Low dietary fish-oil threshold for myocardial membrane n-3 PUFA enrichment independent of n-6 PUFA intake in rats

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Abstract Long chain n-3 PUFA docosahexaenoic acid (DHA) is important for heart and brain function. Investigations of biologically plausible mechanisms using animal models associate cardioprotection with DHA incorporation into myocardial membranes that are largely derived from supra-physiological fish oil (FO) intake. We measured the incorporation of DHA into myocardial membranes of rats from low dietary FO intake within human dietary range and quantitatively assessed the influence of dietary n-6 PUFA. With rats fed diets containing 0.16%–5% FO, equal to 0.12%–8.7% energy (%en) as eicosapentaenoic acid (EPA) and DHA (EPA+DHA), and either 1.5%en or 7.5%en n-6 PUFA (linoleic acid) for four weeks, dietary n-6:n-3 PUFA ratios ranged from 74 to 0.3. Myocardial DHA concentration increased in a log-linear fashion with a dietary threshold of 0.019%en as EPA+DHA and half maximal dietary [EPA+DHA] equal to 0.29%en (95% CI, 0.23–0.35). Dietary linoleic acid intake did not influence myocardial DHA. Myocardial membranes are sensitive to absolute dietary intake of long chain n-3 PUFA at low %en in the rat, equivalent to a human intake of one meal of fatty fish per week or less. The dietary ratio of n-6:n-3 PUFA has no influence on long chain n-3 PUFA cellular incorporation from dietary fish oil.—Slee, E. L., P. L. McLennan, A. J. Owen, and M. L. Theiss. **Low dietary fish-oil threshold for myocardial membrane n-3 PUFA enrichment independent of n-6 PUFA intake in rats.** ***J Lipid Res.*** **2010.** 51: **1841–1848.**

Supplementary key words myocardial membrane • dietary lipid • docosahexaenoic acid • omega-3 fatty acid • polyunsaturated fatty acid • n-6:n-3 PUFA ratio

Regular consumption of long chain n-3 polyunsaturated fatty acids (PUFA) from fish is associated with a low incidence of premature mortality from cardiovascular disease. The greatest reductions in relative risk for premature mortality are achieved through regular intake of 1–2 meals of n-3 PUFA–rich fatty fish per week. Only small incremental advantages are evident as intakes increase (1–5). Cardiovascular benefits ascribed to regular fish consumption include slowed resting heart rate (6–8) and reduced risk for cardiac arrest (9, 10), heart failure (11, 12), and atrial fibrillation (13). These can all be attributed to direct effects in the heart on the basis of experimental observations (14).

Animal studies show that the protective effects of dietary fish oil are achieved by n-3 PUFA incorporation into myocardial cell membrane phospholipids (15, 16). Several biologically plausible mechanisms of n-3 PUFA action on cardiac function have been proposed (17). Effects observed using hearts ex vivo after prior dietary intervention (but free of neural or hormonal input or circulating n-3 PUFA at time of study) support an obligatory role for myocardial membrane incorporation of docosahexaenoic acid (22:6n-3) (18). However, extrapolation from animal to human must consider that n-3 PUFA presentation in animal diets and infusions are often well above the equivalent intakes found beneficial in clinical or epidemiological studies (17). The membrane incorporation of low, supplemental intake has not been examined. Furthermore, contemporary Western diets are exceedingly rich in n-6 PUFA, and competition between n-3 PUFA and n-6 PUFA for incorporation into the heart may be of substantial importance (19, 20), posing the dietary ratio of n-6:n-3 PUFA as a potential determinant of cardiac effects (21).

The present study addressed two challenges to linking human disease risk to the biological mechanisms of n-3 PUFA action derived from animal studies. First, it aimed to extend the concentration-effect relationship for membrane incorporation of n-3 PUFA to dietary concentrations within the range of human intake (22) and to estimate a

None of the authors had a personal or financial conflict of interest.

Manuscript received 16 November 2009 and in revised form 2 March 2010.

Published, JLR Papers in Press, March 2, 2010
DOI 10.1194/jlr.M004069

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; LA, linoleic acid; LNA, α-linolenic acid; OA, oleic acid; OO, olive oil.

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threshold for incorporation. As concurrent intake of n-6 PUFA may predicate against n-3 PUFA effectively modulating myocardial composition, a second aim of the study was to quantify the effect of dietary n-6 PUFA on membrane incorporation of long chain n-3 PUFA.

MATERIALS AND METHODS

Animals and diets

Ten-week old Sprague-Dawley rats were fed for two weeks on a control, fabricated diet containing olive oil as the fat source, followed by four weeks on a diet containing one of the following concentrations of fish oil (0.16, 0.31, 0.63, 1.25, 5.0%; n = 4 per diet) (high DHA tuna fish oil, NuMega Ingredients, Australia). Control diets were prepared containing no fish oil (n = 4 per diet). These diets contained either low n-6 PUFA (fish oil with remainder as olive oil; n = 24) or high n-6 PUFA (fish oil plus 5% sunflower seed oil with remainder as olive oil; n = 24) providing a range of dietary n-6:n-3 PUFA ratios manipulated by independently changing either n-3 PUFA content or n-6 PUFA content (**Fig. 1**). Fabricated diets were based on the AIN-93 M diet (23) containing (% dry weight) 57% cornstarch, 10% sucrose, 9% casein, 5% gelatin, 5% cellulose, 10% oil, 3.5% mineral mix, and 1% vitamin mix (24). The diet provided 64.6% of energy (%en) as carbohydrate, 13.6%en as protein, and 22%en as fat. The fatty acid profile of each diet is shown in **Table 1**. Experiments were conducted according to the NHMRC Australia, Guidelines for the Use of Experimental Animals.

