Docosahexaenoic acid lowers phosphatidate level in human activated lymphocytes despite phospholipase D activation

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Abstract N–3 polyunsaturated fatty acids from marine oil have been shown to decrease T cell-mediated immune function both in animals and humans, and to inhibit the mitogen-induced lymphoproliferative response when added to lymphocyte culture medium. As phosphatidic acid (PA) is a key mediator of the mitogenic process, the present study aims to investigate whether docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids, the main n–3 fatty acids from fish oil, are able to alter the mitogen-induced synthesis of PA, when added to the culture medium of human peripheral blood mononuclear cells (PBMC). Incubation of PBMC in a medium containing 5 μM DHA bound to 5 μM human delipidated serum albumin induced a 2–fold increase in the basal PA mass whereas incubation with EPA, in the same conditions, had no effect. In contrast, both fatty acids markedly reduced the concanavalin A (ConA)-induced production of PA as compared with untreated cells. Paradoxically, phospholipase D (PLD) activity, evidenced by the synthesis of phosphatidylbutanol, was only detected in DHA-treated cells further stimulated by ConA, indicating that both DHA and ConA are required for PLD activation. Similarly, an increased diacylglycerol (DAG) mass was only observed in DHA-treated cells stimulated by ConA, whereas no modification occurred in control or EPA-treated cells stimulated or not by ConA. Furthermore, 1-butanol suppressed the ConA-induced increase of DAG mass observed in DHA-treated cells, indicating that phosphatidate was the source of the newly synthesized diacylglycerol. Altogether, these results show that, in concanavalin A-activated human peripheral blood mononuclear cells, docosahexaenoate stimulates both phospholipase D and phosphatidate phosphohydrolase activities, which ultimately results in an increased diacylglycerol production at the expense of phosphatidate.—**Bechoua, S., M. Dubois, G. Némoz, M. Lagarde, and A-F. Prigent.** Docasahexaenoic acid lowers phosphatidate level in human activated lymphocytes despite phospholipase D activation. *J. Lipid Res.* 1998. **39:** 873–883.

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Epidemiologic observations as well as animal and human dietary studies have suggested an anti-inflammatory effect of n–3 fatty acids (1). The beneficial effects of n–3 fatty acids from marine oil on inflammatory diseases seem to be mainly due to their ability to reduce the synthesis of pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), especially in humans (2, 3). In animal studies more conflicting results have been reported, describing either a reduced (4) or an increased (5) cytokine synthesis upon n–3 fatty acid supplementation of the diet, depending on the cell population studied and the animal species used for the experimentation (2). Numerous reports have also described an effect of n–3 fatty acids on cell-mediated immunity. Fish oil supplementation of human and animal diets has been shown to decrease interleukin-2 (IL-2) production, T cell mitogen-induced proliferation, and delayed-type hypersensitivity skin response (DTH) indicating an overall decreased T cell-mediated immune function (6–10). In addition, polyunsaturated fatty acids either from the n–6 or the n–3 families have been shown to inhibit the mitogen-induced lymphocyte proliferation when added in vitro to the lymphocyte culture medium, whatever the tissue or species origin of the lymphocyte preparations, including murine splenic or thymic lymphocytes (9) or human peripheral blood lymphocytes (11–13). However, the

Abbreviations: AA, arachidonic acid; ConA, concanavalin A; DAG, diacylglycerol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NEM, N-ethylmaleimide; PBMC, peripheral blood mononuclear cells; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; TG, triacylglycerols.

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biochemical mechanisms involved in the immunosuppressive effects of polyunsaturated fatty acids remain unclear. Several reports have shown that free fatty acids can interact with signalling pathways involved in stimulus–response coupling mechanisms (14, 15). Ion channels, G proteins, protein kinases, and phospholipases are among the main targets of polyunsaturated fatty acids recently described. In Jurkat lymphocytes both n–6 and n–3 fatty acids induced a rapid and transient increase of cytosolic free calcium from internal stores, but simultaneously decreased the antiCD3-induced calcium entry through receptor-operated channels (16). This inhibition of receptor-mediated calcium influx might be involved in the decrease of IL-2 synthesis after addition of free fatty acids to lymphocyte culture medium. The protein kinase C (PKC) family, which plays a key role in the control of cell growth and differentiation, is another recognized target of free fatty acids. Interestingly, fatty acids such as oleate activate protein kinase C in vitro by a mechanism that is distinct from PS/DAG, with a preference for soluble over membrane-bound enzymes (17) and for calcium-independent and DAG unresponsive isoforms (18). However, different regulatory patterns have been observed when fatty acids were added to cell culture medium. May, Southworth, and Calder (19) have reported that culture of rat lymph node lymphocytes with n–6 as well as n–3 fatty acids resulted in a reduction in total PKC activity, the highest inhibitory effect being observed for the n–3 fatty acids, eicosapentaenoic and docosahexacnoic acids. In contrast, Rossetti et al. (20) did not observe any influence of eicosapentaenoic acid on the PKC activity of human T lymphocytes after an overnight incubation in the presence of the fatty acid bound to albumin, whereas n–6 fatty acids seemed to facilitate the translocation of PKC from cytosol to membrane.

Another important signalling event involved in the early steps of mitogenesis is the activation of phospholipase Cγ1, leading to cleavage of phosphatidylinositol bisphosphate into inositol trisphosphate and diacylglycerol. In lymphocytes, diacylglycerol, whose primary function is the activation of protein kinase C, is rapidly phosphorylated to phosphatidic acid (PA) by diacylglycerol kinase, so that in the first 30 min following mitogenic activation, only PA increase can be detected (21, 22). Phosphatidic acid itself is thought to play a crucial role in the control of the mitogenic process through specific interactions with key enzymes such as PLCγ1 (23), PKCζ isoenzyme (24), and cyclic AMP phosphodiesterase (PDE) belonging to the PDE4 family (25). Furthermore, phosphatidic acid is also a potent mitogen for several types of cells including fibroblasts (26) and T-cell clones (27). We have already demonstrated that in thymic lymphocytes as well as in human peripheral lymphocytes, the PA synthesized after mitogenic activation was almost exclusively derived from phosphoinositides through the PLCγ1–diacylglycerol kinase pathway and that phospholipase D was not involved in normal lymphocytes (22, 28).

