Neutrophil Migration Inhibitory Properties of Polyunsaturated Fatty Acids

The Role of Fatty Acid Structure, Metabolism, and Possible Second Messenger Systems

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Abstract

The n-3 polyunsaturated fatty acids (PUFA) appear to have antiinflammatory properties that can be partly explained by their biological activity on leukocytes. Since leukocyte emigration is an essential component of the inflammatory response, we have examined the effects of the n-3 PUFA (eicosapentaenoic and docosahexaenoic acids) on neutrophil random and chemotactic movement. Preexposure of neutrophils for 15–30 min to 1–10 μg/ml PUFA reduced the random and chemotactic migration to both FMLP- and fungi-activated complement. The inhibitory effect diminished with increasing saturation and carbon chain length, and methylation abolished this activity. Arachidonic and docosahexaenoic acids were the most active fatty acids. The PUFA concentration required to inhibit migration was dependent on cell number, suggesting that the fatty acid effects on leukocyte migration in vivo may be governed by the stage of the inflammatory response. It was concluded that the PUFA rather then their metabolites were responsible for the inhibition since: (*a*) antioxidants did not prevent the PUFA-induced migration inhibition and the hydroxylated intermediates were less active, and (*b*) inhibitors of the cyclooxygenase and lipoxygenase pathways were without effect. Inhibitors of protein kinases and calmodulin-dependent enzyme system did not prevent the PUFA-induced migration inhibition, which was also independent of phospholipase D–catalyzed hydrolysis of phospholipids. It is also shown that PUFA decrease the FMLP-induced Ca^{2+} mobilization. (*J. Clin. Invest.* 1994. 93:1063–1070.) Key words: chemotaxis • chemokinesis • phospholipase D • protein kinases • lipoxygenases / cyclooxygenases

Introduction

The use of diets containing certain n-3 polyunsaturated fatty acids (PUFA)[1] continues to attract major interest because of their potential clinical benefits for preventing or treating a range of diseases, including cardiovascular diseases, rheumatoid arthritis, cystic fibrosis, asthma, and eczema. For example, it has been claimed that the lower incidence of cardiovascular diseases in some human populations is due to fish diets rich in n-3 fatty acids (1, 2), and some successful attempts have been made to treat rheumatoid arthritis (3) and asthma (4) with these fatty acids. Although the beneficial effect of PUFA is believed to be a function of their antiinflammatory properties (by interfering with prostaglandin and leukotriene metabolism), there are a number of activities reported, such as stimulation of oxygen radical production in neutrophils, that would be expected to increase the inflammatory response (5–11). Thus, further investigation into the biological properties of PUFA is necessary to identify their potential use as antiinflammatory agents.

Accumulation of phagocytic cells at inflammatory foci is dependent on cell locomotion, which is regulated by a range of mediators that include complement products, cytokines, and eicosanoids (12–14). At present there is little known of the effects of PUFA on leukocyte locomotion. As this function is crucial to cell accumulation at sites of inflammation, we examined the ability of PUFA to alter neutrophil locomotion, paying particular attention to fatty acid structure required for activity and the mechanism of PUFA-induced effects. The data show that PUFA are strong inhibitors of neutrophil locomotion. This cell migration inhibitory activity is highly dependent on the structure of the PUFA itself, rather than a metabolite, and is independent of protein kinase C.

Methods

Fatty acids. Arachidonic ($C_{20:4,n-6}$), eicosapentaenoic ($C_{20:5,n-3}$), docosahexaenoic ($C_{22:6,n-3}$), arachidic ($C_{20:0}$), oleic ($C_{18:1,n-9}$), linoleic ($C_{18:2,n-6}$), and linolenic ($C_{18:3,n-3}$) acids as well as docosahexaenoic acid methyl ester were purchased from Sigma Chemical Co. (St. Louis, MO). Dotriacontatetraenoic acid methyl ester ($C_{32:4,n-6}$) was synthesized by elongation of arachidonic acid methyl ester ($C_{20:4,n-6}$) using the conjunctive reagent tosylmethylisocyanide as described previously (15, 16). The ester ($\leq$ 1 mg) was converted to the free acid by hydrolysis in 2 ml of a mixture of 3 M LiOH-1,2 dimethoxyethane (1:1 [vol/vol]) at 60°C for 2 h. The hydrolyzate was diluted with water (2 ml), the pH adjusted to 3, and the free acid extracted into hexane (3 × 3 ml). Under these conditions there was negligible change in fatty acid structure as determined by gas chromatography/mass spectrometry, with ~ 95% cleavage of the ester bond occurring (data not presented).