At completion of the feeding period, animals were anesthetized (pentobarbitone sodium 60 mg/kg ip), exsanguinated via the abdominal aorta, and the heart was removed. The heart was dissected free, ventricles were rinsed in ice-cold saline (0.9% NaCl), blotted dry, snap frozen, and stored at −80°C.

Fatty acid extraction and analysis

Total lipids were extracted from 100–200 mg samples of myocardium using a modification of the Folch method (25). Phospholipids were isolated from the total muscle lipid by solid phase extraction using silica Sep-pak™ cartridges (Waters, Australia). Fatty acid methyl esters were prepared by direct transesterification (26) and analyzed by gas chromatography using a Shimadzu GC-17A with flame ionization detection using a 30 m × 0.25 mm, 0.25 μm FAMEWAX column (J and W Scientific, US) with hydrogen as carrier gas and a step temperature program rising from 150°C to 260°C over 27 min and held for 6 min. Individual fatty acids were identified from authentic fatty acid methyl ester standards (Sigma-Aldrich, Australia) and expressed as a percentage of total fatty acids.

Statistics

A factorial experimental design was used to investigate the effect of dietary n-3 PUFA concentration and n-6 PUFA background on tissue fatty acid composition, analyzed by two-way ANOVA with FO dose and n-6 PUFA as the main effects and for interactions between the main effects. Tukey HSD post hoc analyses were used to compare individual means of dietary groups. Power calculation showed that n = 4 per group would have 80% power to detect a 12.5% change in the DHA concentration of myocardial membranes from a control concentration of 10.34 ± 0.72% (mean ± SD). A previous study showed 1.25% FO feeding over four weeks increased DHA content by 100% (to 20.06 ± 2.98%; mean ± SD) (24). Statistical analyses were performed using Statistix software, version 8 (Analytical Software, US). Linear regression analysis with Pearson's correlation was performed to determine linear associations between diet and heart n-3 PUFA concentrations using GraphPad Prism (version 4.03) for Windows (GraphPad Software, US). Data were expressed as mean ± SEM. Statistical significance was accepted at $P < 0.05$.

RESULTS

Energy intake, body weight, body weight gain, and liver and heart weights did not differ between diets (data not shown).

Fatty acid composition of heart membranes with control diets

Myocardial membranes of rats fed the low n-6 PUFA control diet contained 31.6% saturated fat, 12.6% monounsaturated fatty acids (MUFA), and 54.7% PUFA (**Table 2**). The main membrane PUFA were arachidonic acid (AA) (20:4n-6); linoleic acid (LA) (18:2n-6); and n-3 PUFA DHA. Hearts from rats fed the high n-6 PUFA control diet had significantly less MUFA, significantly more total PUFA, and slightly (but significantly) more saturated fatty acids (all $P < 0.001$) (**Table 3**). The high n-6 PUFA control diet increased myocardial LA ($P < 0.05$), but AA was unchanged ($P = 0.72$).

Fish oil feeding and n-6 PUFA effects on myocardial membrane

Myocardial n-3 PUFA concentration was significantly different at every dietary concentration of fish oil (Tables 2, 3). Incorporation of DHA was dose-related and hyperbolic in nature ($r^2 = 0.937$) with half maximal incorporation associated with a FO concentration in the diet of

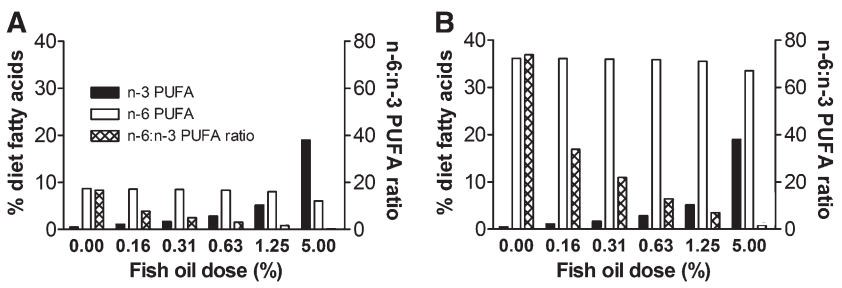

Fig. 1. Total n-3 PUFA and n-6 PUFA concentrations as percent of dietary fat, and n-6:n-3 PUFA ratio in rat diets. A: Low n-6 PUFA diet. B: High n-6 PUFA diet. Filled bars: n-3 PUFA; Open bars: n-6 PUFA; Cross-hatched bars: n-6:n-3 PUFA ratio. PUFA, polyunsaturated fatty acid.

