Effect of dietary supplementation with increasing doses of docosahexaenoic acid on neutrophil lipid composition and leukotriene production in human healthy volunteers

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n-3 PUFA supplementation helps in the prevention or treatment of inflammatory diseases and CVD. However, many supplementations reported so far are either a combination of *n*-3 PUFA or used large daily amounts of *n*-3 PUFA dosages. The present study investigated the influence of increasing dose intake of DHA on the fatty acid composition of phospholipids in neutrophils and on their capability to produce leukotrienes (LT) B_4 and B_5 *in vitro*. Twelve healthy volunteers were supplemented with increasing daily doses of DHA (200, 400, 800 and 1600 mg, each dose in TAG containing DHA as the only PUFA and for a 2-week period). At the end of each supplementation period, neutrophil fatty acid composition, and LTB_4 and LTB_5 production were determined by GC and liquid chromatography–tandem MS, respectively. The DHA/arachidonic acid ratio increased in a dose-dependent manner with respect to the increasing doses of DHA supplementation and was significantly different from baseline after supplementation with either 400, 800 or 1600 mg DHA. The LTB_5/LTB_4 ratio was significantly increased compared to baseline after supplementation with 800 and 1600 mg DHA. LTB_5/LTB_4 and DHA/arachidonic acid ratios were correlated (r 0.531, $P < 0.0001$). The present data suggest that both changes in neutrophil lipid composition and LT production occurred with daily supplementation with 800 and 1600 mg DHA. The clinical benefits associated with these doses of DHA in inflammatory diseases remain to be investigated.

Dietary DHA: Leukotrienes: Polymorphonuclear cells

In the past century, dietary and other environmental changes have been considered to be among the major causes of the rapid expansion of chronic diseases. In western countries, the increased incidence of inflammatory diseases has partly been attributed to the abundant consumption of *n*-6 PUFA with lower ingestion of *n*-3 PUFA. In this context, nutritional manipulations help in the prevention and/or treatment of various inflammatory diseases[1].

Linoleic and α-linolenic acids are essential fatty acids and must therefore be supplied in the diet. They are the precursors of the *n*-6 and *n*-3 series of fatty acids, respectively.

Linoleic acid may be converted to arachidonic acid (AA), which is subsequently incorporated into cell membrane phospholipids. Further metabolism of released AA by 5-lipoxygenase yields the four-series leukotrienes (LT; e.g. LTB_4), which possess potent inflammatory properties. Conversely, α-linolenic acid may be converted into EPA and DHA, which are also subsequently incorporated into cell membrane phospholipids.

It may be stressed, however, that the conversion of linolenic acid into long-chain derivatives is weak in men[2], which means that EPA and especially DHA must be consumed as preformed acids, i.e. from marine food. EPA yields the five-series eicosanoids, including LTB_5, through the 5-lipoxygenase pathway. Interestingly, many of the eicosanoids produced from EPA possess markedly reduced inflammatory properties compared with the AA-derived lipid mediators and may even exert antagonistic functions (for a review, see Calder[3]).

Dietary supplementations reported so far are a combination of DHA/EPA[4–9]; consequently, there is a lack of clarity regarding the differential biological effects of EPA and DHA. In addition, and although the clinical benefits of *n*-3 PUFA in CVD are observed with sub-gram doses[10], the underlying cellular changes induced by DHA or EPA supplementation have been investigated using daily amounts of *n*-3 PUFA greater than 1 g/d[4–9,11]. Few studies have assessed the effects of supplementation with lower doses of DHA and

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the data reported are conflicting. In platelets[12] or lymphocytes[13] from elderly people, the incorporation of DHA in lipid membranes occurs after daily supplementation with 150 mg DHA + 30 mg EPA. In contrast, Di Stasi *et al.*[8] found that 12-week oral supplementation with 1 g *n*-3 PUFA failed to change DHA content in platelet and mononuclear cell membranes. To our knowledge, the influence of low dosages of *n*-3 PUFA on the lipid content of neutrophils and their capability to synthesize LTB_4 and LTB_5 remains to be determined. The present study investigated the dose-dependent incorporation of DHA in neutrophils from healthy volunteers and the subsequent *ex vivo* production of LTB_4 and LTB_5.

Materials and methods

Clinical study

The protocol was approved by the local ethic committee in accordance with the Declaration of Helsinki, and all the participants gave written informed consent.

