n-3 PUFAs modulate T-cell activation via protein kinase C-α and -ε and the NF-κB signaling pathway

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Abstract **We elucidated the mechanisms of action of two n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in Jurkat T-cells. Both DHA and EPA were principally incorporated into phospholipids in the following order: phosphatidylcholine < phosphatidylethanolamine < phosphatidylinositol/phosphatidylserine. Furthermore, two isoforms of phospholipase A_2 (i.e., calcium-dependent and calcium-independent) were implicated in the release of DHA and EPA, respectively, during activation of these cells. The two fatty acids inhibited the phorbol 12-myristate 13-acetate (PMA)-induced plasma membrane translocation of protein kinase C (PKC)-α and -ε. The two n-3 PUFAs also inhibited the nuclear translocation of nuclear factor κB (NF-κB) and the transcription of the interleukin-2 (IL-2) gene in PMA-activated Jurkat T-cells. Together, these results demonstrate that DHA and EPA, being released by two isoforms of phospholipase A_2, modulate IL-2 gene expression by exerting their action on two PKC isoforms and NF-κB in Jurkat T-cells.—** Denys, A., A. Hichami, and N. A. Khan. **n-3 PUFAs modulate T-cell activation via protein kinase C-α and -ε and the NF-κB signaling pathway. *J. Lipid Res.* 2005.** 46: **752–758.**

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Several studies have shown beneficial effects of n-3 PUFAs in rheumatoid arthritis (1), cardiovascular diseases (2), and diabetes (3). It has been shown that diets enriched with n-6 PUFAs stimulate the growth of different cell types and promote metastasis, whereas diets containing fish oil rich in n-3 PUFAs inhibit cell growth and, consequently, exert curative effects in autoimmune diseases (4). Hence, it has been suggested that diets enriched with eicosapentaenoic acid (EPA; 20:5, n-3) or/and docosahexaenoic acid (DHA; 22:6, n-3) exert their effects by substituting arachidonic acid (20:4, n-6) in plasma membrane phospholipids (5). Additionally, several authors have reported that n-3 PUFAs modulate T-cell functions such as T-cell proliferation and cytokine secretion (6, 7). n-3 PUFAs may exert their action by interfering with T-cell signaling. T-cell activation has been divided into early and late events. One of the early events during T-cell stimulation, via the T-cell receptor, is the activation of several protein tyrosine kinases, leading to the phosphorylation of mitogen-activated protein kinases (MAPKs) via protein kinase C (PKC)-dependent and PKC-independent pathways (8, 9). During the last decade, it has been shown that MAPKs, like extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2), which belong to the serine/threonine protein kinase family, are involved in cell proliferation and differentiation (10, 11). Nuclear factor κB (NF-κB), first discovered in the nuclei of mature B cells, seems to be a substrate of ERK1/ERK2 (12). In unstimulated cells, NF-κB is sequestered, in inactive form, in the cytosol by the action of inhibitor κB (I-κB) (13). After exposure of T-cells to mitogens such as phorbol 12-myristate 13-acetate (PMA), NF-κB is unmasked as a result of the sequential phosphorylation and degradation of I-κB (14). The free NF-κB is translocated to the nucleus, where it activates interleukin-2 (IL-2) gene transcription by binding to the NF-κB binding site present on the IL-2 promoter. It has been demonstrated that the MAPKK-kinase (Raf)/MAPK kinase (MEK)/ERK1/ERK2 pathway acts on NF-κB activation (15, 16). We have shown that PMA-induced activation of ERK1/ERK2 is suppressed by n-3 PUFAs in Jurkat T-cells (17–19) as well as in fibroblast NIH 3T3 cells (20). However, it remains to be ascertained whether inhibitory actions of n-3 fatty acids on ERK1/ERK2 phosphorylation are attributable to the inhibition of PKC and NF-κB translocation in human T-cells.

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Abbreviations: AACOCF3, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone; BpB, 4-bromo-phenacyl-bromide; $cPLA_2$, cytosolic phospholipase A_2; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; I-κB, inhibitor κB; IL-2, interleukin-2; $iPLA_2$, calcium-independent phospholipase A_2; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI/PS, phosphatidylinositol/phosphatidylserine; PKC, protein kinase C; PLA_2, phospholipase A_2; PMA, phorbol 12-myristate 13-acetate; $sPLA_2$, secreted phospholipase A_2.