TABLE 1. Dietary fatty acid composition for diets with different concentrations of fish oil and n-6 PUFA

	Low n-6 PUFA Background					
	0% FO 10% OO	0.16% FO 9.84% OO	0.31% FO 9.69% OO	0.63% FO 9.37% OO	1.25% FO 8.75% OO	5.0% FO 5.0% OO
Fatty Acid (% total fat)						
14:0	0	0.05	0.09	0.19	0.38	1.52
16:0	10.42	10.57	10.72	11.01	11.61	15.16
18:0	2.82	2.86	2.90	2.97	3.12	4.02
18:1 (OA)	75.79	74.85	73.90	72.02	68.24	45.60
18:2 n-6 (LA)	8.32	8.21	8.11	7.89	7.46	4.88
18:3 n-3 (LNA)	0.52	0.52	0.52	0.52	0.53	0.55
20:4 n-6 (AA)	0.12	0.15	0.17	0.23	0.33	0.97
20:5 n-3 (EPA)	0	0.11	0.22	0.43	0.87	3.48
22:5 n-3 (DPA)	0	0.02	0.03	0.07	0.14	0.55
22:6 n-3 (DHA)	0	0.45	0.90	1.80	3.61	14.43
Σ n-6 PUFA	8.65	8.57	8.49	8.33	8.00	6.05
Σ n-3 PUFA	0.52	1.10	1.68	2.83	5.14	19.00
Ratio n-6:n-3	16.63	7.79	5.05	2.94	1.56	0.32
LA %en	1.83	1.81	1.78	1.74	1.64	1.07
EPA %en	0	0.02	0.05	0.10	0.19	0.76
DHA %en	0	0.10	0.20	0.40	0.79	3.15
	High n-6 PUFA Background					
	0% FO 5.0% OO 5.0% SSO	0.16% FO 4.84% OO 5.0% SSO	0.31% FO 4.69% OO 5.0% SSO	0.63% FO 4.37% OO 5.0% SSO	1.25% FO 3.75% OO 5.0% SSO	5.0% FO 0% OO 5.0% SSO
Fatty Acid (% total fat)						
14:0	0	0.05	0.09	0.19	0.38	1.52
16:0	8.08	8.23	8.38	8.67	9.27	12.82
18:0	3.63	3.66	3.70	3.78	3.93	4.83
18:1 (OA)	49.97	49.03	48.08	46.20	42.42	19.78
18:2 n-6 (LA)	36.00	35.89	35.78	35.57	35.14	32.56
18:3 n-3 (LNA)	0.49	0.49	0.49	0.49	0.50	0.52
20:4 n-6 (AA)	0.06	0.09	0.11	0.17	0.27	0.91
20:5 n-3 (EPA)	0	0.11	0.22	0.43	0.87	3.48
22:5 n-3 (DPA)	0	0.02	0.03	0.07	0.14	0.55
22:6 n-3 (DHA)	0	0.45	0.90	1.80	3.61	14.43
Σ n-6 PUFA	36.16	36.08	36.00	35.84	35.51	33.56
Σ n-3 PUFA	0.49	1.07	1.65	2.80	5.11	18.97
Ratio n-6:n-3	73.8	33.72	21.82	12.80	6.95	1.77
LA %en	7.92	7.90	7.87	7.83	7.73	7.16
EPA %en	0	0.02	0.05	0.10	0.19	0.76
DHA %en	0	0.10	0.20	0.40	0.79	3.15

Values are expressed as percentage of total lipids by weight. Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPA docosapentaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; LA, linoleic acid; LNA, α-linolenic acid; OA, oleic acid; OO, olive oil; PUFA, polyunsaturated fatty acid; SSO, sunflower seed oil; %en, % of dietary metabolizable energy.

0.37% (95% CI; 0.29%–0.45%), equal to EPA+DHA 0.29%en (95% CI; 0.23%–0.35%) (**Fig. 2A**). There was no significant effect of background dietary n-6 PUFA on the incorporation of DHA into cardiac cell membranes ($P = 0.65$). Log transformation of the fish-oil dose revealed a linear relationship between [fish oil]$\log_{10}$ and myocardial membrane DHA (slope $P < 0.001$; $r^2 = 0.88$) (Fig. 2B). The regression line extrapolated to intersect with the residual DHA concentration of control hearts at a fish -oil dose of 0.027% (95% CI; 0.013%–0.044%) equal to EPA+DHA 0.021%en (95% CI; 0.010%–0.034%). Interpolation of the regression line predicted that a fish-oil dose of 0.31 ± 0.08% (EPA+DHA: 0.24 ± 0.01%en) would double the myocardial membrane content of DHA. A statistically significant concentration-related increase in EPA was observed with FO feeding, but it never exceeded 1.25% of membrane fatty acids (Fig. 2B and Tables 2, 3).

Total membrane n-6 PUFA decreased with FO feeding (Tables 2, 3). Myocardial AA decreased significantly with each fish-oil dose ($P < 0.001$) in a log-linear fashion (low n-6 PUFA: $r^2 = 0.79$, $P < 0.0001$ for slope; high n-6 PUFA: $r^2 = 0.88$, $P < 0.0001$ for slope) (Tables 2, 3). Myocardial LA content was unchanged by fish oil (low n-6 PUFA: $r^2 = 0.002$, $P = 0.88$ for slope; high n-6 PUFA: $r^2 = 0.022$, $P = 0.53$ for slope), except for a significant lowering with the 5% dietary FO concentration in the low n-6 PUFA diet ($P < 0.001$) (Table 2).