Hydroperoxy arachidonic acid (containing a mixture of isomeric forms) was prepared by DL-α-tocopherol (vitamin E)-controlled autooxidation of arachidonic acid (modified according to Peers and Coxon [17] and Kuhn et al. [18]). Arachidonic acid (25 mg) and DL-α-tocopherol (2.5 mg) were dissolved in 5 ml of chloroform in a 250-ml round-bottomed flask. The solvent was evaporated under reduced pressure so that the flask wall was covered with a thin lipid film. After flushing with oxygen, the flask was sealed and left in the dark at room temperature for 72 h. The residue was subsequently dissolved in 4 ml of petroleum spirit (boiling point range, 40–60°C)/diethyl ether (9:1 [vol/vol]) and applied to a silicic acid column (4 g) that was made

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1. *Abbreviations used in this paper:* DPC, DL-α-dipalmitoylphosphatidylcholine; PUFA, polyunsaturated fatty acids; SOD, superoxide dismutase.

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up in the same solvent. Unreacted arachidonic acid and DL-α-tocopherol were eluted with 120 ml of petroleum spirit/diethyl ether (9:1 [vol/vol]) and hydroperoxy arachidonic (HPETE) acid was eluted with 120 ml of petroleum spirit/diethyl ether (1:1 [vol/vol]). Hydroxy arachidonic acid (HETE) (a mixture of isomeric forms) was prepared from the hydroperoxy acid by reduction with triphenylphosphine (1.5 mol triphenylphosphine/mol acid) in diethyl ether [3 ml] at 4°C) and purification by silicic acid chromatography as described above. The excess triphenylphosphine eluted in the petroleum spirit diethyl ether (9:1 [vol/vol]) fraction and the hydroxy acid in the petroleum spirit diethyl ether (1:1 [vol/vol]) fraction. Fatty acids and their derivatives were dissolved in redistilled chloroform (2–10 mg/ml) and stored under N_2 at −20°C. Thin-layer chromatography and high pressure liquid chromatography indicated that the lipids were of high purity.

Other reagents. The tripeptide, FMLP, bovine erythrocyte superoxide dismutase (SOD), BSA, bovine liver catalase, vitamin E, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), staurosporine, and DL-α-dipalmitoylphosphatidylcholine (DPC) were purchased from Sigma Chemical Co. FURA-2 AM was from Calbiochem (San Diego, CA).

Preparation of fatty acid suspension. The fatty acid (100 μg) and DL-α-dipalmitoyl phosphatidylcholine (400 μg) were placed in 10-ml glass tubes and the solvent evaporated under N_2 at 30°C. The mixture was then reconstituted in 1 ml of HBSS by sonication for 2 min using an ultrasonicator Ystrom System, (power setting 8, tune setting 4; Westwood, NJ). Under these conditions the mixture formed a clear suspension.

Preparation of neutrophils. Neutrophils were prepared from the blood of healthy volunteers using the rapid single-step method (19). Briefly, freshly collected blood was layered onto a Hypaque-Ficoll medium of density 1.114 and centrifuged at 400 *g* for 30 min at room temperature. After centrifugation the leukocytes resolved into two distinct bands. The neutrophils that collected in the second band were harvested. In the majority of cases the preparation of neutrophils was > 99% pure and > 99% viable, as judged by the ability to exclude trypan blue.

Treatment of neutrophils with fatty acids. Neutrophils were pretreated with the specified concentrations of fatty acids for 30 min at 37°C unless indicated otherwise.