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Phospholipase A_2 (PLA_2) belongs to a family of isoenzymes known essentially for their capacity to release fatty acids from the *sn*-2 position of plasma membrane phospholipids. Several isoforms of PLA_2 have been identified in Jurkat T-cells: *i*) secreted PLA_2 ($sPLA_2$), among which are pancreatic type IB and type V; and *ii*) cytosolic PLA_2 ($cPLA_2$), including calcium-dependent type IV and calcium-independent PLA_2 ($iPLA_2$) type VI (21). Tessier, Hichami, and Khan (22) have shown that three isoforms of PLA_2 (i.e., types IB, V, and VI) are involved in T-cell proliferation. However, no study is available on the role of different isoforms of PLA_2 in the release of n-3 fatty acids in human T-cells.

Keeping in view the paucity of information on the release of n-3 fatty acids and their subsequent action on PKC and NF-κB translocation, it was thought worthwhile to investigate the involvement of different isoforms of PLA_2 in the release of DHA and EPA and their effects on PKC and NF-κB translocation in human Jurkat T-cells.

MATERIALS AND METHODS

Chemicals

Anti-I-κBα antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-α-tubulin antibodies were from Calbiochem. PMA, DHA, and EPA were procured from Sigma. PLA_2 inhibitors, arachidonyl trifluoromethyl ketone (AACOCF3), and bromoenol lactone (BEL) were from Cayman Chemical (Ann Arbor, MI). Aristolochic acid was from Sigma-Aldrich (Saint Quentin Fallavier, France). [^{14}C]DHA (53 mCi/mmol) was purchased from New England Nuclear (Boston, MA), and [^{3}H]EPA (100–200 Ci/mmol) was obtained from ICN Biomedicals (Orsay, France). SuperScript II Reverse Transcriptase, Platinum *taq* DNA Polymerase, and primers were purchased from Invitrogen Life Technologies (Cergy Pontoise, France). Agarose and T4 polynucleotide kinase were from Promega (Charbonnière, France).

Cell culture

Jurkat T-cells were routinely cultured in RPMI 1640 medium supplemented with L-glutamine and 10% fetal calf serum at 37°C in a humidified chamber containing 95% air and 5% CO_2. Cell viability was assessed by trypan blue exclusion. Cell numbers were determined by hemocytometer.

Incorporation of DHA and EPA into phospholipids

Jurkat T-cells were serum starved for 6 h and then incubated for 2 h with [^{14}C]DHA or [^{3}H]EPA at 1.5 μCi/3 × 10^8 cells. PUFAs were dissolved in RPMI 1640 serum-free medium supplemented with 0.2% fatty acid-free BSA. At the end of the incubation, total lipids were extracted from Jurkat T-cells according to the method of Bligh and Dyer (23). Phospholipid classes were separated by TLC using silica G60 and the solvent chloroform-methanol-acetic acid (35:14:2.7, v/v/v). Phospholipid classes comigrating with authentic standards were scraped off, and radioactivity was quantified by adding 2 ml of scintillation cocktail in a liquid scintillation analyzer (Packard 1900 TR).

DHA and EPA release

The release of DHA and EPA was determined as described elsewhere (24). In brief, after incubation of Jurkat T-cells with radiolabeled EPA or DHA for 2 h, cells were washed twice with RPMI 1640 serum-free medium containing 0.2% BSA and suspended in 500 μl of RPMI 1640 medium supplemented with 0.5% BSA. Cells were then treated with 15 μM PLA_2 inhibitors or vehicle (dimethyl sulfoxide, 0.1% final concentration) for 30 min followed by a 20 min stimulation with PMA (200 nM) and ionomycin (500 nM). Cells were centrifuged (1,250 *g*, 3 min), and 0.4 ml of supernatant was saved and added to 2 ml of scintillation cocktail to determine radioactivity in a liquid scintillation analyzer (Packard 1900 TR).

Western blot detection of different isoforms of PKC and I-κBα

PUFAs were dissolved in ethanol (0.1%, v/v). Jurkat T-cells were incubated for 6 h in RPMI 1640 medium without serum. Cells (5 × 10^6/ml) were further incubated for 5 min in the presence of EPA or DHA at 20 μM and then stimulated with PMA (200 nM) for 20 min, essentially according to Nel et al. (9). Control cells were treated with vehicle only [final concentration of ethanol did not exceed 0.2% (v/v)]. Cell stimulation was stopped by centrifugation (1,500 *g*, 10 min), and then cells were lysed with buffer containing the following: 7.5 mM Tris-HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 0.25 M sucrose, and 0.5 μl/ml antiprotease cocktail. Cells were sonicated for 15 s at 4°C three times and then centrifuged (500 *g*, 10 min) to remove nuclear and cell debris. The supernatant was used to isolate cytosolic and plasma membrane-enriched fractions by centrifugation (100,000 *g*, 90 min), essentially according to Tsutsumi et al. (25). The plasma membrane and cytosolic fractions were used to detect PKC translocation after protein separation by SDS-PAGE (10%) and transfer onto polyvinylidine difluoride membranes. Later, nonspecific binding sites were blocked by 5% nonfat milk, and immunodetection was performed using anti-PKC antibodies and secondary anti-rabbit antibodies at 1:1,000 dilution. The different isoforms of PKC were visualized by detecting peroxidase activity using the ECL system.