Effects of dietary n-6:n-3 ratio on n-3 PUFA and n-6 PUFA tissue incorporation

Myocardial total n-3 PUFA increased and total n-6 PUFA decreased significantly with FO feeding, resulting in a significant FO concentration-related decrease in the membrane n-6:n-3 PUFA ratio. The dietary n-6:n-3 PUFA ratio decreased with increasing dietary FO concentration (Fig. 1),

TABLE 2. Fatty acid composition of heart membranes as percentage of total fatty acids for various FO doses

	Low n-6 PUFA Background					
	0% FO 10% OO	0.16% FO 9.84% OO	0.31% FO 9.69% OO	0.63% FO 9.37% OO	1.25% FO 8.75% OO	5% FO 5% OO
Fatty Acid						
16:00	9.27 ± 0.10^{a}	$10.18 \pm 0.11^{a,b}$	10.70 ± 0.36^{b}	11.07 ± 0.18^{b}	10.99 ± 0.16^{b}	10.65 ± 0.32^{b}
18:00	$21.77 \pm 0.14^{a,b}$	$21.06 \pm 0.22^{a,b}$	$21.01 \pm 0.25^{a,b}$	20.77 ± 0.31^{b}	20.63 ± 0.22^{b}	22.00 ± 0.32^{a}
18:1n-9 (OA)	8.11 ± 0.14^{a}	6.68 ± 0.55^{b}	6.83 ± 0.23^{b}	6.74 ± 0.19^{b}	6.56 ± 0.23^{b}	6.51 ± 0.14^{b}
18:1n-7	4.08 ± 0.07	4.14 ± 0.10	3.97 ± 0.30	3.95 ± 0.19	3.77 ± 0.09	3.50 ± 0.05
18:2 n-6 (LA)	17.95 ± 0.94^{a}	17.00 ± 1.08^{a}	18.39 ± 0.70^{a}	16.98 ± 0.10^{a}	17.32 ± 0.30^{a}	10.44 ± 0.63^{b}
18:3 n-3 (LNA)	0.01 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.02
20:4 n-6 (AA)	24.79 ± 0.39^{a}	22.44 ± 0.32^{b}	19.59 ± 0.53^{c}	$18.12 \pm 0.33^{c,d}$	$16.78 \pm 0.48^{d,e}$	16.09 ± 0.41^{e}
20:5 n-3 (EPA)	0.01 ± 0.01^{a}	$0.08 \pm 0.01^{a,b}$	$0.18 \pm 0.01^{b,c}$	0.22 ± 0.02^{c}	0.39 ± 0.04^{d}	1.13 ± 0.03^{e}
22:5 n-3 (DPA)	0.79 ± 0.04^{a}	$0.88 \pm 0.05^{a,b}$	$0.92 \pm 0.07^{a,b}$	$0.88 \pm 0.08^{a,b}$	1.04 ± 0.05^{b}	1.05 ± 0.03^{b}
22:6 n-3 (DHA)	7.69 ± 0.55^{a}	12.75 ± 0.57^{b}	14.91 ± 0.38^{b}	17.90 ± 0.44^{c}	19.29 ± 0.41^{c}	24.54 ± 1.01^{d}
Σ n-6 PUFA	46.18 ± 0.74^{a}	41.52 ± 0.68^{b}	39.61 ± 0.49^{b}	36.43 ± 0.31^{c}	35.33 ± 0.47^{c}	27.15 ± 0.98^{d}
Σ n-3 PUFA	8.55 ± 0.58^{a}	13.79 ± 0.60^{b}	16.05 ± 0.44^{b}	19.04 ± 0.49^{c}	20.76 ± 0.34^{c}	26.78 ± 0.98^{d}
Σ PUFA	54.73 ± 0.22^{a}	$55.31 \pm 0.14^{a,b}$	$55.66 \pm 0.20^{a,b}$	$55.48 \pm 0.30^{a,b}$	56.08 ± 0.16^{b}	54.70 ± 0.41^{a}
Σ MUFA	12.56 ± 0.13^{a}	$11.37 \pm 0.50^{a,b}$	11.28 ± 0.29^{b}	11.11 ± 0.19^{b}	10.72 ± 0.15^{b}	10.63 ± 0.22^{b}
Σ Saturated fat	31.65 ± 0.13^{a}	$31.86 \pm 0.23^{a,b}$	$32.27 \pm 0.24^{a,b}$	32.44 ± 0.15^{b}	$32.29 \pm 0.14^{a,b}$	33.64 ± 0.11^{c}
Ratio n-6:n-3	5.50 ± 0.45^{a}	3.03 ± 0.18^{b}	$2.48 \pm 0.10^{b,c}$	$1.92 \pm 0.07^{c,d}$	$1.71 \pm 0.05^{c,d}$	1.05 ± 0.08^{d}

Dietary oils (FO and OO) are percentage of diet dry weight. Values are mean ± SEM of FA as percentage of total FA measured (n = 4). Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FO, fish oil; LA, linoleic acid; LNA, α-linolenic acid; MUFA, monounsaturated fatty acid; OA, oleic acid; OO, olive oil; PUFA, polyunsaturated fatty acid. Within rows, values not sharing a common superscript are significantly different from one another, $P < 0.05$ (two-way ANOVA with Tukey's posthoc test for multiple comparison of means).