Twelve healthy males (mean age 58 years; age range 53–65 years) were included. Volunteers were excluded if they were taking any anti-inflammatory medication, and had been diagnosed as having CVD, diabetes, liver or endocrine dysfunction, or cancer. All participants supplemented their usual diet with daily consumption of capsules of Pro-Mind (Decoma, Belgium). Each capsule contained 200 mg DHA in TAG from algal oil, 0·125 mg DL-α-tocopherol and 0·125 mg ascorbic palmitate. The supplementation consisted of the daily ingestion of successively 200, 400, 800 and 1600 mg DHA for 2 weeks for each dose, without interruption. The volunteers were instructed to keep their usual diet regimens throughout the study.

Blood samples were collected after overnight fasting 2 weeks before DHA supplementation started (DHA 0 mg), and at the end of each DHA dose supplementation period (200, 400, 800 and 1600 mg). Blood samples were also collected 5 weeks after supplementation was arrested.

Isolation of human polymorphonuclear cells

Venous blood was collected on citrate as anticoagulant. Polymorphonuclear cells were isolated by dextran sedimentation, followed by Ficoll-Paque centrifugation as previously described[14]. Cellular viability was greater than 98 % as judged by the Trypan blue exclusion method.

Polymorphonuclear cells (2×10^6 cells/ml) were suspended in 450 μl Tyrode-HEPES buffer (pH 7·4) containing 0·133 g/l $CaCl_2$ and 0·1 g/l $MgCl_2$ and incubated for 5 min at 37°C with the calcium ionophore A23187 at 0·5 μmol/l, or vehicle (ethanol 0·05 %). The reaction was stopped by the addition of 500 μl cold methanol–acetonitrile (v/v). The supernatants were stored at −80°C for later LT quantification as well as the cell residues for fatty acid composition.

Analysis of total lipids in neutrophils

Determination of mole quantities of total lipids was accomplished by GC as previously described[15].

Leukotrienes B_4 and B_5 quantification

Quantifications of LTB_4 and LTB_5 were performed on 800 μl centrifuged supernatant by liquid chromatography–tandem MS. LTB_4-d_4 (2 ng) was added on each sample as an internal standard. Solid-phase extraction was performed by using C18 (EC) cartridge (100 mg/10 ml) purchased from International Sorbent Technology (UK). Methanolic extracts were dried under nitrogen flow at room temperature and reconstituted in 40 μl mobile phase: methanol–ammonium formate (10 mmol/l; 80:20, v/v). After centrifugation, 10 μl were injected into the liquid chromatography–tandem MS system previously described[16]. The chromatographic separation was obtained on a Chromasil C8 5 μm (125×2 mm) column (Macherey-Nagel, France) maintained at 30°C.

MS acquisitions were made in the negative-ion mode using multiple reaction monitoring with the transitions m/z 335·0 → 195·1 for LTB_4, m/z 339·1 → 197·1 for LTB_4-d_4 and m/z 333·2 → 195·1 for LTB_5. The lower limit of quantification was 30 pg/ml for both LT.

Materials

Reagents used and their sources were: A23187 and LTB_5 from Sigma Aldrich, and LTB_4 and LTB_4-d_4 from Cayman.

Statistical analysis

Fatty acid content was calculated as a percentage of total fatty acids in the neutrophil lipids. LT production was expressed as ng/2×10^6 cells. LTB_5/LTB_4 ratio was expressed as LTB_5/LTB_4 ratio $\times$ 100. Data are presented as median (10th to 90th percentiles). Comparisons between baseline and each dose of DHA supplementation were performed using a Friedman test. Subsequent pair-wise comparisons were made with the Wilcoxon test adjusted with the Bonferonni correction for multiple comparisons. Correlations were analysed using the Spearman rank test. $P < 0.05$ was considered significant.

Results

Cell fatty acid composition

The fatty acid composition of total lipids in neutrophils at baseline and after supplementation with increasing doses of DHA is displayed in Table 1.

The major changes result in a dose-dependent increase of DHA proportions that are significantly different from baseline with the daily supplementation with 400, 800 and 1600 mg DHA, respectively. The proportion of 16:0, 18:0, 18:1*n*-9 and 18:2*n*-6 remained unchanged (data not shown).

The DHA/AA ratio increased in a dose-dependent manner with respect to the increasing doses of DHA supplementation and was significantly different from baseline for the daily supplementations with 400, 800 and 1600 mg DHA, respectively.

Five weeks discontinuation of DHA supplementation led to a decrease of DHA proportion and DHA/AA ratio, which were similar to those observed at baseline (Table 1).