The dissociation of I-κBα and NF-κB in the cytosolic fractions was assessed in Western blotting using mouse monoclonal anti-I-κBα antibodies (1:2,000 dilution) and secondary peroxidase-conjugated anti-mouse antibodies. Peroxidase activity was detected using ECL reagents. The same quantity of protein was subjected to Western blotting and probed by antibodies directed against α-tubulin to ensure equal loading and transfer of protein.

Nuclear extracts and electrophoretic mobility shift assay

Jurkat T-cells were serum starved for 6 h and then either treated with PUFAs for 5 min before PMA stimulation as described for Western blotting or incubated for 2 h in the presence of PUFAs bound to 0.2% BSA to allow their incorporation into plasma membrane phospholipids. At the end of the PUFA treatment, cells were incubated with PLA_2 inhibitors (15 μM) or GF109203X (500 nM) for 30 min before stimulation with PMA for 20 min.

Nuclear extracts were prepared essentially as described by Dignam, Lebovitz, and Roeder (26) with some modifications. After treatment, cells (50 × 10^6) were washed with PBS without calcium and magnesium salts by centrifugation (250 *g*, 10 min) at room temperature. Cell pellets were resuspended in 5 volumes of ice-cold cell homogenization buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 2 μl/ml protease inhibitor cocktail), left on ice for 10 min, and then centrifuged (250 *g*, 10 min) at room temperature. The cell pellets were suspended in 3 volumes of ice-cold cell homogenization buffer containing 0.05% (v/v) Nonidet P-40, then cells were lysed with 20 strokes of a tight-fitting Dounce homogenizer. Nuclei were collected by centrifugation (250 *g*, 10 min) at 4°C. Pellets of nuclei were resuspended first in 300 μl of hypotonic buffer (40 mM HEPES-KOH, pH 7.9, 0.4 M KCl, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, and 2 μl/ml protease inhibitor cocktail), then NaCl was added to a final concentration of 300 mM. The mixture

was left at 4°C for 30 min. After centrifugation (100,000 *g*, 20 min), the supernatant was divided into aliquots of 50 μl and stored at −80°C. The amount of protein was determined with Bradford reagent. The same quantity of protein (6 μg) was incubated with ^{32}P end-labeled DNA fragments containing the NF-κB protein binding site. The sequence of the double-stranded oligonucleotide used for detection of NF-κB was 5′-AGTTGAGGGGACTTTCCCAGG-3′. Oligonucleotides were end-labeled with [α-^{32}P]CTP by T4 polynucleotide kinase. For the binding reaction, 6 μg of nuclear extract was added to a reaction mixture containing 4 μg of poly(dI-dC), 4 μl of binding buffer (6 mM HEPES-KOH, pH 7.9, 120 mM NaCl, 0.4 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM DTT, 150 μM PMSF, and 7% glycerol), and 20,000 dpm of ^{32}P-labeled oligonucleotide in a final volume of 15 μl, and this was incubated at room temperature for 20 min. Unlabeled competitor oligonucleotide was added in a 50-fold excess to confirm the specificity of the binding reaction. The DNA-protein complexes were separated by 4% polyacrylamide nondenaturing gel electrophoresis in 0.5× TBE (45 mM Tris, 1 mM EDTA, and 45 mM boric acid, pH 8.3) running buffer. The gels were dried and exposed to Biomax Light-2 film.

RNA isolation and semiquantitative RT-PCR analysis of IL-2 mRNA

For RT-PCR analysis, Jurkat T-cells were seeded on 24-well plates (1.5–2 × 10^6 cells/well) and incubated for 12 h in RPMI 1640 serum-free medium. PUFAs were added to cells for 5 min, then cells were activated by the addition of PMA (200 nM) for 4 h. At the end of the experiment, cells were centrifuged (1,500 *g*, 10 min) and total RNA was purified from the cell pellet using Trizol® reagent (Invitrogen Life Technologies) as described by Tessier, Hichami, and Khan (22). Total RNA (0.5 μg) was reverse-transcribed using SuperScript II Reverse Transcriptase. At the end of the RT reaction, the cDNA was either used immediately for PCR or stored at −20°C until use. The conditions for PCR amplification have been described elsewhere (22). Reaction products were electrophoresed on a 1% agarose gel containing ethidium bromide. The RNA pattern was visualized by ultraviolet transillumination.