and there was an inverse relationship between dietary ratio and the incorporation of EPA+DHA in myocardial membranes within both the low and the high n-6 PUFA diets (**Fig. 3**). A plot of dietary ratio against the myocardial membrane ratio revealed a linear relationship (Fig. 3, inset). The background concentration of n-6 PUFA in the diet significantly influenced the slope of the relationship (low n-6 PUFA diet: $r^2 = 0.936$; high n-6 PUFA: $r^2 = 0.956$; difference in slopes: $P < 0.0001$). However, neither the myocardial membrane EPA+DHA concentration nor its n-6:n-3 PUFA ratio differed between high and low n-6 PUFA diets at any individual dietary FO concentration (Fig. 3) despite large differences in dietary ratio. Only for the FO-free control diets was there a significant difference in the membrane ratio between high and low n-6 PUFA. There was a direct relationship between the n-6:n-3 PUFA ratio in the diet and AA concentrations in the heart. However, at each fish-oil dose, AA was displaced from heart membranes independently of the dietary ratio of n-6:n-3 PUFA (Tables 2, 3).

TABLE 3. Fatty acid composition of heart membranes as percentage of total fatty acids for various FO doses

	High n-6 PUFA Background					
	0%FO 5% OO 5% SSO	0.16% FO 4.84% OO 5% SSO	0.31% FO 4.69% OO 5% SSO	0.63% FO 4.37% OO 5% SSO	1.25% FO 3.75% OO 5% SSO	5% FO 5% SSO
Fatty Acid						
16:00	9.38 ± 0.21^{a}	$9.56 \pm 0.19^{a,b}$	$10.31 \pm 0.27^{a,b}$	$10.42 \pm 0.24^{a,b}$	$10.32 \pm 0.40^{a,b}$	10.69 ± 0.25^{b}
18:00	22.11 ± 0.32	22.45 ± 0.32	21.55 ± 0.20	21.86 ± 0.31	21.69 ± 0.28	21.93 ± 0.31
18:1n-9 (OA)	5.60 ± 0.10^{a}	$4.86 \pm 0.20^{a,b}$	$4.96 \pm 0.09^{a,b}$	4.34 ± 0.11^{b}	4.79 ± 0.35^{b}	3.09 ± 0.06^{c}
18:1n-7	3.36 ± 0.05^{a}	$3.30 \pm 0.03^{a,b}$	$3.27 \pm 0.14^{a,b}$	$3.18 \pm 0.18^{a,b}$	$3.18 \pm 0.20^{a,b}$	2.75 ± 0.06^{b}
18:2 n-6 (LA)	21.13 ± 0.50	18.76 ± 1.06	20.70 ± 0.70	19.87 ± 0.78	18.93 ± 1.08	18.86 ± 0.59
18:3 n-3 (LNA)	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00
20:4 n-6 (AA)	23.88 ± 0.33^{a}	22.40 ± 0.27^{a}	20.05 ± 0.45^{b}	$18.36 \pm 0.54^{b,c}$	16.64 ± 0.27^{c}	14.42 ± 0.64^{d}
20:5 n-3 (EPA)	0.02 ± 0.01^{a}	0.05 ± 0.00^{a}	$0.09 \pm 0.01^{a,b}$	0.15 ± 0.02^{b}	0.25 ± 0.01^{c}	0.79 ± 0.04^{d}
22:5 n-3 (DPA)	0.67 ± 0.04	0.88 ± 0.22	0.78 ± 0.07	0.86 ± 0.04	1.02 ± 0.06	1.02 ± 0.07
22:6 n-3 (DHA)	6.70 ± 0.21^{a}	13.00 ± 0.82^{b}	$14.29 \pm 0.52^{b,c}$	$17.16 \pm 1.09^{c,d}$	$19.36 \pm 0.95^{d,e}$	22.25 ± 0.35^{e}
Σ n-6 PUFA	49.85 ± 0.14^{a}	43.54 ± 0.76^{b}	$42.56 \pm 0.40^{b,c}$	$39.73 \pm 1.13^{c,d}$	$36.79 \pm 1.24^{d,e}$	34.56 ± 0.49^{e}
Σ n-3 PUFA	7.47 ± 0.23^{a}	13.99 ± 0.97^{b}	$15.22 \pm 0.50^{b,c}$	$18.23 \pm 1.06^{c,d}$	20.68 ± 0.95^{d}	24.12 ± 0.33^{e}
Σ PUFA	57.32 ± 0.16^{a}	$57.53 \pm 0.21^{a,b}$	$57.78 \pm 0.24^{a,b}$	$57.96 \pm 0.09^{a,b}$	$57.47 \pm 0.49^{a,b}$	58.68 ± 0.33^{b}
Σ MUFA	9.46 ± 0.12^{a}	$8.57 \pm 0.23^{a,b}$	$8.62 \pm 0.12^{a,b}$	7.98 ± 0.24^{b}	8.45 ± 0.26	6.40 ± 0.18^{c}
Σ Saturated fat	32.21 ± 0.17^{a}	32.82 ± 0.15^{a}	32.59 ± 0.13^{a}	$33.06 \pm 0.18^{a,b}$	$32.89 \pm 0.41^{a,b}$	33.79 ± 0.07^{b}
Ratio n-6:n-3	6.69 ± 0.22^{a}	3.17 ± 0.27^{b}	$2.81 \pm 0.11^{b,c}$	$2.21 \pm 0.18^{c,d}$	1.80 ± 0.13^{d}	1.43 ± 0.04^{d}