Measurement of neutrophil chemotactic and random movement. Neutrophil locomotion was measured by the agarose technique (20) with minor modification (21). Molten 2% agarose (3 ml) was mixed with 3 ml of double-strength Medium 199 containing 10% FCS and poured into 60 × 15-mm tissue culture petri dishes (Lux; Miles Laboratories Inc., Naperville, IL). Sets of three wells of 2.5 mm diameter, each 3.0 mm apart were cut in the agarose. To the center, inner, and outer wells of each set was added either 5 μl of 4×10^6 neutrophils/ml, 5 μl of the chemoattractant (10^{-7} M FMLP), or 5 μl of control medium, respectively. The plates were incubated for 90 min at 37°C in an atmosphere of 5% CO_2 and high humidity, and the migration distance was measured with an inverted microscope under phase contrast illumination. Neutrophil random migration was measured by the same technique except that single wells were cut in the agarose instead of sets of three.

Studies were also conducted using fungi-activated complement as chemotactic factors (22). This was generated by treating human serum with *Torulopsis glabrata.*

Determination of intracellular free calcium concentration. The $C_{22:6,n-3}$ solutions used for the Ca^{2+} mobilization studies were prepared as follows. An aliquot was removed from a stock solution (2–3 mg/ml) in chloroform stored in the dark at −20°C and the solvent evaporated to dryness under a stream of nitrogen at 30°C. The residue was resuspended in redistilled ethanol to give a final concentration of 6 mg/ml. A working dilution in water of 1 mg/ml $C_{22:6,n-3}$ was prepared from the 6-mg/ml solution in ethanol just before use, kept in the dark at 2°C, and always used within 2 h of preparation. The final ethanol concentration present in the assay using this method (0.17% [vol/vol]) had no effect on either the cell viability, the basal intracellular Ca^{2+} concentration, or the magnitude or duration of the Ca^{2+} changes induced by other agonists (data not shown).

To load the cells with FURA-2, neutrophils (10^7 cells/ml) in HBSS were incubated with FURA-2 AM (1 μM) for 30 min at 37°C as previously described (10). Changes to the intracellular free Ca^{2+} concentration were determined using a luminescence spectrometer and Fluorescence Data Manager software (LS 50B; Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, England) as previously described (10), except that the emission wavelength was changed to 510 nm. FURA-2-loaded neutrophils (2 ml, 3×10^6 cells/ml) in HBSS were prewarmed for 5 min at 37°C. Baseline fluorescence was determined for 2 min before $C_{22:6,n-3}$ (5 μg/ml) or the appropriate ethanol control was added (20 μl), and readings were taken for a further 5 min. The maximum and minimum values for FURA-2 banded cells (F_{max} and F_{min}) were determined as previously described (10).

The intracellular Ca^{2+} concentration was calculated using the method of Grynkiewicz et al. (23).

Results

Effect of PUFA on cell migration. Random and chemotactic migration was assessed in neutrophils pretreated for 30 min with 10 μg/ml of $C_{20:0}$, $C_{20:4,n-6}$, $C_{20:5,n-3}$, and $C_{22:6,n-3}$. The results show that while $C_{20:0}$ had no significant effect, the unsaturated fatty acids $C_{20:4,n-6}$, $C_{20:5,n-3}$, and $C_{22:6,n-3}$ completely inhibited neutrophil locomotion (Fig. 1). Both random cell migration and migration towards a gradient of FMLP were inhibited by the PUFA. When activated complement was used as the chemotactic agent similar results were observed (data not presented). These concentrations of fatty acid had no effect on neutrophil cell viability based on trypan blue uptake and release of the cytoplasmic enzyme, lactate dehyrogenase (data not shown). In addition, these concentrations of fatty acids stimulated the respiratory burst (9, 10) and adhesion (24) of neutrophils.

The ability of fatty acids to inhibit neutrophil locomotion was found to be concentration dependent (Fig. 2). Depression of both random and chemotactic cell movement was observed at $C_{20:4,n-6}$, $C_{20:5,n-6}$, and $C_{22:6,n-3}$ concentrations ≤ 4 μg/ml. It was also evident that $C_{20:4,n-6}$ and $C_{22:6,n-3}$ showed greater migration inhibitory activity than $C_{20:5,n-3}$.