Statistical analysis

Results are shown as means ± SD. Statistical analysis of data was carried out using STATISTICA (version 4.1; Statsoft, Paris, France). The significance of differences between mean values was determined by one-way ANOVA followed by the least significant difference test.

RESULTS

EPA and DHA are incorporated into phospholipids

When Jurkat T-cells are exposed for 2 h to exogenous [^{3}H]EPA or [^{14}C]DHA, they incorporate fatty acids into phospholipids. We observed that the incorporation of DHA was more pronounced in phosphatidylcholine (PC; 56.5 ± 4.7% of total phospholipids) than in phosphatidylethanolamine (PE; 35.4 ± 2.9%) and to a lesser extent in phosphatidylinositol/phosphatidylserine (PI/PS; 7.5 ± 0.8%). The incorporation of EPA was as follows: PC (55 ± 7.2%), PE (25 ± 0.6%), and PI/PS (19.9 ± 0.6%).

EPA and DHA are released by the action of two isotypes of PLA_2

We observed that the release of DHA and EPA was significantly higher in PMA- and ionomycin-stimulated cells than in control (unstimulated) cells (**Fig. 1**). AACOCF3, an inhibitor of type IV $cPLA_2$, significantly decreased [^{14}C]DHA release induced by PMA and ionomycin (Fig. 1). BEL, an $iPLA_2$ inhibitor, significantly inhibited the PMA-plus ionomycin-induced [^{3}H]EPA release. Aristolochic acid, a nonspecific $sPLA_2$ inhibitor (27), and 4-bromo-phenacylbromide (BpB), which selectively inhibits $sPLA_2$ by inducing an alkylation of the His-48 group located close to the active site of this enzyme (28), exerted no significant effect on the release of [^{14}C]DHA and [^{3}H]EPA in these cells.

EPA and DHA inhibit plasma membrane translocation of PKCα and PKCε

As we have previously reported that EPA and DHA curtailed PMA-induced MAPK activation (17–19), we assessed the effect of DHA and EPA in the presence or absence of PMA on translocation of three PKC isoforms (PKCα, PKCδ, and PKCε) from cytosol to plasma membrane. **Figure 2** shows that PMA induced the translocation of PKCα, PKCδ, and PKCε from cytosol to the plasma membrane. In our study, PKCδ appears as a doublet, as found in NIH 3T3 cells (29). Furthermore, DHA alone did not induce the translocation of any isoform of PKC, although EPA induced PKCδ translocation. As illustrated in Fig. 2, EPA and DHA completely inhibited the PMA-induced translocation of PKCα and PKCε but not of PKCδ.

EPA and DHA inhibit NF-κB activation

The NF-κB transcription factor is a heterodimeric complex containing two DNA binding subunits, p50 and RelA, which belong to the Rel family (27). In resting T-cells, NF-

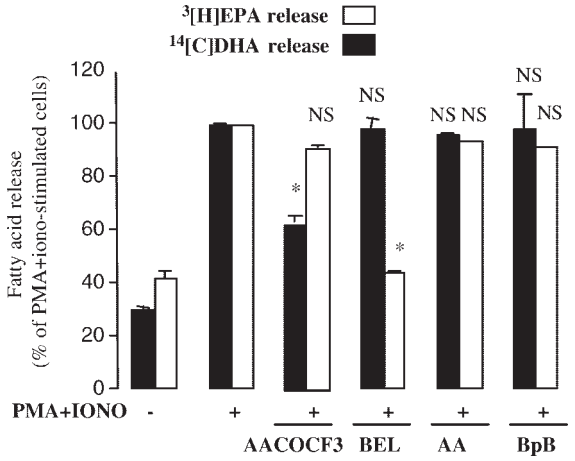

Fig. 1. Effects of phospholipase A_2 (PLA_2) inhibitors on [^{3}H]eicosapentaenoic acid (EPA) and [^{14}C]docosahexaenoic acid (DHA) release induced by phorbol 12-myristate 13-acetate (PMA) and ionomycin. Jurkat T-cells were incubated with [^{3}H]EPA and [^{14}C]DHA. The cells were then treated with 15 μM arachidonyl trifluoromethyl ketone (AACOCF3), aristolochic acid (AA), 4-bromo-phenacyl-bromide (BpB), bromoenol lactone (BEL), or vehicle (DMSO, 0.1% final concentration) for 30 min, followed by 20 min of stimulation with PMA (200 nM) and ionomycin (500 nM) (PMA+IONO). EPA and DHA release was determined as described in Materials and Methods. Results are expressed as means ± SD of three independent experiments. Data are expressed as the percentage of the PMA+IONO-stimulated value, which was considered to be 100%. Asterisks indicate significant differences ($P < 0.001$) compared with the PMA+IONO group. NS, insignificant values.