Dietary oils (FO, OO, and SSO) are percentage of diet dry weight. Values are mean ± SEM of FA as percentage of total FA measured (n = 4). Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FO, fish oil; LA, linoleic acid; LNA, α-linolenic acid; MUFA, monounsaturated fatty acid; OA, oleic acid; OO, olive oil; PUFA, polyunsaturated fatty acid; SSO, sunflower seed oil. Within rows, values not sharing a common superscript are significantly different from one another, $P < 0.05$ (two-way ANOVA with Tukey's posthoc test for multiple comparison of means).

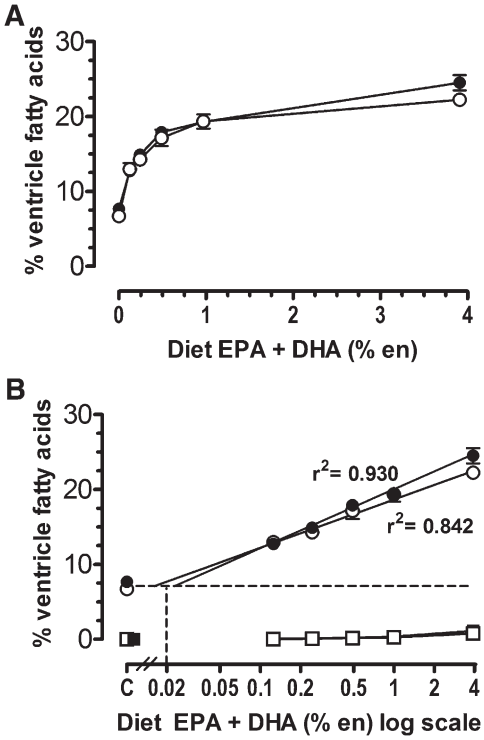

Fig. 2. Effects of dietary fish oil plotted as %en EPA+DHA and background n-6 PUFA on major phospholipid n-3 content of rat heart. A: Ventricle DHA concentration. B: Ventricle EPA and DHA concentrations with dietary fish oil plotted on a $\log_{10}$ scale (horizontal broken line represents the control DHA concentration, and the vertical broken line represents the predicted threshold for membrane incorporation). Open symbols (□○) represent low n-6 PUFA diet. Closed symbols (□●) represent high n-6 PUFA diet. Squares (□□) represent EPA. Circles (○●) represent DHA. C represents control diet with no added fish oil (0% FO). Values are mean ± SEM; n = 4 per data point. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; PUFA, polyunsaturated fatty acid; %en, % of dietary metabolizable energy.

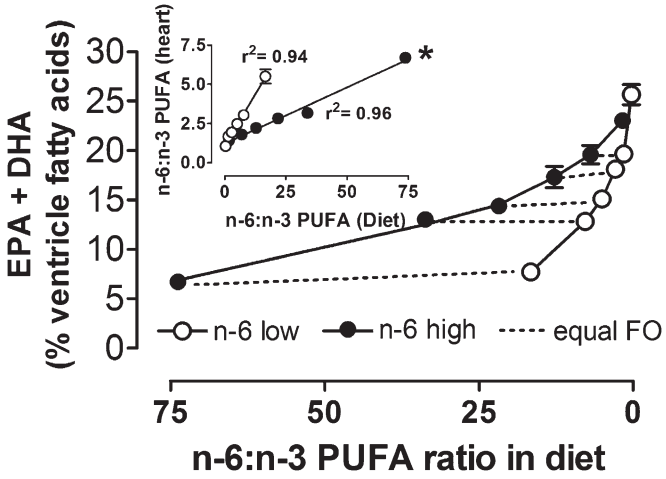

Fig. 3. Effect of n-6:n-3 PUFA ratio of rat diet on EPA+DHA concentrations of rat ventricle phospholipid fatty acids. Inset: The n-6:n-3 PUFA ratio of the ventricle plotted against the n-6:n-3 PUFA ratio of the diet. Open circles (○) represent low n-6 PUFA diet. Closed circles (●) represent high n-6 PUFA diet. Dotted lines join data points of equal dietary FO. Values are mean ± SEM; n = 4 per data point. * indicates significantly different slope ($P < 0.0001$). DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; PUFA, polyunsaturated fatty acid.

DISCUSSION

This study found that very small intakes of fish oil can markedly increase the myocardial membrane long chain n-3 PUFA concentration. We identified a dietary threshold for the myocardial membrane incorporation of long chain n-3 PUFA, predicting that increased incorporation can be achieved when fish-oil intake exceeds 0.019% of dietary metabolizable energy as EPA+DHA (0.027% of the diet by weight as FO). This is similar to the intake (0.013%en) of EPA+DHA that marginally but significantly raises heart DHA concentration in n-3 PUFA–deficient rats (27). In terms of relative human intake, we calculated this estimated threshold to be equivalent to a single 1 g capsule of fish oil per week (**Table 4**). Moreover, long chain n-3 PUFA intake in the rat, equivalent in humans to only two meals of fatty fish per week, was sufficient to double the myocardial concentration of DHA, the principle myocardial n-3 fatty acid in the heart of many species (14). This equates to an n-3 PUFA intake consistently associated with low risk of cardiovascular disease mortality (28, 29).

