Polyunsaturated fatty acids stimulate translocation of protein kinase Cα, -βI, -βII and -ε and enhance agonist-induced NADPH oxidase in macrophages

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INTRODUCTION

Cellular activation during the inflammatory reaction leads to the activation of phospholipid Arachidonic acid (AA) and other fatty acids [1]. Stimulation of leucocytes with a heterogeneous group of mediators, which includes opsonized particles and cytokines, induces the production of AA [2,3]. AA and related fatty acids may then be involved in a series of biological events that amplify/mediate the inflammatory response and cell activation. For example, AA has been shown to activate the neutrophil oxygen-dependent respiratory response [4–7], induce degranulation [8], increase expression of CR3 receptors [9] and regulate chemotaxis [10]. Although limited studies have been conducted with macrophages, evidence shows that AA also activates a respiratory burst in macrophages [11]. We have extended these studies on the mononuclear phagocyte by investigating the effect of the n-6 fatty acid AA on the agonist-induced respiratory burst in human macrophages. Since other fatty acids of the n-3 series, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are metabolized to yield different products, their effects were compared with those of AA. The results show that although these polyunsaturated fatty acids (PUFA) induce a poor respiratory burst response in monocytes and macrophages, they prime or act in synergy with other macrophage agonists. Evidence is presented that this priming response involves protein kinase C (PKC).

EXPERIMENTAL

Preparation of monocytes and macrophages

Monocytes or macrophages were prepared by adherence of mononuclear leucocytes to cytodex microcarriers, as previously described by Kumaratilake and Ferrante [12]. Briefly, mononuclear leucocytes were separated from heparinized blood from healthy donors by centrifugation of blood on Hypaque-Ficoll medium. Monocytes were purified by adherence of mononuclear leucocytes to preswollen cytodex microcarriers (Pharmacia Fine Chemicals, NSW, Australia) on a rotating platform at 37 °C for 2–4 h. Then the microcarriers with adhered monocytes were cultured in RPMI 1640 medium containing 10% foetal-calf serum at 37 °C in a humidified atmosphere of air/CO₂ (19:1) for 1–7 days. Monocytes/macrophages were immediately released by vortexing the microcarriers followed by two washes. The purity of monocytes and macrophages by this method is approx. 95%. Cell viability measured by Trypan Blue dye exclusion was > 95%. Differentiation of macrophages was monitored by cytocentrifuged smear, stained with May–Grunwald Giemsa and examined for the loss of myeloperoxidase activity [12].

Differentiation of HL-60

HL-60 cells (ATCC, Atlanta, GA, U.S.A.) were grown in RPMI 1640 containing 10% foetal-calf serum, 2 mM L-glutamine, penicillin and streptomycin at 37 °C in air/CO₂ (19:1). The HL-
60 cells were differentiated to monocyte/macrophages according to Levy et al. [13]. Cells were treated with $1 \times 10^{-8}$ M 1-$\alpha$,25-dihydroxyvitamin D$_3$ for 6 days. Differentiation and appearance of macrophage-like cells was confirmed by morphology and the expression of CD14 antigen. The viability of the differentiated cells was > 98%.

Fatty acids

Dipalmitoylphosphatidylcholine (DPC), the non-esterified fatty acids, AA, EPA, DHA and arachidic acid (20:0), and methyl arachidonate were purchased from Sigma (St. Louis, MO, U.S.A.). The methyl ester, and the hydroxy and hydroperoxy derivatives, of AA were prepared as described previously [14]. Fatty acids were checked for purity by silica-gel TLC in diethyl ether/hexane/acetic acid (60:40:1 by vol.). DPC was used as a vehicle (DPC–fatty acid micelles) for the non-esterified fatty acids and their derivatives [7]. Control incubations using the appropriate concentrations of DPC were included in the experiments.

