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Abstract

N-3 polyunsaturated fatty acid (*n*-3 PUFA) supplementation helps in the prevention or treatment of inflammatory and cardiovascular diseases. 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 docosahexaenoic acid (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 mg, 400 mg, 800 mg and 1600 mg, each dose in triglycerides 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 gas chromatography and liquid chromatography - tandem mass spectrometry, respectively. The DHA/arachidonic acid (AA) 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 mg, 800 mg or 1600 mg of DHA. The LTB_5/LTB_4 ratio was significantly increased compared to baseline after supplementation with 800 mg and 1600 mg of DHA. LTB_5/LTB_4 and DHA/AA ratios were correlated ($r = 0.531$, $P<0.0001$). Our data suggest that both changes in neutrophil lipid composition and LT production occurred with daily supplementation with 800 mg and 1600 mg of DHA. The clinical benefits associated with these doses of DHA in inflammatory diseases remain to be investigated.

Introduction

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 polyunsaturated fatty acids (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 alpha-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 4-series leukotrienes (LT) (e.g. LTB_4), which possess potent inflammatory properties. Conversely, alpha-linolenic acid may be converted into eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, 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 5-series eicosanoids, including LTB_5 through the 5-lipoxygenase pathway. Interestingly, many of the eicosanoids produced from EPA possess markedly reduced inflammatory compared with the AA-derived lipid mediators and may even exert antagonistic functions (for review see (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 PUFAs in cardiovascular diseases 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 per

day (4; 5; 11; 6-9). Few studies have assessed the effects of supplementation with lower doses of DHA and 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 of DHA + 30 mg of EPA. In contrast, Di Stasi *et al* (8) found that 12-week oral supplementation with 1 g of *n*-3 PUFAs 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 (58 years old, age range: 53-65 years) were included. Volunteers were excluded if they were taking any anti-inflammatory medication, and had been diagnosed as having cardiovascular disease, 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 of DHA in a triglyceride from algal oil, 0.125 mg of DL-α-tocopherol and 0.125 mg of ascorbic palmitate. The supplementation consisted of the daily ingestion of successively 200, 400, 800 and 1600 mg of DHA for two weeks each dose, without interruption. The volunteers were instructed to keep their usual diet regimens all over the study.

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

Isolation of human PMNs

Venous blood was collected on citrate as anticoagulant. PMNs 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.

PMNs ($2x10^6$ cells/mL) were suspended in 450 µl of Tyrode-Hepes buffer pH 7.4 containing 0.133 g/L $CaCl_2$ and 0.1g/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 of 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 gas chromatography as previously described (15).

LTB_4 and LTB_5 quantification

Quantifications of LTB_4 and LTB_5 were performed on 800 µL of centrifuged supernatant by liquid chromatography - tandem mass spectrometry. 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-10 mmol/L ammonium formate (80:20, v/v). After centrifugation, 10 µL were injected into the LC-MS/MS system previously described (16). The chromatographic separation was obtained on a Chromasil C8, 5 µm (125x2 mm) column (Macherey-Nagel, France) maintained at 30°C.

Mass spectrometry 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 LTs.

Materials

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

Statistical analysis

Fatty acid content was calculated as a percentage of total fatty acids in the neutrophil lipids. Leukotriene production was expressed as ng/2x10^6 cells. LTB_5/LTB_4 ratio was expressed as LTB_5/LTB_4 ratio x 100. Data are presented as median (10^{th}-90^{th} 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 mg, 800 mg and 1600 mg of 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 mg, 800 mg and 1600 mg of 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 DHA 1600 mg (Table 1).

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

Leukotriene production

As shown in Figure 1, the LTB_5/LTB_4 ratio increased as a function of the doses of DHA and was significantly different from baseline after 800 mg and 1600 mg of 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 induced 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 LTs 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 per day of DHA, 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/d of DHA ethyl ester 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-ethylester (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 of 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 determining which dose of DHA initiates the retroconversion of DHA to EPA, our data suggest that it might occur only for high doses of DHA. *Last*, and unexpectedly, the proportion of 22:5 *n*-3 (docosapentaenoic acid, (DPA)) was significantly decreased compared to baseline after supplementation with 800 mg and 1600 mg of DHA and remained significantly lowered five

weeks after the arrest of DHA. DPA 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 DPA 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 ratio that reached statistical significance from 800 mg of DHA and over. Consistent with the conversion of DHA to EPA as above discussed, 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, our 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 5 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 LTs 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, our data suggest that changes in total lipid content occurred within 2 weeks and therefore earlier than that reported up to now.

In addition, DHA incorporation and *ex vivo* LT production returned to baseline levels five-weeks after that the dietary supplementation with the highest dose of DHA was arrested, indicating a high turn over 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, our data demonstrate that changes in neutrophil lipid composition and LTB_5/LTB_4 ratio occurred after daily supplementation with 800 mg and 1600 mg of 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 cardiovascular diseases.