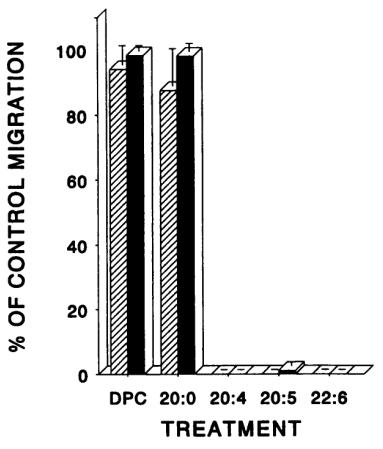

Figure 1. The effect of PUFA on neutrophil locomotion. Neutrophils were pretreated with 10 μg fatty acid/10^6 cells per ml for 30 min and then examined for their ability to migrate randomly (■) or in a chemotactic gradient (■) generated with FMLP. The results are expressed as percent changes in migration induced by the vehicle DPC or the fatty acid compared with the control treatment (HBSS) of neutrophils. The values represent the mean±SEM of four experiments, each conducted in triplicate using cells from a different individuals.

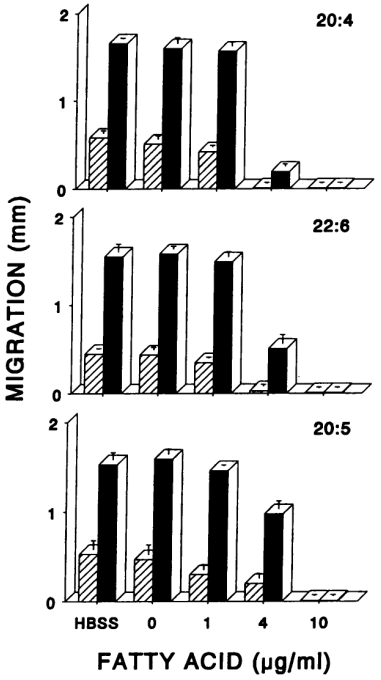

Figure 2. Fatty acid concentration–related effects on neutrophils locomotion. Neutrophils (10^6/ml) were pretreated with the indicated concentrations of fatty acid for 30 min and then tested for their ability to migrate randomly (■) or in a chemotactic gradient (■) generated with FMLP. The results are the mean±SEM of triplicates, representative of two other experiments.

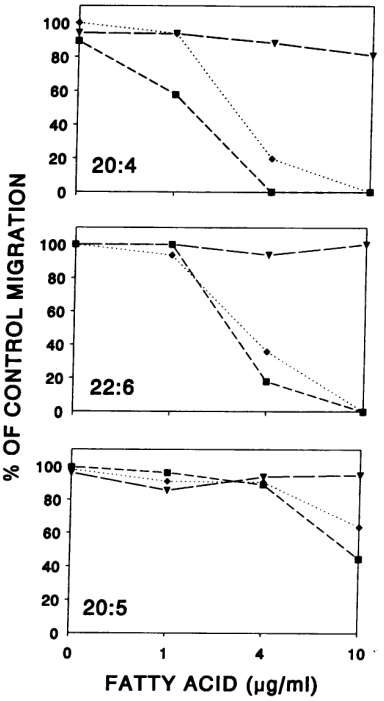

Figure 4. The relationship between fatty acid concentration and neutrophil concentration, and inhibition of neutrophil migration in response to FMLP. Neutrophils were treated with the indicated concentration of fatty acids. Neutrophil concentrations used were 2.5×10^5 cells/ml (■), 10^6/ml (♦), and 10^7/ml cells (▼). Comparisons have been made with cells from one donor in quadruplicates. A similar trend was seen in two other experiments.

The ability of the fatty acids $C_{20:4,n-6}$ and $C_{22:6,n-3}$ to inhibit neutrophil migration was also found to be dependent on the length of time the neutrophils were preexposed to the fatty acids. Thus, the extent of migration inhibition increased with the fatty acid preincubation time, with pretreatment periods of ≥ 30 min resulting in complete inhibition of cell locomotion (Fig. 3).