Treatment of cells with fatty acids, N-formylmethionyl-leucyl-phenylalanine (fMLP), phorbol ester, calcium ionophore and inhibitors

Leucocytes were treated simultaneously (unless specified otherwise) with fatty acids at 10 µg/ml (unless specified otherwise) and either $5 \times 10^{-6}$ M fMLP (Sigma), $10^{-7}$ M PMA (Sigma) or $10^{-7}$ M calcium ionophore (A23187; Sigma).

In some experiments, the cells were treated with $10^{-7}$ M PMA for 18 h at 37 °C to deplete PKC. To inhibit the activity of PKC, cells were pretreated with 0.2 µM GF-109203X (Biomol, Plymouth Meeting, PA, U.S.A.) for 10 min before stimulation with agonists.

Chemiluminescence assay

Superoxide production was measured by the reduction of the fluorescent probe lucigenin (Sigma) essentially as described previously [15,16]. Briefly, $10^9$ monocytes or macrophages in 100 µl of Hanks Balanced Salt solution (HBSS) were treated with stimulus and the reaction mixture made up to a total volume of 400 µl. Then 500 µl of 250 µg/ml lucigenin was added. The tubes were immediately transferred to the luminometer chamber (Model 1251, Bio-Orbit Oy, Turku, Finland) and the light output recorded through the interface of the computer with the multiscaler software, and presented as the peak initial rate (mV) of each activity unless indicated otherwise. We have previously shown that the chemiluminescence produced is totally inhibited by superoxide dismutase [6]. In these investigations we have avoided using the measurement of superoxide by the cytochrome c reduction assay since cytochrome c can inhibit the activity of fatty acids [16].

PKC translocation

The cells were washed in HBSS and incubated with different concentrations of fatty acids for the times indicated in the Figure legends and Results section. After incubation, cells were washed again with HBSS and sonicated in buffer containing 20 mM Tris, pH 7.5, 5 mM EGTA, 2 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml benzamidine, 10 µg/ml pepstatin A, 10 mM PMSF and 2 mM mercaptoethanol, and the sonicate centrifuged at 100000 g for 30 min. The pellet was resuspended in sonication buffer supplemented with 2% (v/v) Triton X-100. After 30 min (1 °C), the extract was centrifuged at 100000 g for 30 min. The supernatant was adsorbed on to DE-52 (Bio-Rad Labs., Richmond, CA, U.S.A.), which was pre-equilibrated in 20 mM Hepes (pH 7.5). After washing (2 × 0.5 ml of Hepes buffer), PKC was eluted with 0.2 M NaCl. PKC activity was assayed by using histone type III-S (Sigma) as substrate, according to the method of Hardy et al. [17]. The reactions were terminated by spotting aliquots on to P81 papers (Whatman Ltd., Maidstone, Kent, U.K.). After extensive washing with 85 mM orthophosphoric acid, the radioactivity was determined by liquid-scintillation spectrometry.

For immunoblotting [18], Laemmli buffer was added to the DE-52-purified samples, and aliquots (20 µg) were subjected to SDS/PAGE (10 %, gels). The proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) and probed with isoenzyme-specific PKC antibodies (Boehringer Mannheim). The immune complexes were detected by enhanced chemiluminescence (DuPont–NEN).

[γ-32P]ATP (400 Ci/mmol) was obtained from Bresatec (Adelaide, South Australia). All other reagents, unless indicated otherwise, were from Sigma.

Statistics

Results are means ± S.E.M. of the number of experiments indicated in the Figure legend. Differences between treatments were compared by the two-tail Student’s $t$-test for paired data (Statwork, Philadelphia, PA, U.S.A.) and analysis of variance (Fisher’s least-significant test, pairwise Systat, Evanston, IL, U.S.A.).