The present study was undertaken to investigate whether the main n–3 fatty acids from fish oil, docosahexaenoic and eicosapentaenoic acids, known to inhibit the lymphoproliferative response, were able to alter the mitogen-induced synthesis of phosphatidic acid when added to the culture medium of human peripheral blood mononuclear cells (PBMC) as free fatty acids bound to human delipidated albumin.

MATERIALS AND METHODS

Preparation of human PBMC

Peripheral blood was obtained from healthy subjects who had not taken any medication for 2 weeks prior to blood donation (Centre de Transfusion Sanguine, Lyon). Venous blood was drawn into citrate–phosphate–dextran anticoagulant. PBMC were separated by dextran sedimentation and density gradient centrifugation through Histopaque 1077 (Sigma, L'Isle d'Abeau, France) and then washed three times with RPMI 1640 by low speed centrifugation in order to more thoroughly eliminate the contaminating platelets. PBMC were then adjusted to a concentration of 2×10^7 cells/ml in RPMI 1640 (with HEPES and bicarbonate) medium. All steps were carried out at room temperature. Under such conditions, cell viability established by the trypan blue exclusion test was always greater than 95%. Flow cytometry analyses of cell preparations after staining with specific monoclonal antibodies showed that about 65–70% of the isolated cells were $CD3^+$ T cells (T_3 Coulter clone), 4–6% were $CD19^+$ B cells (B_4 Coulter clone), 16–24% were $CD11b^+$ monocytes (MO1 Coulter clone), and 4–6% were $CD41a^+$ platelets (GP IIb IIIa, Immunotech, Marseille, France).

Preparation of fatty acid–albumin complexes

Free docosahexaenoic and eicosapentaenoic acids (Sigma, L'Isle D'Abeau, France) were stored at −20°C in ethanol solution under nitrogen. Aliquots of the ethanolic solution were evaporated to dryness under reduced pressure. Human delipidated serum albumin (5 μm) in RPMI 1640 medium was added to give a final fatty acid concentration of 5 μm and an albumin to fatty acid ratio of 1. The mixtures were incubated under nitrogen for 4 h at 37°C.

Radiolabeling of the mononuclear cell suspensions

Freshly isolated cells were incubated for 1 h at 37°C either in the presence of [^{3}H]arachidonic acid (37 kBq/ml, specific activity 7.4 MBq/mmol, Amersham, Les Ulis, France) or [^{3}H]docosahexaenoic acid (9.25 kBq/ml, specific activity 2.22 MBq/mmol, DuPont NEN) in 0.1% ethanol. In some experiments, [^{3}H] docosahexaenoic acid was mixed with 5 μm unlabeled docosahexaenoic acid bound to HSA. After the incubation, the radiolabeled cells were washed three times in RPMI 1640 medium.

Mitogenic activation of radiolabeled PBMC

Labeled cells were further incubated for 60 min at 37°C in RPMI medium containing 5 μm docosahexaenoic or eicosapentaenoic acid bound to 5 μm delipidated HSA. Control cells were incubated without fatty acid in the presence of 5 μm HSA alone. Fatty acid-treated and control cells were then incubated for 30 min at 37°C in the absence (unstim.) or presence of 5 μg/10^6 cells concanavalin A (+ConA). In experiments designed to investigate the influence of primary alcohols on the synthesis of phosphatidylalcohol and the formation of PA and DAG, ConA activation of the cells (5 μg ConA/10^6 cells for 30 min) was performed in the presence of 1% ethanol or 1-butanol.

Lipid extraction

Incubations were terminated by addition of ethanol and acidification of the medium to pH 3–4 with 2 N HCl. Lipids were extracted with chloroform–ethanol 6:3 (by vol.) according to Boukhchache and Lagarde (29) in the presence of 50 μm butylhydroxylated toluene.

Thin-layer chromatography (TLC) analyses of lipid extracts

Lipid extracts were separated on thin-layer plates (20 × 20 × 0.25 silica gel G60 plates, Merck, Darmstadt, Germany). DAG was separated on monodimensional TLC with the solvent system hexane–diethylether–acetic acid 50:50:1 (by vol) which allows for separation of the various lipid classes (free fatty acids, DAG, TG, and steryl esters) from total phospholipids. PA was separated on bidimensional TLC using chloroform–methanol–40% ammonia 65:35:5.5 (by vol) for the first migration, and chloroform–acetone–methanol–acetic acid–water 30:40:10:10:5 (by vol) for the second migration (30). This TLC system separates the major phospholipids including PI and PS, as well as minor components such as PA, lysophosphatidylethanolamine (lysoPE), and lysophosphatidylcholine (lysoPC). Phosphatidylalcohols were separated on bidimensional TLC using chloroform–methanol–40% ammonia 65:35:5.5 (by vol) for the first migration, and ethyl acetate–isooctane–acetic acid 9:5:2 (by vol) for migration in the second dimension. Spots stained by Coomassie brillant blue R (see below) were scraped, mixed with Picofluor (Packard, Rungis, France) and the radioactivity was determined by liquid scintillation counting. The radioactivity associated with PA, DAG, or phosphatidylalcohols was expressed as percentage of the radioactivity incorporated in total phospholipids.

Quantitative determination of the PA and DAG mass

PA was quantitated on TLC plates as described by Nakamura and Handa (31). Briefly, plates were first developed using the basic solvent system described above, until 6 cm below the top, dried under reduced pressure for 2 h. A standard curve of 1-stearoyl, 2-arachidonoyl-PA in the linear range (0.5–3 μg) was spotted on the undeveloped area of each plate (6 cm top in the first dimension). Then plates were developed in the second dimension with the acidic solvent system. After drying for 30 min, the developed plates were immersed for 20 min in a staining solution of 0.03% Coomassie brillant blue R in 20% methanol and 150 mm NaCl. After 20 min, the plates were removed from the staining solution, immersed in 20% methanol and 150 mm NaCl (destaining solution) for 15 min. Plates were then air dried and the density of each spot was measured with a videodensitometer (Bioprofil Vilber Lourmat/Fröbel, Lindau, Germany). DAG was quantitated on TLC by the same method. In this case, a standard curve of dipalmitoylglycerol in the linear range (0.25–4 μg) was spotted on the plate before development with the acidic solvent system hexane–diethylether–acetic acid 50:50:1 (by vol). After development, plates were processed as described above and the density of each spot was measured by videodensitometry.

