipase activities that were responsible for releasing 20:4n–6. Indeed, it has been previously reported that alteration of membrane structure by the modification of the fatty acyl chain modulates phospholipase and acyltransferase activities (34). As these enzymes are also involved in maintaining the level of non-esterified 20:4n–6 in living cells (35), the changes in the membrane 20:4n–6 and non-esterified 20:4n–6 would occur in an opposite direction.

It has been indicated that 12-LO products modulate ion channel activities in aplysia (36), membrane polarization in rat hippocampal neurons (37), endothelial cell proliferation (38) and homosynaptic long-term depression of the rat hippocampus (39). However, the biological implication of 12-HETE production in the pineal gland is not well defined. It has been previously reported that lipoxygenase metabolites as well as lipoxygenase inhibitors affect melatonin synthesis and release in the cultured rat pineal glands (40). An altered 12-LO production observed under the n–3-deficient condition may in turn contribute to the modification of pineal melatonin level. In the present study, we observed that the endogenous production of pineal melatonin and 12-HETE was indeed altered concomitantly during n–3 fatty acid deficiency, suggesting a close link between the two biochemical processes in the rat pineal gland. However, it is also possible that these two processes are modulated separately by the status of n–3 fatty acids.

Melatonin, an indoleamine hormone, plays a central role in a variety of physiological processes, including development, seasonal control of reproduction, body weight, and metabolism (41, 42). Melatonin has also been indicated as a potent immunomodulator (43). It has been well documented that serum and pineal melatonin levels follow the same diurnal variation with the peak during the nighttime (41). There has been evidence suggesting that fatty acid status can influence melatonin synthesis (44). Wainwright and Wainwright (44) reported that fatty acids, such as arachidonic, oleic, and palmitic acids, significantly altered the cycle of serotonin N-acetyltransferase activity in the cultured chick pineal gland. As serotonin N-acetyltransferase is a key enzyme in melatonin synthesis (45), alteration in its activity may inevitably affect melatonin production. Recently, Gazzah et al. (9) reported that there was a significant reduction in adenosine-dependent melatonin release in cultured pineal glands from rats raised on an n–3-deficient diet, while the basal melatonin level remained unchanged in those animals. In the present study, we observed that the endogenous daytime melatonin level was significantly elevated in pineal glands from the n–3-deficient rats (Table 5). This increase in the endogenous melatonin level in the n–3-deficient pineal glands may have resulted from changes in the n–6/n–3 ratio, as modification of membrane unsaturation affects activities of membrane-bound proteins including enzymes, receptors, and transporter proteins (34, 46, 47). Such changes in receptor activity, for example, may affect melatonin production, as melatonin synthesis in the pineal gland is a β-adrenergic receptor-mediated process (41). Alternatively, the increased pineal melatonin level during n–3 deficiency may be due to an altered release process, although the circulating levels of melatonin were not determined in the present study. As discussed above, it is also possible that the modified 12-HETE production due to n–3 deficiency contributed to the increase in the endogenous melatonin level. In any event, this altered melatonin production due to n–3 deficiency is likely to affect melatonin-mediated physiological processes.

In summary, we found that n–3 essential fatty acid status affects lipid profiles and biochemical functions in the rat pineal gland. In pineal lipids from the n–3-deficient rats, 22:6n–3 content and 22:6n–3-containing PL species were significantly lower than that of the n-3-adequate animals. The loss of 22:6n–3 and 22:6n–3-containing PL species was compensated with substantial increases in n–6 LCPUFA, particularly 22:4n–6 and 22:5n–6. N–3 deficiency did affect pineal biochemical functions as evidenced by the altered endogenous levels of 12-HETE and melatonin in the n–3-deficient rats. The endogenous melatonin level was negatively correlated with the levels of non-esterified 20:4n–6 and 12-HETE, suggesting that the two key pineal biochemical functions, lipoxygenation and melatonin synthesis, may be synergistically regulated by the status of n–3 essential fatty acids. The mechanism underlying such regulation as well as its physiological implication are the subjects of our future investigation.

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