RESULTS

Synergistic responses of monocytes/macrophages to PUFA and fMLP/PMA

Monocytes were treated with 10 µg/ml of AA, EPA or DHA alone or with each of the PUFA and fMLP, and the production of superoxide was determined in a chemiluminescence assay. The results showed that treatment of monocytes with all three PUFA resulted in a small but significant respiratory burst (Table 1). The

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chemiluminescence (mV)</th>
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<tbody>
<tr>
<td>Monocytes</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Control</td>
<td>1.429 ± 0.376</td>
</tr>
<tr>
<td>AA</td>
<td>4.529 ± 0.472</td>
</tr>
<tr>
<td>EPA</td>
<td>5.897 ± 1.135</td>
</tr>
<tr>
<td>DHA</td>
<td>4.787 ± 0.933</td>
</tr>
<tr>
<td>fMLP</td>
<td>2.924 ± 2.904</td>
</tr>
<tr>
<td>fMLP + AA</td>
<td>22.600 ± 5.036</td>
</tr>
<tr>
<td>fMLP + EPA</td>
<td>46.933 ± 10.35</td>
</tr>
<tr>
<td>fMLP + DHA</td>
<td>42.625 ± 11.584</td>
</tr>
<tr>
<td>PMA</td>
<td>23.795 ± 3.419</td>
</tr>
<tr>
<td>PMA + AA</td>
<td>59.801 ± 10.792</td>
</tr>
<tr>
<td>PMA + EPA</td>
<td>81.267 ± 21.419</td>
</tr>
<tr>
<td>PMA + DHA</td>
<td>64.021 ± 9.470</td>
</tr>
</tbody>
</table>
combined addition of fMLP and each PUFA gave rise to a synergistic response, i.e. a response that was significantly greater than the sum of each of the stimulators. Monocytes that had been differentiated into macrophages by in vitro culture responded to these fatty acids in a similar fashion (Table 1).

The saturated equivalents of these PUFA, such as 20:0 arachidic acid, did not show any effects, and the methyl ester and the hydroxy and hydroperoxy derivatives had no effect on superoxide production (results not shown).

To determine whether the synergistic effects of fatty acids also occurred with agonists that acted downstream of receptor-binding, we examined the effects of PUFA in combination with PMA, an agonist that directly activates PKC. A marked synergistic response was observed between PMA and each of the PUFA tested with both monocytes and macrophages (Table 1).

When the above data were expressed as peak initial rate of chemiluminescence produced, it was evident that the synergistic effects occurred over the complete incubation period with both fMLP and PMA (Figure 1). This is reflected in the data presented in Table 2, which summarizes the total chemiluminescence produced over the incubation period, averaged to mV/s.

Synergistic responses between fatty acids and PMA were observed in a concentration-dependent manner from 1 to 10 µg/ml of AA, EPA or DHA (Figure 2).

Figure 1  Synergistic responses of monocytes to PUFA (DHA) + fMLP (A) and PUFA (DHA) + PMA (B) as a function of rate of chemiluminescence produced over time

Time (sec) = Time (s).

Table 2  Synergistic response of monocytes to PUFA and either fMLP or PMA as the total chemiluminescence accumulated over the incubation period

Human monocytes were treated with 30 µM DHA or vehicle in the presence or absence of either 5 µM fMLP or 100 nM PMA. The superoxide production from these cells was measured by the lucigenin-dependent chemiluminescence assay. Results are means ± S.E.M. of four experiments, each conducted with cells from different individuals. *P < 0.05 for the difference between the sum of the value of DHA and fMLP/PMA effects versus the values for the co-treatment with these agents.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial peak rate (mV)</th>
<th>Accumulated chemiluminescence (mV/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.540 ± 0.488</td>
<td>1.348 ± 0.082</td>
</tr>
<tr>
<td>DHA</td>
<td>4.787 ± 3.051</td>
<td>2.628 ± 0.507</td>
</tr>
<tr>
<td>fMLP</td>
<td>10.882 ± 1.669</td>
<td>11.833 ± 0.811</td>
</tr>
<tr>
<td>fMLP + DHA</td>
<td>34.442 ± 2.160</td>
<td>33.600 ± 2.245</td>
</tr>
<tr>
<td>PMA</td>
<td>21.775 ± 5.367</td>
<td>20.544 ± 2.942</td>
</tr>
<tr>
<td>PMA + DHA</td>
<td>56.600 ± 5.352</td>
<td>54.705 ± 6.949</td>
</tr>
</tbody>
</table>

Figure 2  PUFA-concentration-related effects on the PMA-induced chemiluminescence response in monocytes

Monocytes were treated with PMA and varying concentrations of PUFA. The results are the mean of five experiments. a, *P < 0.05 and b, *P < 0.01 for comparison between the values for the sum of the individual effects of PUFA and PMA versus the effect of the co-treatment with both agents.