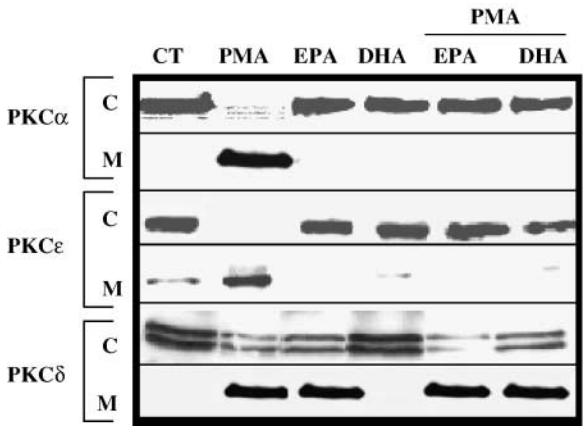

Fig. 2. Effects of EPA and DHA on PMA-induced translocation of protein kinase C (PKC) isoforms. Jurkat T-cells (5×10^6/ml) were incubated for 6 h in RPMI 1640 medium without serum. Later, cells were treated or not [control (CT)] with EPA or DHA at 20 μM for 5 min before stimulation with PMA (200 nM) for 20 min. After incubation at 37°C, cells were lysed and the three isoforms of PKC were detected in plasma membrane (M) and cytosolic (C) fractions by immunoblotting as described in Materials and Methods. The figure shows a representative blot from an experiment that was reproduced at least four times independently.

κB remains cytosolic, as its translocation toward the nucleus is prevented because of the high-affinity association of its RelA subunit with the cytoplasmic inhibitor, I-κBα (12). During T-cell stimulation, I-κBα is rapidly degraded and NF-κB is translocated toward the nucleus, and this phenomenon can be detected by electrophoretic mobility shift assay of nuclear fractions (12).

To assess the effects of EPA and DHA on the nuclear translocation of NF-κB, we stimulated Jurkat T-cells with PMA. EPA and DHA inhibited both the PMA-induced nuclear translocation of NF-κB and I-κBα degradation (**Fig. 3A**, **C**). EPA or DHA alone exerted no significant effect on either NF-κB translocation or I-κBα dissociation.

As we observed that EPA and DHA were released, respectively, by the activation of $iPLA_2$ and $cPLA_2$, we were tempted to assess whether the inhibition of activation of theses phospholipases could block the suppressive effects of EPA and DHA with respect to NF-κB activation. We observed, at first in PUFA-untreated cells, that BEL exerted a weak inhibitory effect, whereas AACOCF3, aristolochic acid, and BpB exerted a moderate inhibitory effect on PMA-induced NF-κB activation (Fig. 3B). Furthermore, enrichment of plasma membrane phospholipids with EPA or DHA significantly inhibited NF-κB activation. It is important to note that AACOCF3 reversed the suppressive effects of DHA, whereas BEL failed to block the inhibitory effect of EPA on PMA-induced NF-κB activation. In fact, PKC activation is implicated in the nuclear translocation of NF-κB, as GF109203X, the PKC inhibitor, curtailed PMA-induced NF-κB activation (Fig. 3B).

EPA and DHA inhibited IL-2 mRNA expression induced by PMA

IL-2, a cytokine that plays a crucial role in T-cell activation and proliferation, is regulated by several transcrip-