RESULTS

Influence of docosahexaenoic and eicosapentaenoic acids on the basal and ConA-induced production of phosphatidic acid

ConA activation of human PBMC, prelabeled with a tracer amount of [^{3}H]arachidonate, for 5 and 30 min in a medium containing 5 μm human delipidated albumin (HSA), significantly increased the relative amount of radiolabeled phophatidic acid above control level. When labeled cells were first incubated in the presence of 5 μm docosahexaenoic or eicosapentaenoic acid bound to the HSA for 1 h at 37°C (albumin to fatty acid ratio = 1) and then stimulated, the proportion of [^{3}H] arachidonate-labeled phosphatidic acid was also signifi-

cantly increased as compared with that found in the corresponding unstimulated cells (**Fig. 1**). However, the ConA-induced increase in radiolabeled phosphatidic acid tended to be lower in docosahexaenoic and eicosapentaenoic acid-treated than in control cells, mainly due to a higher basal proportion of radiolabeled phosphatidic acid in fatty acid-treated cells, whatever the duration of ConA activation. Thus after 30 min incubation, ConA increased the level of radiolabeled phosphatidic acid by 5.24-fold in control cells and by 3.77- and 3.56-fold in DHA- and EPA-treated cells, respectively.

As we have previously reported (22), ConA activation of control cells was also accompanied by a huge increase in the mass of phosphatidic acid whatever the duration of the activation period (**Fig. 2**). One hour incubation of cells with docosahexaenoic acid led to a doubling of the PA mass in the absence of mitogen

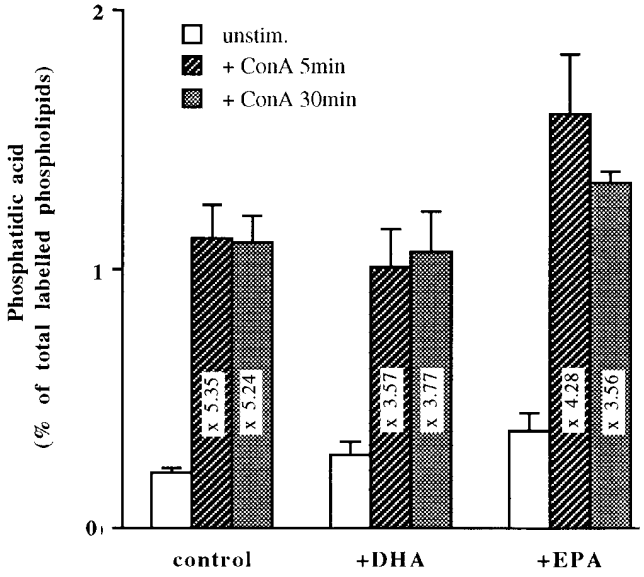

Fig. 1. Influence of docosahexaenoic and eicosapentaenoic acids on the basal and ConA-induced production of radiolabeled phophatidic acid. [^{3}H]arachidonate-labeled human PBMC were preincubated for 1 h at 37°C either in the presence of 5 μm HSA alone (control) or in the presence of 5 μm docosahexaenoic (+DHA) or 5 μM eicosapentaenoic (+EPA) acid bound to HSA (fatty acid to albumin ratio = 1). Cells were further incubated at 37°C in the absence (unstim.) or presence of ConA (5 μg/10^6 cells) for 5 min (+ConA 5 min) or 30 min (+ConA 30 min). At the end of the incubation period, lipids were extracted from cells plus medium and the lipid extracts were separated by TLC as described in Materials and Methods. Results are expressed relative to the radioactivity incorporated in total phospholipids and are means ± SE of six separate experiments for control cells and of four and three separate experiments for DHA- and EPA-treated cells, respectively. Data were analyzed by ANOVA. Stimulated levels were significantly different from unstimulated ($P < 0.05$) whatever the treatment used.

whereas eicosapentaenoic acid treatment had no significant effect. Unexpectedly, both fatty acids markedly impaired the ConA-induced increase in the PA mass. In stimulated cells, docosahexaenoic acid significantly reduced the mass of PA by 44 and 32%, after 5 and 30 min of ConA activation, respectively. The lowering effect of eicosapentaenoic acid was significant only after 30 min of ConA activation (−37%). Thus, the first set of experiments clearly indicated that both docosahexaenoic and eicosapentaenoic acids were able to modulate early signalling events involved in the mitogenic process leading to the synthesis of phosphatidic acid.

Time-course of [^{3}H]docosahexaenoate incorportation in the different lipid classes of human PBMC

To get further insights into the possible mechanisms involved, we next examined the uptake of 5 μm [^{3}H] docosahexaenoic acid bound to 5 μm HSA into the different lipid classes as a function of time. Human PBMC very efficiently incorporated [^{3}H]DHA, 54% of the amount present in the culture medium being already taken up as early as from 30 min incubation (**Fig. 3**). Further incubation slightly increased the amount of [^{3}H]DHA taken up by the cells up to 70% after 150 min incubation. Under the same experimental conditions, [^{3}H]arachidonic acid was incorporated at a similar rate. Most radiolabeled docosahexaenoic acid was incorporated into cell phospholipids and to a lesser extent in triacylglycerols and sterol esters (**Fig. 4A**). The radiolabel associated with diacylglycerol did not exceed 1% of total (not shown). Docosahexaenoic acid incorporation into triacylglycerols and sterol esters was maximal at the earliest time-point studied (30 min) and did not vary significantly further. In contrast, [^{3}H]DHA incorporation into total phospholipids (Fig. 4A), mainly PC and PE (Fig. 4B) continued to increase with time, in close parallel to the cellular uptake. However, when considering the distribution of the radiolabel among the various phospholipid classes, total phospholipid radioactivity being taken as 100 at each time point, no drastic variation was observed during the considered period of time. Thus, the radiolabel associated with PC, PE, and PI/PS varied from 63, 31 and 6%, respectively, at 30 min to 68, 29, and 3% at 150 min.