The effect of varying the ratio of PUFA to cell number. During the inflammatory response the cell concentration may, at sites of inflamation, change by several orders of magnitude. It was therefore of interest to determine whether the effective concentration of fatty acid required to inhibit neutrophil migration was dependent on cell concentration. Varying concentrations of fatty acids were preincubated (30 min at 37°C) with either 2.5×10^5, 1×10^6 or 1×10^7 neutrophils/ml before assessing cell migration. The results showed that the cell concentration had a marked effect on whether the fatty acids were able to inhibit either the random or chemotactic migration of the neutrophils (Fig. 4). It was observed that as the cell concentration declined the concentration of fatty acid required to inhibit neutrophil migration also decreased (Fig. 4). The most likely explanation for these data is that as the cell number decreases the amount of fatty acid available to each cell effectively increases. It should also be noted that reducing the cell concentration dramatically altered the effective inhibitory concentration of the various fatty acids. For example, for chemotactic migration at a concentration of 10^6 cells/ml, 10 μg/ml of $C_{22:6,n-3}$ completely inhibited movement, but when the cell concentration was increased to 10^7 neutrophils/ml, no effect of $C_{22:6,n-3}$ was observed (Fig. 4).

Effects of PUFA on FMLP-induced chemokinesis. Pretreatment of neutrophils with FMLP increases the cells' migration activity (chemokinesis). We were therefore interested to know whether the chemokinetic response of the cells was modulated by PUFA. Neutrophils pretreated for 20 min with 10 μg/ml $C_{20:4,n-6}$ showed inhibition of FMLP-induced migration (Fig. 5). However, if the FMLP and PUFA were added simulta-

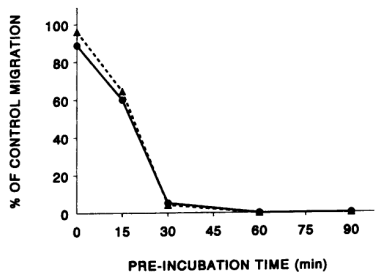

Figure 3. Effects of varying the fatty acid–neutrophil preincubation time. Neutrophils (10^6 cells/ml) were pretreated with 10 μg/ml of fatty acids, $C_{20:4,n-6}$ (●) and $C_{22:6,n-3}$ (▲), for the times indicated and then tested for ability to migrate in a chemotactic gradient generated with FMLP. The results are the mean of quadruplicates.

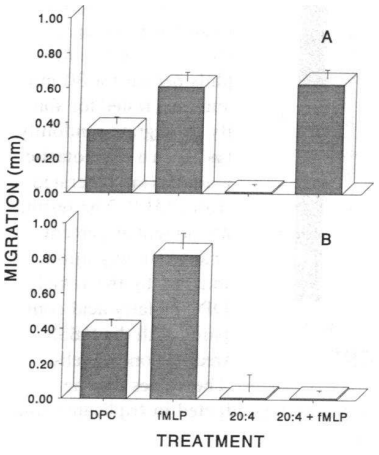

Figure 5. Effect of $C_{20:4,n-6}$ (10 μg/ml) on FMLP (10^{-7} M)-induced chemokinesis. (*A*) Effects of pretreating neutrophils simultaneously with either FMLP and $C_{20:4,n-6}$. (*B*) Effects of pretreating neutrophils for first 15 min with either DPC or $C_{20:4,n-6}$ and then each with either medium or FMLP for a further 15 min before assaying for cell migration. The results are the mean±SEM of three experiments.

neously at the beginning of the pretreatment period the FMLP-induced enhanced migration was not inhibited (Fig. 5).

Determination of fatty acid structure required for migration inhibition activity. A range of fatty acids were examined for their ability to inhibit neutrophil migration to identify which features of the fatty acid molecule were important in determining the biological potency. Carbon chain length was found to be important as dotriacontatetraenoic acid ($C_{32:4,n-6}$) was ineffective for migration inhibition even though it has the same number and positioning of double bonds as $C_{20:4,n-6}$ (Fig. 6).