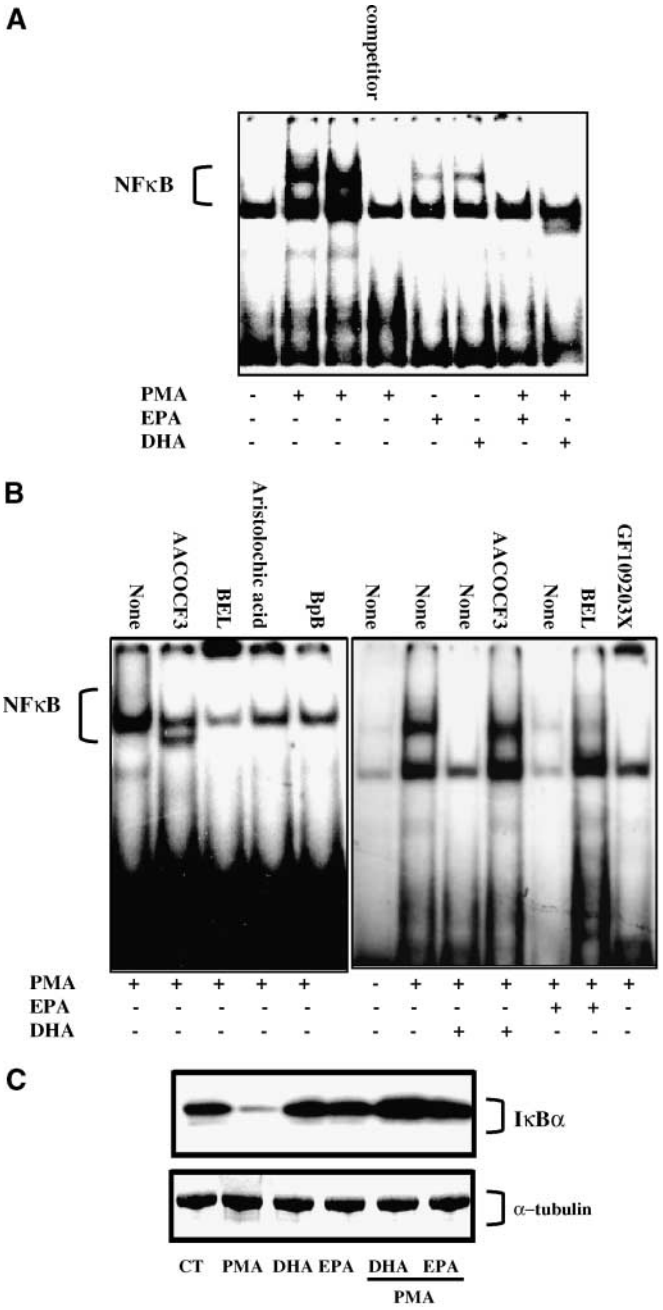

Fig. 3. Effects of EPA and DHA on nuclear factor κB (NF-κB) activation. A, B: Electrophoretic mobility shift assay of NF-κB activation in Jurkat T-cells. Cells were serum-starved for 6 h in RPMI 1640 medium. A: Later, cells were incubated with EPA or DHA for 5 min at 20 μM and then stimulated with PMA for 20 min. B: Cells were incubated for 2 h with EPA or DHA in the presence of BSA (as described for Fig. 1). Control cells were incubated only in the presence of 0.2% BSA. At the end of the incubation, cells were washed and treated for 30 min with PLA_2 inhibitors (each at 15 μM) or GF109203X (500 nM) before stimulation with PMA for 20 min. At the end of the treatments, nuclear extracts were prepared and aliquots (6 μg of each) were combined with the ^{32}P-labeled NF-κB oligonucleotide probe. C: Cells were incubated with EPA or DHA for 5 min at 20 μM and then further stimulated for 20 min with PMA. Later, cells were lysed and proteins were subjected to SDS-PAGE and probed with antibodies directed against inhibitor κB (I-κBα) or α-tubulin to ensure equivalent protein loading. CT, control. The figure shows a representative blot from an experiment that was reproduced at least four times independently.

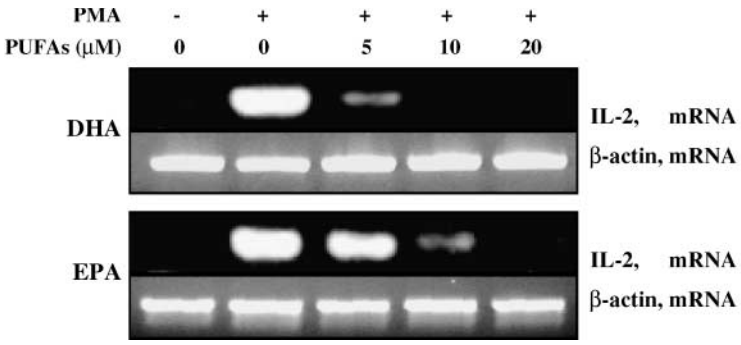

Fig. 4. Effects of EPA and DHA on interleukin-2 (IL-2) mRNA expression. Jurkat T-cells (2×10^5 cells/ml) were treated with DHA or EPA at 5, 10, or 20 μM for 5 min before stimulation with PMA during 4 h as described in Materials and Methods. The figure shows a representative blot from an experiment that was reproduced at least four times independently.

tion factors, including NF-κB. As noted above, EPA and DHA diminished NF-κB nuclear translocation. We wanted to assess the effects of EPA and DHA on the transcription of the IL-2 gene. We observed that these fatty acids dose-dependently inhibited IL-2 mRNA expression induced by PMA (**Fig. 4**).

DISCUSSION

Recently, we demonstrated that in NIH 3T3 fibroblasts and Jurkat T-cells, EPA and DHA inhibited ERK1/ERK2 activation (17–20). The present study was designed to elucidate the molecular mechanisms by which these PUFAs inhibited T-cell activation, especially IL-2 gene expression, when the PKC-dependent signaling pathway was activated.