The proportion of EPA was significantly correlated with the proportion of DHA (r 0·364, $P = 0.0018$) and was significantly increased compared to baseline after the daily supplementation with 1600 mg DHA (Table 1).

Table 1. Fatty acid composition of total lipids in neutrophils at baseline (DHA 0mg) and after supplementation with increasing doses of DHA (200, 400, 800 and 1600 mg/d, each dose for a 2-week period)‡

DHA dose (mg/d)	20:4n-6		20:5n-3		22:5n-3		22:6n-3		AA/DHA ratio	
	Median	10th to 90th percentiles	Median	10th to 90th percentiles	Median	10th to 90th percentiles	Median	10th to 90th percentiles	Median	10th to 90th percentiles
0	12.0	10.2–15.1	0.34	0.15–0.65	1.40	0.81–3.0	1.24	0.59–1.87	0.10	0.04–0.16
200	11.0	9.6–14.4	0.36	0.12–0.54	1.23	0.43–1.66	1.52	0.9–2.27	0.13	0.09–0.19
400	12.2	10.2–13.9	0.42	0.21–0.69	1.34	0.71–2.14	1.84*†	1.33–3.12	0.15*†	0.12–0.24
800	11.1	9.6–12.8	0.46	0.20–0.70	1.20**	0.64–1.65	2.32*†	1.74–3.27	0.22*†	0.15–0.29
1600	10.8	6.2–12.8	0.56*†	0.36–1.10	0.93***	0.05–1.41	3.19*†	1.77–4.16	0.31*†	0.19–0.40
WO period	11.7	9.5–13.7	0.38	0.12–0.49	1.0***	0.08–1.67	1.46	0.71–1.79	0.13	0.01–0.16

AA, arachidonic acid; WO, washout.

Median values were significantly different from those of the 0mg DHA dose: *P<0.0005, **P<0.02, ***P<0.004.

Median values were significantly different from those of the washout period: †P<0.0005.

‡Data are expressed as mol percentages and are presented as median and 10th to 90th percentiles from twelve experiments for each dose of DHA and nine experiments for the WO period (5 weeks after the arrest of DHA supplementation). For details of procedures, see Materials and methods.

The proportion of 22:5n-3 was significantly decreased compared to baseline after supplementation with 800 and 1600 mg DHA and remained significantly lower 5 weeks after the arrest of DHA supplementation (Table 1).

Leukotriene production

As shown in Fig. 1, the LTB_5/LTB_4 ratio increased as a function of the doses of DHA and was significantly different from baseline after 800 and 1600 mg DHA.

The LTB_5/LTB_4 ratio was correlated with the dose of DHA (r 0.532, $P<0.0001$). In addition, the LTB_5/LTB_4 ratio was significantly correlated with EPA content (r 0.525, $P<0.0001$), EPA/AA ratio (r 0.510, $P<0.0001$) and DHA/AA ratio (r 0.531, $P<0.0001$).

Discussion

The present study is the first to demonstrate that dietary supplementation with increasing doses of DHA as the only PUFA induces a dose-dependent incorporation of DHA in neutrophil lipids, and a subsequent dose-dependent increase in the ratio of LTB_5/LTB_4 production by neutrophils in response to calcium ionophore challenge. Before DHA supplementation started, the proportion of DHA in total lipids and the production of LT remained stable during a 2-week period (data not shown), attesting both the reliability and the reproducibility of biochemical measurements.

The dietary supplementation with DHA induced major changes in the lipid content from neutrophils. First, the proportion of DHA in neutrophil lipids was significantly increased after 2-week supplementation with 400 mg DHA/d, attesting that incorporation of DHA in lipids from neutrophils could occur after daily supplementation with this low dose of DHA. Conversely, a previous study suggested that 1.8 g DHA ethyl ester/d was required to significantly increase DHA proportion in neutrophil lipids[8]. This discrepancy could be explained by higher bioavailability of DHA-containing glycerides compared to DHA ethyl ester[17]. However, with respect to the slow DHA incorporation[18], it could not be excluded that a longer intervention would have been more effective with the lowest dose of DHA. Second, the proportion of EPA significantly increased after the daily intake of 1600 mg DHA, and was significantly correlated to DHA content. Since the dietary supplementation was free of EPA, the data suggest that DHA was likely retroconverted to EPA as previously described[19]. Although the design of the present study did not allow determination of which dose of DHA initiates the retroconversion of DHA to EPA, the present data suggest that it might occur only for high doses of DHA. Lastly, and unexpectedly, the proportion of 22:5n-3 (docosapentaenoic acid) was significantly decreased compared to baseline after supplementation with 800 and 1600 mg DHA and remained significantly lowered 5 weeks after the arrest of DHA. With docosapentaenoic acid being an intermediate of EPA and DHA, which both increased in response to highest doses of dietary DHA, it may be speculated that DHA could compete with endogenous docosapentaenoic acid to lower it in lipid stores.