In subsequent experiments, PBMC were preincubated with 5 μm [^{3}H]DHA for 60 min and then stimulated or not with ConA for the following 30 min. The mitogenic activation of [^{3}H]DHA-radiolabeled cells induced a significant remodeling of the radioactivity associated with each phospholipid class. First, ConA induced a doubling of the radioactivity associated with PA, indicating the formation of PA molecular species containing docosahexaenoic acid (**Fig. 5A**). Whereas in [^{3}H]arachidonate labeled cells, ConA activation repro-

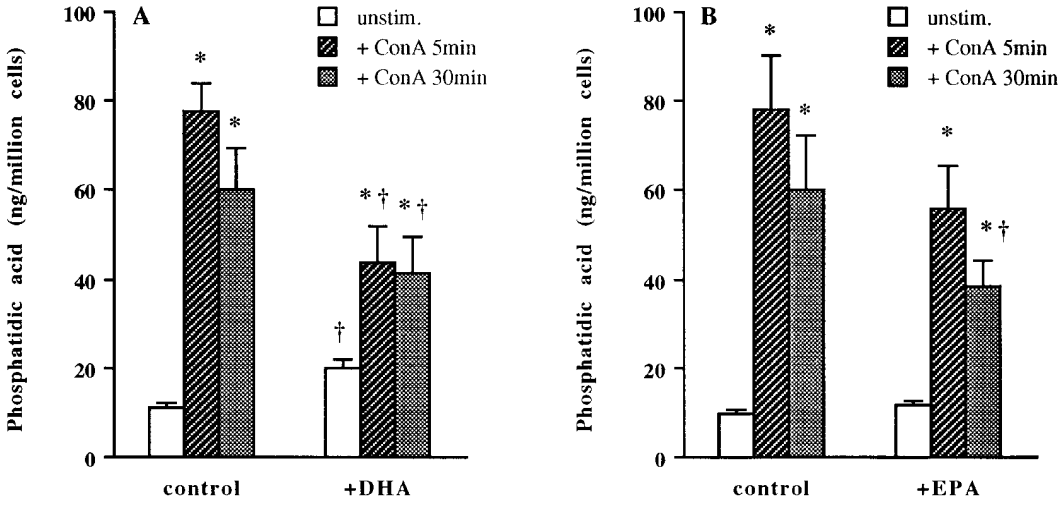

Fig. 2. Influence of docosahexaenoic (A) and eicosapentaenoic (B) acids on the basal and ConA-induced production of phosphatidic acid mass. PBMC were preincubated as described in the legend of Fig. 1. Cells were further incubated at 37°C in the absence (unstim.) or presence of ConA (5 μg/10^6 cells) for 5 min (+ConA 5 min) or 30 min (+ConA 30 min). At the end of the incubation period, lipids were extracted from cells plus medium and the lipid extracts were separated by TLC. Phosphatidic acid mass was quantitated by videodensitometry as described in Materials and Methods. Results are expressed as ng/10^6 cells and are means ± SE of six separate experiments for unstimulated and 30 min ConA-stimulated cells and of four separate experiments for 5 min ConA-stimulated cells. Data were analyzed by ANOVA and the level of statistical significance of the differences was evaluated by means of the Fisher PLSD test. *, different from unstimulated in each group at $P < 0.05$; †, different from the corresponding group in control, $P < 0.05$.

ducibly induced a decrease in the radioactivity associated with PI/PS (22, 32) with no change in the radioactivity of PC, an opposite pattern was observed in [^{3}H]DHA-labeled cells. Indeed, in [^{3}H]DHA-labeled cells, a modest but highly significant ($P < 0.001$) decrease of the radiolabel associated with PC (Fig. 5B) was accompanied by an increased radioactivity in PI/PS (Fig. 5A) and PE (Fig. 5B). Due to the prominent PC compartment, the decreased radioactivity of PC cannot be entirely explained by exchange of docosahexaenoate with the PE compartment by transacylation reactions. The decreased DHA-labeled PC, after ConA stimulation, also suggests that PC might be a precursor of phosphatidic acid through phospholipase D activation.

Phospholipase D activation upon ConA stimulation of docosahexaenoate-treated cells

To test the hypothesis that docosahexaenoate enrichment of the cells might prime phospholipase D activity, control and DHA-treated cells were stimulated by ConA either in the presence or absence of a primary alcohol (**Fig. 6A**). In good agreement with our previous results (22), the addition of ethanol during ConA stimulation of control cells did not alter the ConA-induced increase in PA mass. In contrast, in DHA-treated cells, ethanol significantly lowered (−36%, $P = 0.03$) the mass of PA produced during ConA activation. Because the transphosphatidylation activity of PLD produces phosphatidylalcohol at the expense of PA, these results clearly indicate an activation of phospholipase D in DHA-treated cells. A clear confirmation of PLD activation was brought about by experiments measuring the formation of phosphatidylbutanol in the presence of the primary alcohol 1-butanol (Fig. 6B). In these experiments, cells were first labeled with a tracer amount of [^{3}H]arachidonate, then preincubated in the presence of HSA alone (controls) or in the presence of 5 μm unlabeled docosahexaenoate bound to HSA for 1 h and further stimulated by ConA. A substantial amount of radiolabeled phosphatidylbutanol was measurable only in DHA-treated cells stimulated by ConA.