The number of double bonds as well as their position were also found to be important. Reducing the number from four to one dramatically lowered the fatty acid activity for migration inhibition (Fig. 7). This trend continued as the extent of saturation of the fatty acid increased. Thus, it was observed that: $C_{20:4,n-6}$, $C_{22:6,n-3} > C_{20:5,n-3} > C_{18:3,n-3}$, $C_{18:2,n-6} > C_{18:1,n-9} > C_{20:0}$ for migration inhibition (Figs. 4 and 7).

The carboxyl group of the fatty acid was also found to be an essential requirement for biological activity, as $C_{22:6,n-3}$ methyl ester was ineffective in inducing migration inhibition (Fig. 8).

Mechanisms of PUFA-induced migration inhibition: free acid vs. metabolic products. The PUFA can be metabolized via the lipoxygenase or cyclooxygenase pathways (24). Their conversion to hydroperoxy and hydroxy derivatives may be the first step in the initiation of peroxidation mechanisms that could be responsible for cell migration inhibition. We therefore examined the effects of the $C_{20:4,n-6}$ hydroxy and $C_{20:4,n-6}$ hydroperoxy on neutrophil migration. The $C_{20:4,n-6}$ hydroxy showed much lower activity on cell-random and chemotactic migration than the free acid (Fig. 9). While the hydroperoxy showed good activity it was still less effective than the equivalent concentration of $C_{20:4,n-6}$ (Fig. 9). This difference was more distinct when the concentration-related effects were compared (Fig. 10).

Subsequently, we examined the effects of inhibitors of the cyclooxygenase and lipoxygenase pathways. Neutrophils were pretreated with either 1–100 μM of indomethacin or 1–10 μM of nordihydroguriapetic acid (NDGA), inhibitors of the cyclooxygenase and lipoxygenase pathways, respectively, to determine whether it was the PUFA or some metabolite that was responsible for the migration inhibitory effect. Under the same conditions we confirmed that there is near complete inhibition of neutrophil cyclooxygenase and lipoxygenase activities (data not shown). After a 5-min incubation with these agents the neutrophils were treated for 30 min with the fatty acids and examined for random and chemotactic migration. At these concentrations the inhibitors were found not to affect the PUFA-induced neutrophil migration inhibition (data not presented). These inhibitors at these concentrations had no effect on cell migration in the absence of the PUFA.

We have previously demonstrated that the antimalarial activity of PUFA could be abrogated by the addition of various antioxidants (25). We therefore examined whether the addition of SOD, catalase, and vitamin E affected the migration inhibitory properties of PUFA. The results showed that SOD (100 μg/ml), catalase (10^3 U/ml), and vitamin E (20 μM) did not affect the migration inhibition induced by 7.5 μg/ml of $C_{20:4,n-6}$, $C_{20:5,n-3}$, and $C_{22:6,n-3}$, at a leukocyte concentration of 10^6 cells/ml (data not presented).

Mechanisms of PUFA-induced migration inhibition: signal transduction. Since PUFA have been shown to activate purified protein kinase C, we examined whether the migration inhibitory properties of PUFA were a consequence of activation of protein kinase C by pretreating the cells with two pKC inhibitors, H-7 and staurosporine, before their inclusion in the migration assay. Cells were pretreated with 50 μM of H-7 or 15 nM staurosporine for 5 min and then for a further 20 min with the PUFA before assessing their migration activity. However, neither H-7 nor staurosporine prevented the PUFA from inducing migration inhibition (data not presented).

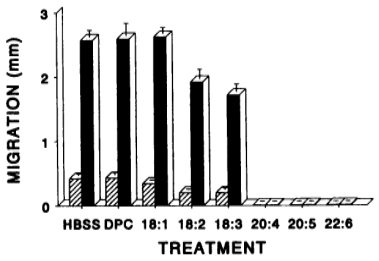

Figure 7. The effect of varying the degree of unsaturation of fatty acids on neutrophil locomotion. Neutrophils were pretreated with 10 μg of fatty acid/10^6 cells per ml ($C_{18:1,n-9}$; $C_{18:2,n-6}$; $C_{18:3,n-3}$; $C_{20:4,n-6}$; $C_{20:5,n-3}$; $C_{22:6,n-3}$) for 30 min and then tested for ability to migrate randomly (▬) or in a chemotactic gradient (▬) generated with FMLP. The results are presented as the mean±SEM of triplicates.