Our previous identification of a linear relationship between fish-oil intake and myocardial n-3 PUFA incorporation (24) must be reconsidered, as the present study has revealed that the previous dose range lay within the asymptotic portion of a hyperbolic curve, well above the minimum requirements for membrane change. A similar hyperbolic relationship has been described for incorporation of DHA from cod-liver oil into heart and other tissues of n-3 PUFA–deficient rats (27).

Studies of physiological effects and mechanisms of action historically use high intakes of fish oil (in the range 5%–12% of diet and up to 25%en) (17). In our previous research, this use of high doses was informed by the need to use high intakes to demonstrate the small cardiovascular effects of n-6 PUFA (14); however, the limited evidence available from studies using low intakes suggests the early high intakes are well above the minimum requirements for physiological effect. For example, prevention of isch-

TABLE 4. How much is that for human consumption?

Rat			Human		
Fish oil % weight	EPA+DHA mg per 100 g	EPA+DHA % energy	EPA+DHA g per day[a]	100 g salmon per week[b]	Fish oil per day[c]
5.0	1791	3.91	9.1	33	28
1.25	448	0.98	2.3	8	6.8
0.63	226	0.49	1.2	4	3.5
0.31	111	0.24	0.57	2	1.7
0.16	57	0.13	0.28	1	<1
0.027[d]	10	0.019	0.04	0.15	0.12

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.
[a] Based on human energy intake of 8700 kJ per day.
[b] Based on salmon n-3 content of 1.9 g per 100 g.
[c] Based on typical fish oil capsule content of 330 mg EPA+DHA.
[d] Extrapolated threshold intake of fish oil from Fig. 2B.

emia-induced ventricular fibrillation first demonstrated with a 12% FO supplement (16) is achieved just as effectively by intake as low as 1.25% FO (0.98%en EPA+DHA) (30) or 0.5% purified DHA (0.39%en) (31). Antiarrhythmic effects of intakes in the lower range used in the current study have not been assessed. Improvements in myocardial oxygen efficiency and reduced ischemia-reperfusion injury, first demonstrated with 10%en as EPA+DHA (18), are still evident with FO intake of 2.75%en as EPA +DHA (32). Lower intakes have not been assessed. Stanley et al. (33) recently reported myocardial incorporation and physiological effects on heart function in vivo in rats fed FO doses in the range 0.7%en–7%en as EPA+DHA, and a similar low intake of 0.7%en as EPA+DHA was earlier shown to increase cardiac DHA and lower heart rate (34). As the principle n-3 PUFA in the heart irrespective of dietary source, DHA is present in all myocardial subcellular membranes (35). Even with high fish-oil intakes, the cardiac phospholipid fatty acid concentration of DHA in the rat rarely exceeds 25% (24, 36–38) as observed here with the 5% FO supplementation (although concentrations well in excess of 30% are observed when fish oil is fed as the sole source of lipid in a fully fabricated diet (24)). The few low-dose evaluations have produced 18%–22% DHA from dietary intakes of 0.39%en (31) or 0.7%en (33, 34), again in the same range as the present study. Therefore, both physiological effects and membrane incorporation have largely been evaluated above the dose required for maximum effect in rats.

The human ventricle contains comparatively less DHA (<3%) and less total PUFA (<40%) (39–41) than the rat heart in this and other studies. This basal level is inversely related to body size and directly related to resting metabolic rate and heart rate between species (42). However, the dietary and adaptive responses in membrane composition are similar. For example, although basal myocardial membrane DHA concentration is markedly higher in rats than humans, DHA remains the main n-3 PUFA in both. It is increased by low intake of fish oil in humans (39) now demonstrated in rats, showing for the first time that both human and rat hearts respond within a similar low dietary range. Other similarities between humans and rats include increased myocardial DHA concentrations associated with chronic stress leading to sudden death (43), lowering sudden death risk with dietary fish oil (14), and reduced heart rate with dietary-induced increases in myocardial DHA concentration (14, 44).

The ready incorporation of DHA into myocardial membranes with the very low dietary intake reported here similarly implies a threshold for effects on cardiac function that is lower than previous doses evaluated, predictably around low dietary intake that produces significant membrane change. Importantly, our observations establish effects on membrane incorporation in the rat within the range of normal human dietary exposure, hitherto one remaining factor limiting confident use of data from animal-experimental models to predict human responses to fish consumption (22). Mozaffarian and Rimm conducted a pooled analysis of prospective and randomized controlled trials to describe a maximal effect on cardiovascular mortality risk achieved with an intake of about 250 $mg.d^{-1}$ EPA+DHA, above which little further risk reduction is observed (28). The threshold intake for initiating incorporation of n-3 PUFA into myocardial membranes of the rat established in the present study was estimated to be equivalent to a human intake of 40 $mg.d^{-1}$ of EPA+DHA, with further marked changes in membrane incorporation (doubling of DHA) achieved with the rat intake equivalent to 250 $mg.d^{-1}$. In the most comparable evaluation of human incorporation, a study of cardiac transplant patients demonstrated a significant increase in myocardial n-3 PUFA (mainly as DHA) with a dietary supplement of 500 $mg.d^{-1}$ EPA+DHA (39), which, according to our estimates, is equivalent to 0.21%en and in the midrange of the dose response curve established using rats.