The increased incorporation of DHA in neutrophil lipids was associated with a trend towards an increased LTB_5/LTB_4
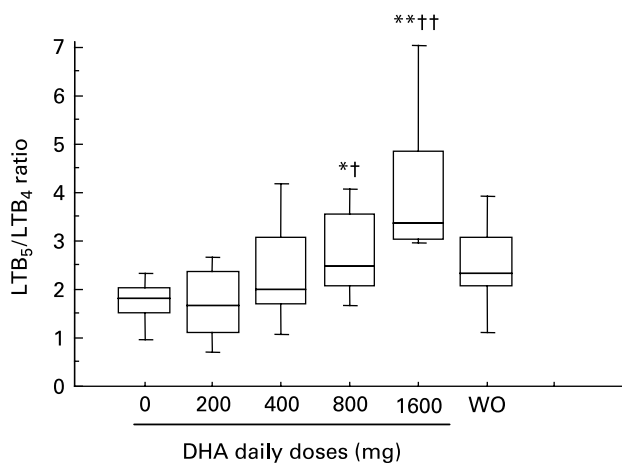
Fig. 1. Changes in leukotriene (LT) production by A23187-stimulated neutrophils from healthy volunteers before and after supplementation with increasing doses of DHA. Data are expressed as LTB_5/LTB_4 ratios. Boxes represent values within the interquartile range; whiskers represent the data range; and the lines across the boxes represent median values. WO, washout period. Median values were significantly different from those of the 0 mg DHA dose: $*P < 0.017$, $**P < 0.0079$. Median values were significantly different from those of the washout period: $†P < 0.017$, $††P < 0.0079$.

ratio that reached statistical significance from 800 mg DHA and over. Consistent with the conversion of DHA to EPA as discussed earlier, LTB_5/LTB_4 ratio was correlated with DHA/AA and EPA/AA ratios. Since the proportion of AA did not significantly decrease during the DHA supplementation period, the present data suggest that DHA increased LTB_5/LTB_4 ratio at least in part through its retroconversion to EPA rather than through an inhibitory effect on AA content.

In the present study, the doses of DHA have not been allocated at random to five separate groups of healthy subjects but have been taken successively by the same subjects. Consequently, it could not be excluded that the increased incorporation of DHA in neutrophil lipids and the subsequent production of LT observed for each dose of DHA may also reflect the effect of cumulative doses of DHA. Results from human studies indeed indicate that the level of DHA reached a plateau after 18 weeks of dietary supplementation[18] and most of the published data reported longer supplementation periods (from 3 weeks to 6 months[5,6,8,9,13]). However, the present data suggest that changes in total lipid content occurred within 2 weeks and therefore earlier than that reported until now.

In addition, DHA incorporation and *ex vivo* LT production returned to baseline levels 5 weeks after that the dietary supplementation with the highest dose of DHA was arrested, indicating a high turnover of DHA in cells.

We must acknowledge some limitations in the present study. The sample size and the lack of placebo group imply that the presented data should be considered as preliminary. Further studies are obviously required to confirm them. In addition, although the dose-dependent increase of DHA content provided indirect evaluation of the compliance of subjects, the latter has not been directly measured.

In conclusion, the present data demonstrate that changes in neutrophil lipid composition and LTB_5/LTB_4 ratio occurred after daily supplementation with 800 and 1600 mg DHA for 2 weeks. Further studies are needed to investigate the clinical benefits of dietary supplementation with these doses of DHA in inflammatory diseases or CVD.