Free fatty acids are released from phospholipids upon the activation of several PLA_2 isoforms. The nature of the plasma membrane phospholipids generally depends upon the polyunsaturated species, as determined by dietary intake. To investigate the signaling pathways, and to give a physiological relevance to our study, we investigated, at first hand, in which class of phospholipids the exogenous fatty acids were incorporated. We observed that EPA and DHA were incorporated into different classes of phospholipids in the following order: PC > PE > PI/PS. Because we reported that Jurkat T-cells constitutively expressed mRNA of four isoforms of PLA_2 (22, 30) [i.e., two secreted (types IB and V), one cytosolic calcium-dependent (type IV), and one cytosolic calcium-independent (type VI)], we attempted to assess which isoform of PLA_2 was involved in the release of these two fatty acids. PLA_2 activation after T-cell receptor aggregation involves both PKC-dependent and -independent pathways (31). In the present study, we used PMA and ionomycin, the respective activators of PKC- and calcium-dependent pathways (22). We used the $sPLA_2$ inhibitors aristolochic acid and BpB (32). We also used AACOCF3, which is known to be a specific inhibitor of $cPLA_2$ (32). AACOCF3, at high concentrations, may also inhibit $iPLA_2$ (32). BEL, a mechanism-based inhibitor of $iPLA_2$, was also used in our study (33).

We noticed that in T-cells activated by PMA and ionomycin, $cPLA_2$ was partly involved in [^{14}C]DHA release from phospholipids, as AACOCF3 significantly inhibited [^{14}C]DHA release. Because AACOCF3 used at its IC_{50} value (15 μM) failed to completely suppress [^{14}C]DHA release up to the level of unstimulated cells, the involvement of other isoforms of PLA_2 could not be ruled out. The release of [^{3}H]EPA is catalyzed by $iPLA_2$, as BEL inhibited the release of this fatty acid. It is interesting that the inhibitors of $sPLA_2$ (i.e., aristolochic acid and BpB) failed to inhibit the release of these two n-3 PUFAs. Hence, we can assume

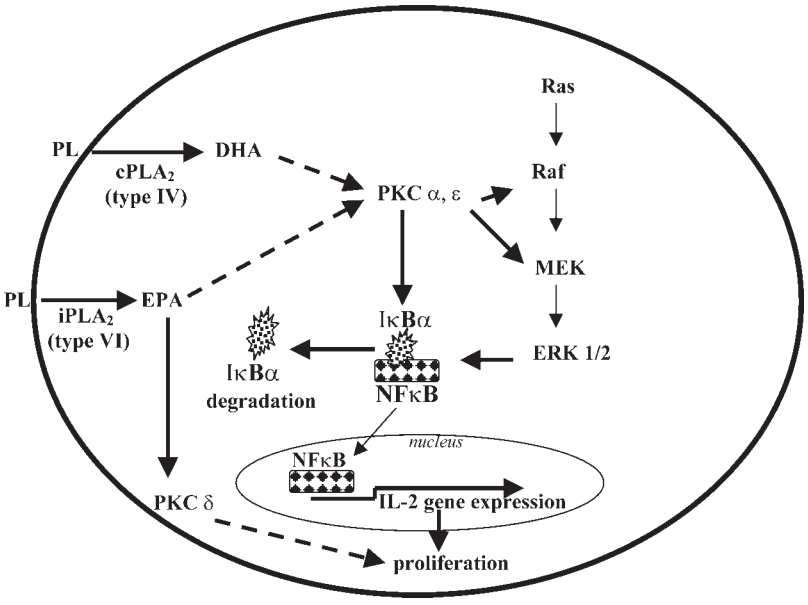

Fig. 5. Schematic representation of the differential effects of EPA and DHA on PKC isoforms and NF-κB activation, leading to the inhibition of IL-2 gene transcription. Continuous and discontinuous arrows show stimulatory and inhibitory actions, respectively. EPA and DHA released by the actions of calcium-independent PLA_2 ($iPLA_2$; type VI) and cytosolic PLA_2 ($cPLA_2$; type IV), respectively, inhibit the translocation of PKCα and PKCε toward the plasma membrane. These PKC isoforms are coupled to mitogen-activated protein kinase activation upstream of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and NF-κB. Inhibition of these PKC isoforms results in the inhibition of nuclear translocation of NF-κB, which is involved in the transcription of the IL-2 gene and, consequently, T-cell proliferation. Simultaneously, EPA induces the translocation of PKCδ, which may also contribute to the immunosuppressive properties of this fatty acid. PL, phospholipids; MEK, MAPK kinase; Ras, Rat sarcoma oncogene, Raf, MAPKK-kinase.

that $iPLA_2$ and $cPLA_2$ may be specific for the respective release of EPA and DHA, as it has been proposed that different isoforms of PLA_2 might be necessary to catalyze the release of different classes of fatty acids (34). This argument is further supported by our previous observations that $iPLA_2$ and $sPLA_2$, but not $cPLA_2$, participate in the release of arachidonic acid from Jurkat T-cells (22, 30).