If the monocytes were pretreated with PUFA, an enhanced response to the subsequent addition of fMLP was observed over a pretreatment period of 30 min (results not shown). With respect to AA, EPA and DHA, maximal enhancement was seen within 5 min of pretreatment time, declining thereafter. Studies examining the PUFA concentration required for priming showed the fatty acids to be effective from 0.1 to 10 µg/ml (results not shown).

Although highly purified preparations of monocytes/macrophages were used in the above studies, it is still possible that the fatty acids could be exerting some of their effects via residual contaminating lymphocytes. We therefore examined the synergistic properties of fatty acids and agonists on HL-60 cells differentiated into macrophages with 1-α,25-dihydroxyvitamin D3. Data from four experiments showed that PMA induced a respiratory burst in these cells (73.18 ± 18.90 mV) but...
DHA elicited a very poor response of 2.33 ± 0.27 mV (basal 1.47 ± 0.21 mV), which is in keeping with the results with monocytes and macrophages. Addition of both PMA and DHA to the differentiated HL-60 macrophages resulted in a synergistic chemiluminescence response of 291.25 ± 57.41 mV.

Role of PKC in PUFA-induced macrophage priming

Previous studies have shown that PUFA can activate PKC directly [17] and translocate cytosolic PKC to a particulate fraction in WB cells [18]. We thus examined whether PUFA translocated the various isoenzymes of PKC, and its role in the synergistic response.

The data in Table 3(A) show that DHA stimulated the translocation of PKC to a particulate fraction of both monocytes and macrophages, with the first few minutes of incubation with the fatty acid. Table 3(B) shows the DHA-stimulated translocation of PKC isoenzymes α, βII, β/III and ε to a particulate fraction.

Examination of the role of PKC in the PUFA-induced priming is made difficult by the fact that the MLPL response is also dependent on PKC activation, and therefore experiments utilizing either PKC inhibitors or PKC depletion cannot resolve this question. Therefore we used A23187, which stimulates cells independently of PKC. Co-addition of DHA and A23187 resulted in a marked synergistic chemiluminescence response in human monocytes (Table 4). Macrophages pretreated with GF-109203X, an inhibitor of PKC, failed to be primed by DHA for a chemiluminescence response to A23187 (Table 4).

In a second set of experiments, we examined whether PKC depletion following a prolonged pretreatment with PMA in macrophages rendered the cells unresponsive to priming by PUFA. The cells were pretreated for 18 h with 100 nM PMA and then incubated with the PUFA and A23187. The data presented in Table 4 show that PKC depletion rendered the monocytes incapable of being primed by PUFA.

**Table 3** Translocation of PKC by DHA

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Histone phosphorylation (of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particulate fraction</td>
</tr>
<tr>
<td>Monocytes</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1</td>
</tr>
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<td></td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 4** Effect of a PKC inhibitor, GF-109203X, or PKC depletion (PMA pretreatment for 18 h) on the ability of DHA to enhance the monocyte chemiluminescence response to A23187

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Pretreatment</th>
<th>Chemiluminescence production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diluent</td>
<td>GF-10923x</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.69 ± 0.10</td>
<td>1.28 ± 0.31</td>
</tr>
<tr>
<td>DHA</td>
<td>3.22 ± 0.57</td>
<td>3.32 ± 0.31</td>
</tr>
<tr>
<td>A23187</td>
<td>2.07 ± 0.24</td>
<td>2.00 ± 0.46</td>
</tr>
<tr>
<td>DHA + A23187</td>
<td>14.84 ± 1.52*</td>
<td>2.34 ± 0.60</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The data show that the respiratory burst in monocytes and macrophages is modulated by long-chain n-3 and n-6 PUFA. Although peripheral blood monocytes, monocyte-derived macrophages and vitamin D3-differentiated HL-60 cells gave very poor respiratory burst responses to AA, EPA and DHA, these fatty acids primed the monocytes/macrophages for a significantly increased respiratory burst to fMLP.