Influence of docosahexaenoic and eicosapentaenoic acids on the basal and ConA-induced production of diacylglycerol

The whole results obtained in this first set of experiments showed that the treatment of human PBMC by docosahexaenoate primed phospholipase D which became activated upon ConA stimulation, whereas no evidence of PLD activation by ConA alone was obtained in control cells. Paradoxically, this PLD activation resulted in a lowering of the PA mass, suggesting an increased

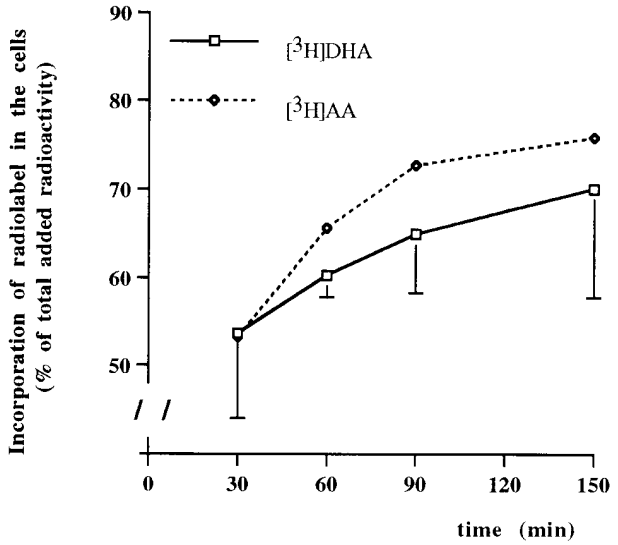

Fig. 3. Incorporation of [^{3}H]docosahexaenoate in human PBMC as a function of time. PBMC suspended in RPMI medium at a concentration of 20 × 10^6 cells/ml were incubated for the indicated period of time in the presence of 5 μm [^{3}H]DHA bound to HSA (fatty acid to albumin ratio = 1). At the end of the incubation period cells were pelleted, washed three times, and the radioactivity associated with the washed pelleted cells was determined by liquid scintillation counting. Results are expressed as percent of the total radioactivity added to the cells and are means ± SE of three separate experiments (solid line). For comparison, data obtained from one representative experiment with 5 μm [^{3}H]arachidonate are indicated (dash line).

catabolism of the newly synthesized PA either through the phosphatidate phosphohydrolase pathway or through phospholipase A_2 hydrolysis. To test the former hypothesis, DAG levels were measured in both control and DHA-treated cells stimulated or not by ConA. As shown in **Fig. 7A**, the relative amount of radiolabeled diacylglycerol was substantially increased in DHA-treated cells as compared with control cells preincubated with HSA alone. Interestingly, no variation in radiolabeled diacylglycerol was observed in cells preincubated with eicosapentaenoic acid. Furthermore, an increase of diacylglycerol mass was only observed in DHA-treated cells further stimulated with ConA (Fig. 7B). As in human PBMC, the mass of diacylglycerol is about 5- to 10-fold higher than that of phosphatidic acid; the 57% increase of diacylglycerol mass observed in DHA-treated cells stimulated by ConA could entirely explain the concomitant decrease of phosphatidic acid mass measured in the same cells. To support further the hypothesis that the increased diacylglycerol mass observed in DHA-treated cells stimulated by ConA might be due to PLD activtion, with a subsequent conversion of phosphatidic acid to diacylglycerol through phosphatidate phosphohydrolase, diacylglycerol was measured in DHA-treated cells stimulated by ConA in the presence of 1% ethanol. Results shown in **Table 1** indicate that the ConA-induced level of diacylglycerol was lowered close to basal level in the presence of ethanol, and thus clearly confirm the above hypothesis.

DISCUSSION

Despite a growing body of evidence in dietary studies showing that n–3 polyunsaturated fatty acids have immunosuppressive effects both in animals and humans, and in vitro when added to lymphocyte culture me-

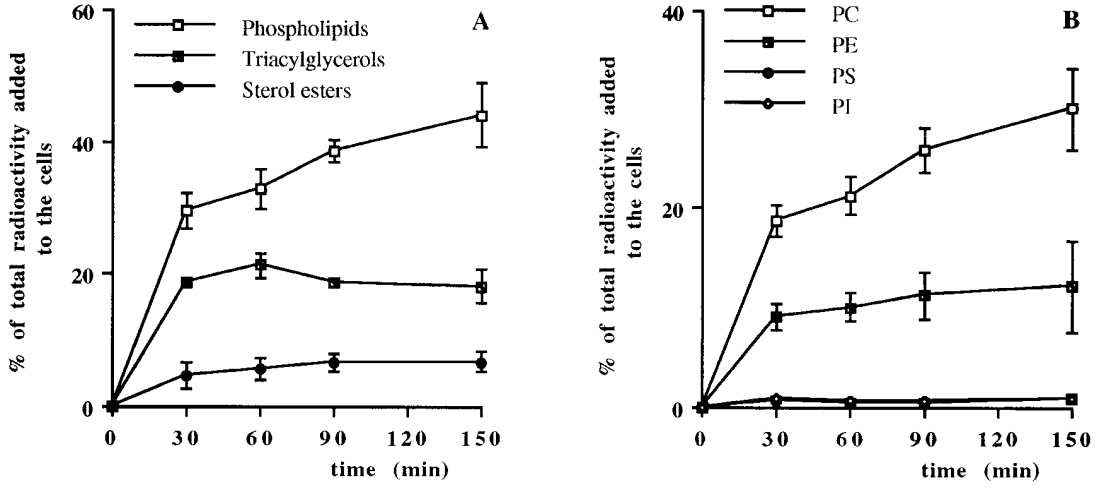

Fig. 4. Incorporation of [^{3}H]docosahexaenoate in the different lipid (A) and phospholipid (B) classes of PBMC as a function of time. PBMC suspended in RPMI medium at a concentration of 20 × 10^6 cells/ml were incubated for the indicated period of time in the presence of 5 μM [^{3}H]DHA bound to HSA (fatty acid to albumin ratio = 1). At the end of the incubation period, lipids were extracted from the washed cells and the lipid extracts were separated by TLC. Results are expressed as percent of the total radioactivity added to the cells and are means ± SE of three separate experiments.