The dietary background concentration of n-6 PUFA had no impact on incorporation of long chain n-3 PUFA into rat heart membranes, except when n-3 PUFA incorporation approached saturation with the highest fish-oil dose, which was well beyond relevant human intake. Nor did it influence the membrane concentration of arachidonic acid, demonstrating that the diets were not n-6 PUFA–deficient. Our study of the literature reveals that the proposal that the ratio of n-6:n-3 PUFA in the diet can be a major influence on the n-3 PUFA concentration or membrane n-6:n-3 PUFA ratio is based, without exception, on modulation of the n-6:n-3 ratios by changing only the amount of n-3 PUFA in the diet with concurrently reduced background of n-6 PUFA (45). Moreover, the ratio is most often altered by the simple presence or absence of a single dietary concentration of fish oil. The present study used contrasting approaches to achieve 12 different n-6:n-3 dietary ratios: modifying the n-3 PUFA content of the diet while maintaining n-6 PUFA content and modifying the n-6 PUFA content while maintaining n-3 PUFA content. The incorporation of long chain n-3 PUFA into the heart was influenced solely by the amount of n-3 PUFA in the diet and was independent of the background of n-6 PUFA, irrespective of the energy contributions of either. Recent epidemiological evidence also demonstrates that the reduced risk of sudden cardiac death attributable to moderate fish consumption is independent of n-6 PUFA dietary background (46). The ratio of n-6:n-3 PUFA only impacts on the incorporation of DHA into the heart from the terrestrial n-3 PUFA α linolenic acid (18:3n-3) in the diet, which is subject to competition with n-6 PUFA for desaturase enzymes in the cascade of metabolism to EPA and DHA (35, 47). Concern raised in some quarters that a diet high in n-6 PUFA may have detrimental effects on health (48, 49) has led some to recommended reductions of n-6 PUFA intake to establish an optimal n-6:n-3 PUFA ratio in the human diet (50–53). If those recommendations are based on concerns about competition for tissue incorporation of preformed, very long chain n-3 PUFA, they are clearly unfounded. The evidence consistently indicates that n-6 PUFA is itself associated with reduced risk of coronary heart disease (19, 54). The present study provides experimental support for the contention that little is likely to

be achieved for human cardiovascular health by simply reducing dietary n-6 PUFA intake (55), supporting recent American Heart Association recommendations regarding the n-6:n-3 PUFA ratio (56). It emphasizes the potential effects of even a small, regular intake of fish or fish oil compared with none.

Our findings showed that extremely low dietary intakes of fish oil in the rat, in the range equivalent to the human intake of 1–2 fish meals per week, produced marked changes in myocardial membrane composition, with a threshold intake for incorporation of n-3 PUFA equivalent to only one fish-oil capsule per week. By using high-DHA tuna fish oil, this study also reproduced a DHA-to-EPA ratio consistent with both common table and canned fish, such as Atlantic and red salmon (57), and the usual intake of n-3 PUFA from fish in the human diet (58) as discussed previously (59). In light of the effects of n-3 PUFA on heart function, especially cardiac arrhythmia and sudden death, these findings support the likelihood of direct myocardial effects contributing to nutritional preconditioning of the heart (59) and associated reduction in cardiovascular mortality.

Previous studies have not tested the utility of the dietary n-6:n-3 PUFA ratio by independently changing both the n-6 PUFA content and the n-3 PUFA content. We have established that the ratio of n-6:n-3 PUFA in myocardial membranes can be significantly increased by increasing n-3 PUFA consumption but not by reducing n-6 PUFA consumption. Our findings show that myocardial cells concentrate n-3 PUFA well above their dietary concentrations and clearly support the contention that this dietary ratio is not useful as a concept for predicting dietary outcomes (55).

This study provides evidence that very low intakes of fish oil (in the human dietary range) can modulate myocardial n-3 PUFA composition, thus providing a biologically plausible basis for associating myocardial incorporation with cardioprotective actions of low human intake. It clearly established effects in rats that are not due to pharmacological or therapeutic doses of fish oil and established that there is no direct competition from dietary n-6 PUFA limiting long chain n-3 PUFA incorporation into the heart.

The fish oil used in this study (high-DHA tuna fish oil) was the generous gift of NuMega Ingredients (Australia). This study was conceptualized by PLM and designed by P.L.M., A.J.O., and E.L.S. E.L.S. conducted the experiment. E.L.S. and M.L.T. analyzed and interpreted the tissue fatty acid records. Statistical analysis was conducted by E.L.S. and P.L.M. Data were interpreted by E.L.S., P.L.M., A.J.O., and M.L.T. P.L.M., A.J.O., and E.L.S. were responsible for the writing of the manuscript. The manuscript was reviewed and approved by all authors.

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