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Statement:

The authors accept the conditions laid down in the Direction to Contributors.

This submission represents original work that has not been published previously, that is not currently being considered by another journal. If accepted for the *British Journal of Nutrition* it will not be published elsewhere in the same form, in English or in any language, without the written consent of the Nutrition Society.

All the authors have seen and approved the contents of the submitted manuscript.

There is no conflict of interest and all the authors adhere to the Committee on Publication Ethics guidelines on research and publication ethics.

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

Data are expressed as mol percentages and are presented as median and 10^{th}-90^{th} percentiles. from 12 experiments for each dose of DHA and 9 experiments for the wash out (WO) period (5 weeks after the arrest of DHA supplementation).

DHA dose	20:4*n*-6		20:5*n*-3		22:5*n*-3		22:6*n*-3		AA/DHA ratio	
mg/d	median	10^{th}-90^{th}	median	10^{th}-90^{th}	median	10^{th}-90^{th}	median	10^{th}-90^{th}	median	10^{th}-90^{th}
0 mg	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 mg	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 mg	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 mg	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 mg	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	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

*: $P<0.0005$ *vs*. 0 mg and vs. WO

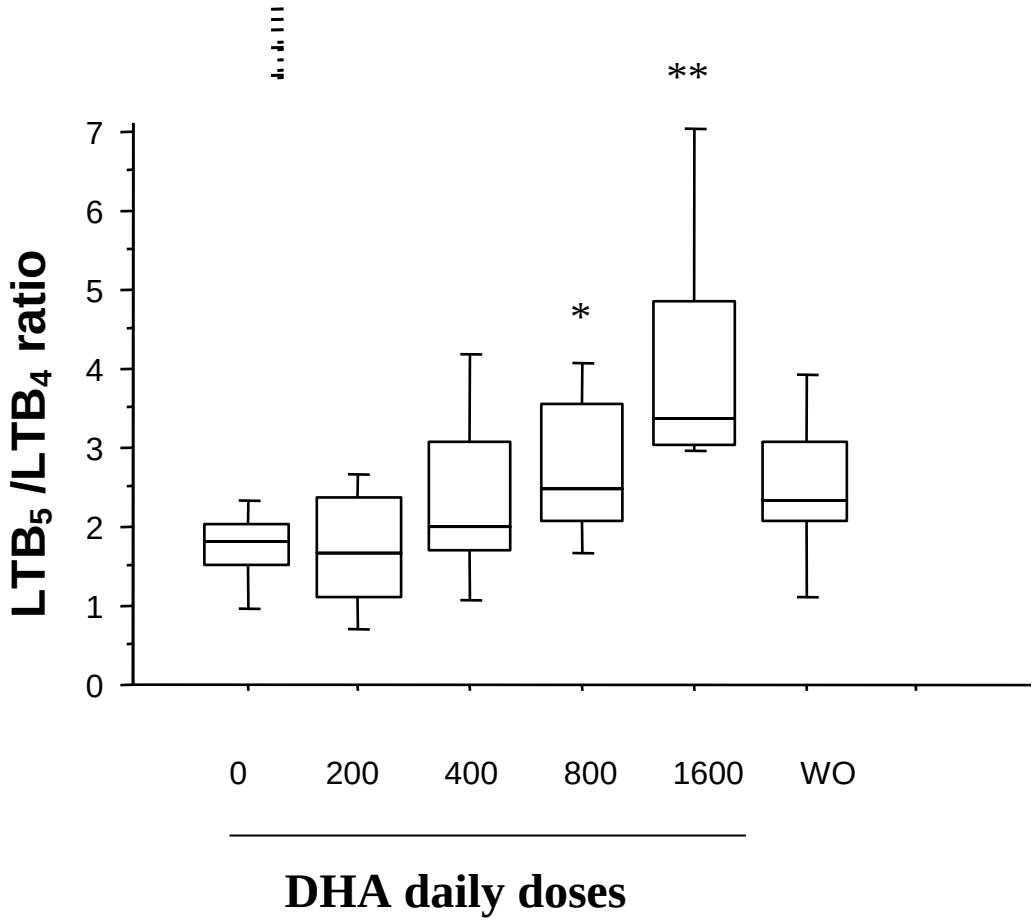
**: $P<0.02$ *vs*. 0 mg

***: $P<0.004$ *vs*. 0 mg

Figure 1: Changes in leukotriene 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, the data range; and the line across the boxes, median values.

•*: $P<0.017$: 800 mg vs 0 mg and vs W0.

**: $P<0.0079$:1600 mg vs 0 mg and vs W0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*
7
6
5
4
3
2
1
0
LTB5 /LTB4 ratio
0
200
400
800
1600
WO
DHA daily doses

Figure 1