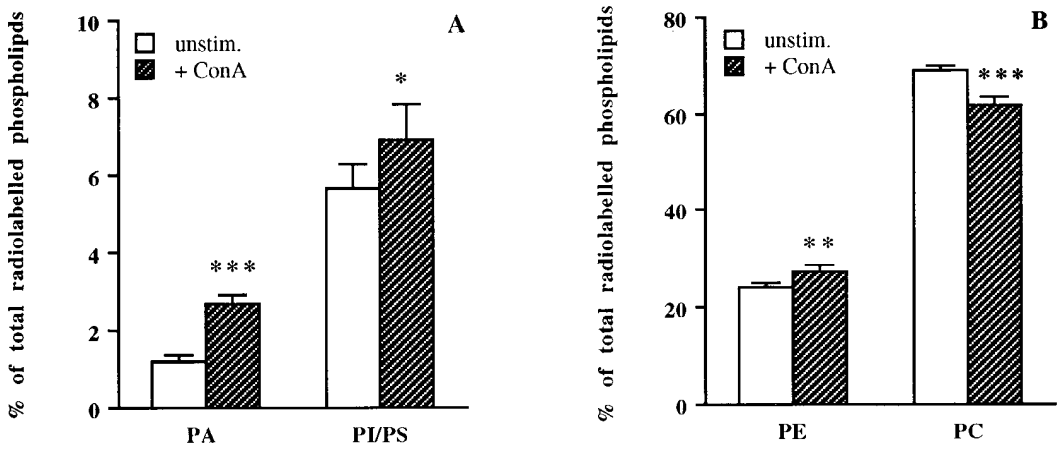

Fig. 5. Influence of ConA stimulation on the distribution of [^{3}H]docosahexaenoate radiolabel in the different phospholipid classes. PBMC were preincubated with 5 μm [^{3}H]DHA bound to HSA (fatty acid to albumin ratio = 1) for 1 h at 37°C and then stimulated by 5 μg ConA/10^6 cells for 30 min (+ConA) or unstimulated (unstim.). At the end of the incubation period, lipids were extracted from cells plus medium and the lipid extracts were separated by TLC. Results are expressed as percent of the radioactivity associated to total phospholipids and are means ± SE of four separate experiments. For each phospholipid class, means are compared by the paired Student's *t* test: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

dium, the mechanisms involved in the modulation of the lymphoproliferative response by fatty acids remain largely unknown. Results from the present study clearly show that both docosahexaenoic and eicosapentaenoic acids, the main polyunsaturated fatty acids from fish oil, are able to affect the early steps of the mitogenic activation of lymphocytes and the production of two crucial lipid second messengers, phosphatidic acid and di-

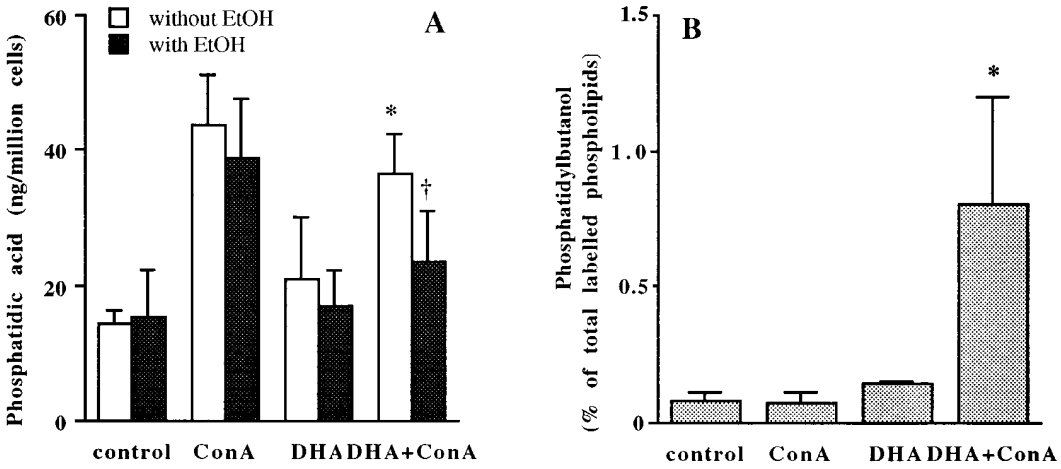

Fig. 6. Phospholipase D activation upon ConA stimulation of docosahexaenoate-treated cells. A: PBMC were preincubated either in the presence of 5 μm HSA alone (control, ConA) or in the presence of 5 μm DHA bound to HSA (DHA, DHA + ConA). Cells were then stimulated with 5 μg ConA/10^6 cells for 30 min (ConA, DHA + ConA) or unstimulated (control, DHA) in the presence (closed bars) or absence (open bars) of 1% ethanol. At the end of the incubation period, lipids were extracted from cells plus medium and the lipid extracts were separated by TLC. Phosphatidic acid mass was quantitated by videodensitometry as described in Materials and Methods. Results are expressed as ng/10^6 cells and are means ± SE of four separate experiments. Means were compared by the paired Student's *t* test; * indicates a significant difference between ConA and DHA + ConA without ethanol, $P < 0.05$; † indicates a significant difference in the DHA + ConA group with and without ethanol, $P < 0.03$. B: PBMC prelabeled with tracer amount of [^{3}H]arachidonate were preincubated either in the presence of 5 μm HSA alone (control, ConA) or 5 μm DHA bound to HSA (DHA, DHA + ConA). Cells were then stimulated by 5 μg ConA/10^6 cells for 30 min (ConA, DHA + ConA) or unstimulated (control, DHA) in the presence of 1% 1-butanol. At the end of the incubation period, lipids were extracted from cells plus medium and the lipid extracts were separated by TLC. The radioactivity associated to phophatidylbutanol was measured by liquid scintillation counting. Results are expressed relative to the radioactivity incorporated in total phospholipids and are means ± SE of three separate experiments. Data were analyzed by ANOVA and the level of statistical significance of the differences was evaluated by means of the Fisher PLSD test. *, different from the other groups at $P < 0.05$.

