n-3 PUFAs modulate T-cell activation via protein kinase C-\(\alpha\) and -\(\varepsilon\) and the NF-\(\kappa B\) 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)-\(\alpha\) and -\(\varepsilon\). The two n-3 PUFAs also inhibited the nuclear translocation of nuclear factor \(\kappa B\) (NF-\(\kappa B\)) and the transcription of the interleukin-2 (IL-2) gene in PMA-activated Jurkat T-cells. Together, these results demonstrate the transcription of the interleukin-2 (IL-2) gene in PMA-activated Jurkat T-cells. Denys, A., A. Hichami, and N. A. Khan. n-3 PUFAs modulate T-cell activation via protein kinase C-\(\alpha\) and -\(\varepsilon\) and the NF-\(\kappa B\) signaling pathway. J. Lipid Res. 2005. 46: 752–758.

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Phospholipase A₂ (PLA₂) 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 PLA₂ have been identified in Jurkat T-cells: i) secreted PLA₂ (sPLA₂), among which are pancreatic type IB and type V; and ii) cytosolic PLA₂ (cPLA₂), including calcium-dependent type IV and calcium-independent PLA₂ (iPLA₂) type VI (21). Tessier-Hichami, and Khan (22) have shown that three isoforms of PLA₂ (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 PLA₂ 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 PLA₂ 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-IκBα antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-α-tubulin antibodies were from Calbiochem. PMH, DHA, and EPA were procured from Sigma. PLA₂ inhibitors, arachidonil trifluoromethyl ketone (AACOCF₃), and bromoeno lactone (BEL) were from Cayman Chemical (Ann Arbor, MI). Aristolochic acid was from Sigma-Aldrich (Saint Quentin Fallavier, France). [¹⁴C]DHA (53 mCi/mmol) was purchased from New England Nuclear (Boston, MA), and [³²P]EPA (100-200 Ci/mmol) was obtained from ICN Biomedicals (Orrsay, 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 10% fetal calf serum at 37°C in a humidified chamber containing 95% air and 5% CO₂. 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 [¹⁴C]DHA or [³²P]EPA at 1.5 μCi/3 × 10⁶ 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 (55:14:2.7, 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 radio-labeled 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 PLA₂ inhibitors or vehicle (dimethyl sulfoxide, 0.1% final concentration) for 30 min followed by a 20 min stimulation with PMH (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 IκBα

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 × 10⁶/ml) were further incubated for 5 min in the presence of EPA or DHA at 20 μM and then stimulated with PMH (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 anti-protease 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 IκBα and NF-κB in the cytosolic fractions was assessed in Western blotting using mouse monoclonal anti-IκBα 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 PMH 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 PLA₂ inhibitors (15 μM) or GF109203X (500 nM) for 30 min before stimulation with PMH for 20 min.

Nuclear extracts were prepared essentially as described by Dignam, Lebovitz, and Roeder (26) with some modifications. After treatment, cells (50 × 10⁶) 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 MgCl₂, 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 5 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 32P 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‘-AGTTAGGGGAGTTCCTCCAGG-3’. Oligonucleotides were end-labeled with [α-32P]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 MgCl2, 0.1 mM EDTA, and 45 mM boric acid, pH 8.3) running 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 [14C]DHA and [3H]EPA in these cells.

**EPA and DHA inhibit plasma membrane translocation of PKCα and PKCe**

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, PKCβ, and PKCe) from cytosol to plasma membrane. Figure 2 shows that PMA induced the translocation of PKCα, PKCβ, and PKCe to cytosol to the plasma membrane. In our study, PKCβ 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 PKCβ translocation. As illustrated in Fig. 2, EPA and DHA completely inhibited the PMA-induced translocation of PKCα and PKCe but not of PKCβ.

**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-κB is sequestered in the cytoplasm and remains inactive. Upon activation by specific stimuli, NF-κB is released from the cytoplasm and translocates to the nucleus, where it binds to specific DNA sequences and activates the transcription of target genes. The release of NF-κB is a complex process involving the activation of various signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK) pathways. The inhibition of NF-κB activation by EPA and DHA suggests that these fatty acids may have anti-inflammatory effects by modulating the expression of pro-inflammatory genes.
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, IκBα (12). During T-cell stimulation, IκBα 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 IκBα degradation (Fig. 3A, C). EPA or DHA alone exerted no significant effect on either NF-κB translocation or IκBα dissociation.

As we observed that EPA and DHA were released, respectively, by the activation of iPLA2 and cPLA2, we were tempted to assess whether the inhibition of activation of these 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-
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 PLA₂ 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 PLA₂ (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 PLA₂ was involved in the release of these two fatty acids. PLA₂ 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 sPLA₂ inhibitors aristolochic acid and BpB (32). We also used AACOCF3, which is known to be a specific inhibitor of cPLA₂ (32). AACOCF3, at high concentrations, may also inhibit iPLA₂ (32). BEL, a mechanism-based inhibitor of iPLA₂, was also used in our study (33).

We noticed that in T-cells activated by PMA and ionomycin, cPLA₂ was partly involved in [¹⁴C]DHA release from phospholipids, as AACOCF3 significantly inhibited [¹⁴C] DHA release. Because AACOCF3 used at its IC₅₀ value (15 μM) failed to completely suppress [¹⁴C]DHA release up to the level of unstimulated cells, the involvement of other isoforms of PLA₂ could not be ruled out. The release of [³H]EPA is catalyzed by iPLA₂, as BEL inhibited the release of this fatty acid. It is interesting that the inhibitors of sPLA₂ (i.e., aristolochic acid and BpB) failed to inhibit the release of these two n-3 PUFAs. Hence, we can assume...
that iPLA2 and cPLA2 may be specific for the respective release of EPA and DHA, as it has been proposed that different isoforms of PLA2 might be necessary to catalyze the release of different classes of fatty acids (34). This argument is further supported by our previous observations that iPLA2 and sPLA2, but not cPLA2, 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 pathophysiologic conditions, large amounts of free fatty acids may be released; this is the case during cardi 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 inhibit 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 physiologic 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 NF-κB (47, 48). In Jurkat T-cells, translocation of NF-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-depen-
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