Major sources of AA are likely to be inflammatory phagocytic cells, neutrophils and macrophages. For example, macrophages treated with mycobacteria, lipopolysaccharide, opsonized zymosan and some chemokines release AA into the extracellular medium [19–21]. A substantial amount of AA can also be released from activated endothelial cells [22,23]. In these and other cell types, the 85 kDa cytosolic phospholipase A2 preferentially cleaves AA from the sn-2 position of phospholipids such as phosphatidylyceroline and inositol-containing phospholipids [1,24,25]. In platelets, the cytosolic phospholipase A2 also liberates EPA but not DHA from n-3 fatty-acid-enriched phospholipids [26–28]. However, DHA can be liberated by the 14 kDa secretory phospholipase A2 [27].

Significant enhancement of the fMLP response was seen at concentrations of 1–2.5 µg/ml (≈ 2–5 µM), which is well within the physiological and pathophysiological concentrations of these fatty acids. For example, in human malaria, the plasma level of AA as a non-esterified fatty acid rises from 5 µmol/l under ischaemic conditions. Others have reported that activation of neutrophils leads to production of AA concentration of the order of 20–30 µmol/l [31], a value that agrees with our findings [2]. Dietary n-3 fatty acids can reach a non-esterified fatty acid concentration of 20–30 µmol/l [32]. The finding suggests that fatty acids generated either as a consequence of the inflammatory reaction through the activation of phospholipase A2, or introduced in diet supplements, can significantly affect a major function in the macrophage, namely the ability to produce oxygen-derived reactive species. These macrophage products are known to be critical not only in the killing of a number of bacteria and parasites, but also in causing tissue damage. Both EPA and DHA have been used as dietary supplements to...
increase the proportion of $n-3$ over $n-6$ fatty acids in membrane phospholipids, in order to achieve an anti-inflammatory effect through the production of substantially less active eicosanoids [33] and, as more recently reported, a decrease in the production of cytokines such as tumour necrosis factor and interleukin-1 [34]. It is, however, evident from our results that EPA and DHA are as active as AA in promoting a key response in macrophages that is associated with promotion of inflammation and tissue damage. This may in part explain the limited success achieved in attempts to treat inflammatory disease with $n-3$ fatty acids [35].

The effects of PUFA were not restricted to the surface-receptor-acting agonist, fMLP. The PUFA acted in a synergistic manner with either PMA, which directly activates PKC, or the Ca$^{2+}$ ionophore, A23187. The PUFA-induced priming could not have involved the synthesis of new protein molecules as the effect on monocytes/macrophages was extremely rapid, with a maximal synergistic response by 5 min. Our results strongly suggest that activation of PKC by PUFA is a possible mechanism for the priming/synergistic response. Human monocytes/macrophages express PKC$\alpha$, $\beta$I and $\beta$II, but little of the $\gamma$ and $\zeta$ isoforms [36], and PKC$\beta$ has been reported to phosphorylate p47$^{phox}$ [37]. We demonstrate, for the first time in human monocytes/macrophages, that PUFA stimulated the translocation of PKC$\alpha$, $\beta$I, $\beta$II and $\zeta$ to a particulate fraction. A role of PKC in the enhancement of superoxide production was confirmed by the use of a selective PKC inhibitor, GF-109203X, and by depletion of PKC through prolonged incubation of cells in the presence of PMA. In using GF-109203X to inhibit the activity of classical PKC$\alpha, \beta$ or $\gamma$ isoforms, it is likely that they could function to amplify the leucocyte response by acting synergistically with other mediators that form part of the network of intercellular active molecules.

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REFERENCES