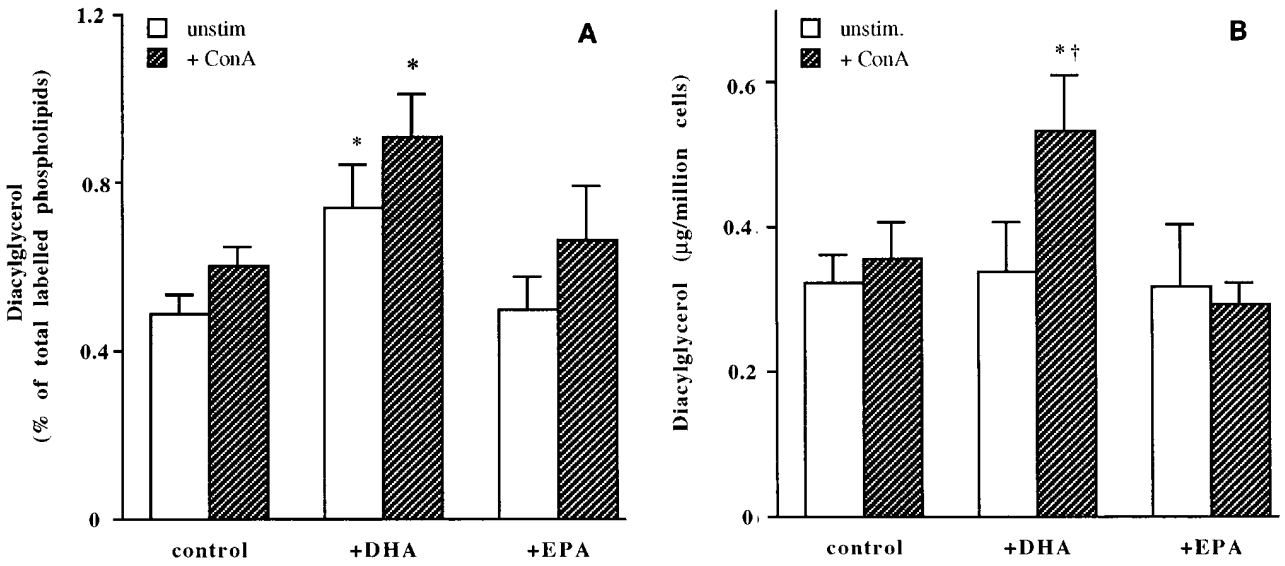

Fig. 7. Influence of docosahexaenoic and eicosapentaenoic acids on the basal and ConA-induced production of radiolabeled DAG (A) and DAG mass (B). A: Human PBMC were prelabeled with tracer amount of [^{3}H]DHA for 1 h 37°C. Cells were then preincubated either in the presence of 5 μm HSA alone (control) or 5 μm docosahexaenoic (+DHA) or 5 μm eicosapentaenoic (+EPA) acids bound to HSA (fatty acid to albumin ratio = 1). Cells were further incubated at 37°C in the absence (unstim.) or presence of ConA (5 μg/10^6 cells) for 30 min (+ConA). At the end of the incubation period, lipids were extracted from cells plus medium and the lipid extracts were separated by TLC, as described in Materials and Methods. Results are expressed relative to the radioactivity incorporated in total phospholipids and are means ± SE of five separate experiments for control and DHA-treated cells and of three experiments for EPA-treated cells. Data were analyzed by ANOVA and the level of statistical significance of the differences was evaluated by means of the Fisher PLSD test: *, different from unstimulated control at $P < 0.05$. B: DAG mass was quantitated by videodensitometry as described in Materials and Methods. Results are expressed as μg/10^6 cells and are means ± SE of five separate experiments for control and DHA-treated cells and of three experiments for EPA-treated cells. Data were analyzed by ANOVA and the level of statistical significance of the difference was evaluated by means of the Fisher PLSD test: * different from unstimulated control at $P < 0.05$; † different from unstimulated in the DHA group at $P < 0.05$.

acylglycerol. This alteration of the mitogenic signal transduction pathway might well explain, in turn, the inhibition of the lymphoproliferative response that we have previously observed with both fatty acids (13). Most in vitro studies measuring the inhibition of the lymphoproliferative responses (11, 33) or aiming to delineate the potential targets of fatty acids (19, 20) were carried out with fatty acid concentrations higher than 30 μm. In the present study, we have chosen a final fatty acid concentration of 5 μm with a fatty acid to albumin ratio of 1, close to that observed under usual human physiological conditions (34) and sufficient to give a measurable enrichment of membrane phospholipids in the considered fatty acid (13). The presence of DHA in cell phospholipids was confirmed in the present study by results of the experiments performed with [^{3}H]DHA. Radiolabeled DHA was incorporated not only in the two main phospholipid compartments, PC and PE, but also in phosphatidic acid which has important signalling functions in lymphocyte activation. Furthermore, the mitogenic activation of DHA-enriched cells significantly increased the relative amount of DHA-labeled PA, suggesting the formation of peculiar PA molecular species containing DHA. This might alter, in turn, the transduction of the mitogenic signal.

Whereas both docosahexaenoic and eicosapentaenoic acids significantly reduced the phosphatidic acid mass in ConA-stimulated mononuclear cells, only docosahexaenoic acid was able to significantly increase its basal level in resting cells. This first observation suggested that docosahexaenoate might stimulate phosphatidic synthesis through PLD activation, in the absence of a mitogenic stimulus. However, the subsequent experiments performed in the presence of a primary alcohol (Fig. 6) showed that alcohol did not modify the synthesis of phosphatidic acid in resting cells treated with DHA alone (Fig. 6B). Unexpectedly, because of the decreased phosphatidic acid level in DHA-treated and ConA-stimulated calls, a large production of phosphatidylalcohol wa observed in DHA-enriched cells stimulated further by ConA. Thus, the activation of phospholipase D, unambiguously demonstrated by the formation of phosphatidylalcohol (35), requires the simultaneous presence of DHA and ConA. These results

TABLE 1. Influence of ethanol on the ConA-induced production of diacylglycerol in docosahexaenoate-treated PBMC

Docosahexaenoate-Treated Cells	Diacylglycerol
	μg/10⁶ cells
Without addition	0.337 ± 0.012
+ConA	0.484 ± 0.066
+ConA +ethanol	0.316 ± 0.041[a]

Human peripheral blood mononuclear cells (PBMC) were preincubated for 1 h at 37°C in the presence of 5 μm DHA bound to HSA (fatty acid to albumin ratio = 1). Cells were further incubated at 37°C in the absence (without addition) or presence of ConA (5 μg/10^6 cells) for 30 min either in the absence (+ConA) or presence of 1% ethanol (+ConA + ethanol). At the end of the incubation period, lipids were extracted from cells plus medium and the lipid extracts were separated by TLC. Diacylglycerol mass was quantitated by videodensitometry as described in Materials and Methods. Results are expressed as μg/10^6 cells and are means ± SE of three separate experiments. Data were analyzed by ANOVA and the level of statistical significance of the differences was evaluated by means of the Fisher PLSD test.