Because three isoforms of PKC (α, δ, and ε) expressed in Jurkat T-cells have been reported to activate the MAPK pathway (35–37) and PUFAs have been shown to modulate PKC activation (38), we attempted to ascertain the effects of EPA and DHA on the translocation of these PKC isoforms. Hence, only PMA was used for cell stimulation to activate the PKC-dependent pathway. The 20 μM concentration of PUFAs is of physiological relevance because, under pathophysiological conditions, large amounts of free fatty acids may be released; this is the case during cardiac ischemia, during which arachidonic acid concentrations are increased up to 50 μM (39). We observed that PMA induced the translocation of three PKC isoforms from the cytosol to the plasma membrane. EPA and DHA inhibited only the translocation of PKCα and PKCε, but not of PKCδ. These observations are in accordance with our previous study, in which we showed that EPA and DHA inhibited the activation of ERK1/ERK2 and the translocation of PKCα and PKCε in NIH 3T3 cells (20). The action of n-3 PUFAs seems to be dependent on the structure of PKC. The regulatory domains of PKCα and PKCε possess two conserved C1 and C2 regions, whereas PKCδ contains only one C1 region and lacks an authentic C2 region (40, 41). The subcellular localization of PKC partially depends on a second messenger bound to the C domain (42). Hence, we postulate that EPA and DHA could bind to the C2 domain of PKCα and PKCε and, consequently, inhibit their translocation toward the plasma membrane. Although n-3 PUFAs alone had no effect on PKCα, PKCε, and PKCδ translocation, EPA, but not DHA, induced the translocation of PKCδ. This observation emphasizes the differences between DHA and EPA. We hypothesize that the structural differences between EPA and DHA may be responsible for the different effect of the former on PKCδ. In fact, EPA contains 20 carbons and 5 double bonds, whereas DHA contains 22 carbons and 6 double bonds. A plausible explanation for EPA-induced PKCδ translocation and its physiological relevance is not available. However, PKCδ differs from other PKC isoforms not only in its structure (see above) but also in its functional properties (26, 43–46). In NIH 3T3 cells, PKCδ arrests cell growth, whereas other isoforms of PKC stimulate this phenomenon (27, 45). In keeping with these observations, we argue that, as in NIH 3T3 cells, EPA-induced PKCδ translocation may contribute to the immunosuppressive properties of this fatty acid (see below).

Several groups have demonstrated that cell proliferation by PKC activation also induces the activation of NK-κB (47, 48). In Jurkat T-cells, translocation of NK-κB into the nucleus is dependent on the activation of the Raf-1/MEK/ERK1/ERK2 pathway (49). We observed that nuclear translocation NF-κB induced by PMA was PKC-dependent, as this phenomenon was sensitive to the PKC inhibitor GF109203X. Furthermore, EPA and DHA inhibited the nuclear translocation of NF-κB and I-κB degradation in PMA-stimulated cells. The inhibitory effect of DHA is reversed by AACOCF3. However, the inhibitory effect of EPA is not reversed by BEL. Hence, we suggest that BEL may also inhibit the release of other fatty acids such as arachidonic acid, which could interfere with molecular mechanisms leading to NF-κB activation. Indeed, it has been reported that BEL, but not AACOCF3, inhibited both the release of arachidonic acid and IL-2 mRNA expression in Jurkat T-cells (22).

In fact, expression of the IL-2 gene is under the control of several nuclear factors, including NF-κB and activating protein complex-1 (AP-1). We were interested in whether n-3 PUFAs, being inhibitors of NK-κB translocation, could inhibit the transcription of the IL-2 gene. We observed that DHA and EPA dose-dependently inhibited the expression of IL-2 mRNA.

In conclusion, we suggest that n-3 PUFAs suppress IL-2 gene expression by inhibiting the membrane recruitment of PKCα and PKCε and blocking the nuclear translocation of NF-κB involved in T-cell proliferation (**Fig. 5**). Because diets enriched with n-3 PUFAs exert immunosuppressive effects (6), one can predict that EPA and DHA may act, in part, on the PKC signaling pathway and, consequently, influence the immune system in health and disease.

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