References

1. Calder PC (2006) n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr* **83**, 1505S–1519S.
2. Burdge GC, Finnegan YE, Minihane AM, Williams CM & Wootton SA (2003) Effect of altered dietary n-3 fatty acid intake upon plasma lipid fatty acid composition, conversion of [^13C]alpha-linolenic acid to longer-chain fatty acids and partitioning towards beta-oxidation in older men. *Br J Nutr* **90**, 311–321.
3. Calder PC (2002) Dietary modification of inflammation with lipids. *Proc Nutr Soc* **61**, 345–358.
4. Chilton FH, Patel M, Fonteh AN, Hubbard WC & Triggiani M (1993) Dietary n-3 fatty acid effects on neutrophil lipid composition and mediator production. Influence of duration and dosage. *J Clin Invest* **91**, 115–122.
5. Sperling RI, Benincaso AI, Knoell CT, Larkin JK, Austen KF & Robinson DR (1993) Dietary omega-3 polyunsaturated fatty acids inhibit phosphoinositide formation and chemotaxis in neutrophils. *J Clin Invest* **91**, 651–660.
6. Kew S, Banerjee T, Minihane AM, Finnegan YE, Muggli R, Albers R, Williams CM & Calder PC (2003) Lack of effect of foods enriched with plant- or marine-derived n-3 fatty acids on human immune function. *Am J Clin Nutr* **77**, 1287–1295.
7. Mayer K, Fegbeutel C, Hattar K, Sibelius U, Kramer HJ, Heuer KU, Temmesfeld-Wollbruck B, Gokorsch S, Grimminger F & Seeger W (2003) Omega-3 vs. omega-6 lipid emulsions exert differential influence on neutrophils in septic shock patients: impact on plasma fatty acids and lipid mediator generation. *Intensive Care Med* **29**, 1472–1481.
8. Di Stasi D, Bernasconi R, Marchioli R, Marfisi RM, Rossi G, Tognoni G & Tacconi MT (2004) Early modifications of fatty acid composition in plasma phospholipids, platelets and mononucleates of healthy volunteers after low doses of n-3 polyunsaturated fatty acids. *Eur J Clin Pharmacol* **60**, 183–190.
9. Gorjao R, Verlengia R, Lima TM, *et al.* (2006) Effect of docosahexaenoic acid-rich fish oil supplementation on human leukocyte function. *Clin Nutr* **25**, 923–938.
10. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (1999) Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. *Lancet* **354**, 447–455.
11. Kelley DS, Taylor PC, Nelson GJ & Mackey BE (1998) Dietary docosahexaenoic acid and immunocompetence in young healthy men. *Lipids* **33**, 559–566.
12. Vericel E, Calzada C, Chapuy P & Lagarde M (1999) The influence of low intake of n-3 fatty acids on platelets in elderly people. *Atherosclerosis* **147**, 187–192.
13. Bechoua S, Dubois M, Vericel E, Chapuy P, Lagarde M & Prigent AF (2003) Influence of very low dietary intake of

marine oil on some functional aspects of immune cells in healthy elderly people. *Br J Nutr* **89**, 523–531.

14. Hosni R, Chabannes B, Pacheco Y, Moliere P, Grosclaude M, Perrin Fayolle M & Lagarde M (1991) Leukotriene B_4 levels from stimulated neutrophils from healthy and allergic subjects: effect of platelets and exogenous arachidonic acid. *Eur J Clin Invest* **21**, 631–637.

15. Diaz O, Berquand A, Dubois M, Di Agostino S, Sette C, Bourgoin S, Lagarde M, Nemoz G & Prigent AF (2002) The mechanism of docosahexaenoic acid-induced phospholipase D activation in human lymphocytes involves exclusion of the enzyme from lipid rafts. *J Biol Chem* **277**, 39368–39378.

16. Hardy G, Boizel R, Bessard J, Cracowski JL, Bessard G, Halimi S & Stanke-Labesque F (2005) Urinary leukotriene E_4 excretion is increased in type 1 diabetic patients: a quantification by liquid chromatography–tandem mass spectrometry. *Prostaglandins Other Lipid Mediat* **78**, 291–299.

17. Visioli F, Rise P, Barassi MC, Marangoni F & Galli C (2003) Dietary intake of fish vs. formulations leads to higher plasma concentrations of n-3 fatty acids. *Lipids* **38**, 415–418.

18. Marangoni F, Angeli MT, Colli S, Eligini S, Tremoli E, Sirtori CR & Galli C (1993) Changes of n-3 and n-6 fatty acids in plasma and circulating cells of normal subjects, after prolonged administration of 20:5 (EPA) and 22:6 (DHA) ethyl esters and prolonged washout. *Biochim Biophys Acta* **1210**, 55–62.

19. Brossard N, Croset M, Pachiaudi C, Riou JP, Tayot JL & Lagarde M (1996) Retroconversion and metabolism of [^{13}C]22:6n-3 in humans and rats after intake of a single dose of [^{13}C]22:6n-3-triacylglycerols. *Am J Clin Nutr* **64**, 577–586.