[a]Different from the stimulated level measured in the absence of ethanol at $P < 0.05$.

are reminiscent of those we have previously obtained with 12(S)-hydroxyeicosatetraenoic acid (22) although in the latter case, phosphatidic acid mass was significantly enhanced above the ConA-induced level. The decreased phosphatidate level in spite of phospholipase D activation suggests that, in DHA-treated cells, the mitogenic activation also induced a rapid catabolism of the newly formed phosphatidate. Several studies have reported a decreased phosphatidate intracellular level resulting from a shift of the cellular balance between active synthesis and degradation. Thus, in Jurkat T cells, the immunosuppressive antibody K20 directed against the CD29 antigen lowered phosphatidate level through an accelerated catabolism evidenced by an increased phosphatidylserine synthesis (36). Chronic EGF stimulation of NIH3T3 fibroblasts, which strongly activated diacylglycerol kinase, only induced a mild increase of phosphatidate level due to a concomitant strong activation of phosphatidate phosphohydrolase (37). Several evidences indicate that the mitogenic activation of DHA-treated cells also stimulated phosphatidate phosphohydrolase activity. First, in DHA-treated and ConA-stimulated cells where phosphatidate level was shown to decrease, diacylglycerol level was actually higher than in the other groups of cells (Fig. 7B). Second, the impairment of phosphatidate synthesis through the use of a primary alcohol (Fig. 6A, column DHA + ConA) totally suppressed the DHA plus ConA-induced increase of diacylglycerol mass. Surprisingly, however, DHA was unable to lower [^{3}H]AA-labeled PA while strongly decreasing the PA mass in ConA-stimulated cells. Several hypotheses may be put forward to explain this apparent discrepancy. It is well known that phosphatidylcholine has a lower content of arachidonate than phosphoinositides. Thus, in the presence of ConA, DHA that stimulated PC hydrolysis through PLD activation presumably led to the formation of PA containing a relatively low amount of arachidonic acid. On the other hand, in cells stimulated by ConA alone, PA was almost exclusively derived from phosphoinositides through PIP_2 hydrolysis and phosphorylation of the resulting DAG by DAG kinase. In DHA-treated cells stimulated by ConA, both pathways are very likely to coexist. Thus, one may speculate that PI-derived and PC-derived PA are produced in separate compartments of the lipid bilayer so that only the PC-derived PA could be in contact with phosphatidate phosphohydrolase and used as a substrate. This might lead to a relative conservation of [^{3}H]AA-containing PA species and explain the apparent lack of DHA effect on [^{3}H]AA-labeled PA in ConA-stimulated cells. Alternatively, PA hydrolase might have some substrate preference for PA molecular species with a low or medium degree of unsaturation as those derived from PC. Some substrate preference has already been demonstrated in rat liver for one form of phosphatidate phosphohydrolase located at the plasma membrane, named PAP2A (38, 39). This enzyme, Mg-independent and NEM-insensitive, was found to be less active toward arachidonoyl–stearoyl–phosphatidate (the most abundant PI-derived PA species) than toward dipalmitoyl-, distearoyl-, and dioleoyl-phosphatidate (38). Whether such a form of phosphatidate phosphohydrolase is present in human PBMC is not known and deserves further investigation.

On the other hand, eicosapentaenoic acid, which also impaired the ConA-induced increase of phosphatidate level, had no significant effect on diacylglycerol production. These results indicate that unlike DHA, EPA has no effect on phosphatidate phosphohydrolase. Although several hypotheses can be put forward such as increased phospholipase A_2 activity or increased de novo phospholipid synthesis, the mechanism of eicosapentaenoate action remains to be elucidated. Results of the present study are in good agreement with those of Sebaldt and Marignani (40) who have reported an increased total diacylglycerol mass in both basal and stimulated macrophages after in vitro supplementation of culture media with docosahexaenoate. However, these authors had also observed an increasing effect for eicosapentaenoate, although lower than that of docosahexaenoate. This discrepancy concerning eicosapentaenoate effect on diacylglycerol level might be explained by the different cellular model used (macrophages vs. lymphocytes) or by the different species used (murine vs. human). In contrast, opposite effects of docosahexaenoate and eicosapentaenoate on diacylglycerol production have been reported in dietary studies in mice

(41). In this study, diacylglycerol production was significantly suppressed in EPA- and DHA-fed mice relative to mice fed saturated fatty acids or arachidonic acid.

Presently, the putative mechanisms involved in the priming effect of docosahexaenoate on phospholipase D and phosphatidate phosphohydrolase activities remain largely speculative. It is well known that polyunsaturated fatty acids may affect protein expression and regulate gene transcription, especially the transcription of specific lipogenic genes (42). In fibroblasts, arachidonate has been shown to stimulate the expression of early response genes such as *C-fos* and Erg-1, but this stimulating effect seemed to be related to its conversion to PGE_2, and n–3 fatty acids that reduced the formation of PGE2 in these cells also reduced the activating effect of arachidonate (43). Another important question concerns the nature of the phospholipase D primed by docosahexaenoate in activated lymphocytes. A fatty acid activatable phospholipase D has been described in membrane fractions from several tissues including rat brain (44, 45), liver (46), and heart (47), and pig lung (48). However, stimulation of this phospholipase D activity, usually described as oleate-sensitive, requires millimolar concentrations of unsaturated fatty acids. Thus, docosahexaenoate that was active at micromolar concentrations on intact human lymphocytes might stimulate the other family of phospholipase D activated by small G proteins (45). Additional experiments are needed to characterize further the phospholipase D target of docosahexaenoic acid.

The stimulating effect of DHA on phospholipase D and phosphatidate phosphohydrolase activities might involve either a direct interaction between the unesterified fatty acid and the target enzymes of modification of the enzyme environment through alterations of the lipid bilayer. Whatever the exact mechanism, the overall effect of DHA in activated lymphocytes results in an increased diacylglycerol production at the expense of phosphatidate cellular level, which might explain its immunosuppressive effect.

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