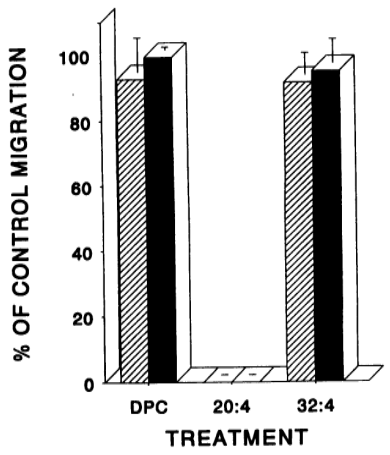

Figure 6. Comparisons of effects of $C_{20:4,n-6}$ and $C_{32:4,n-6}$ on neutrophil locomotion. Cells were treated with 10 μg of fatty acid/10^6 neutrophils per ml for 30 min and then tested for ability to migrate randomly (▬) or in a chemotactic gradient (▬) generated with FMLP. The results are present as percent changes in migration induced by the vehicle DPC or fatty acid compared with the HBSS-treated control cells. The values are the mean±SEM of three experiments, each conducted in triplicate using cells from different individuals.

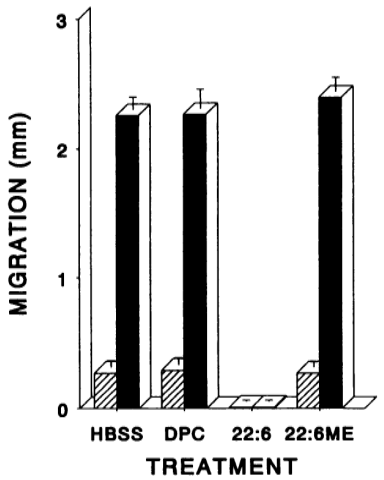

Figure 8. Comparison of the effects of $C_{22:6,n-3}$ and its methyl ester ($C_{22:6,n-3}$ ME) on neutrophil locomotion. Neutrophils were pretreated with 10 μg of fatty acid/10^6 cells per ml for 30 min and then tested for random migration (▬) and chemotactic migration (▬) induced by FMLP. The results shown are the mean±SEM of triplicates and are representative of three experimental runs.

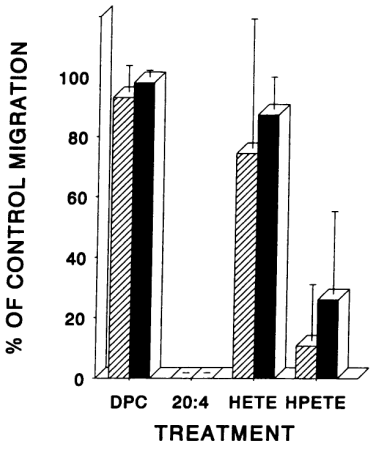

Figure 9. Comparison of the effects of $C_{20:4,n-6}$ with either $C_{20:4,n-6}$ hydroxy (*HETE*) or $C_{20:4,n-6}$ hydroperoxy (*HPETE*). Neutrophils were pretreated with 10 μg fatty acid/10^6 cells per ml for 30 min and then tested for ability to migrate randomly (▬) or in a chemotactic gradient of FMLP (▬). The results are presented as the percent change in cell migration induced by either the vehicle DPC or the fatty acids compared with HBSS-treated control cells. The values are the mean±SEM of four experiments, each conducted in triplicate with cells from different individuals.

We have previously found that the PUFA-induced activation of the neutrophil NADPH oxidase system was sensitive to the calmodulin-dependent enzyme system inhibitor, W-7 (9). This was not the case here, however, as neutrophils pretreated with 50 μM of W-7 were still sensitive to migration inhibition by PUFA (data not shown).

Some authors have proposed that there is a direct correlation between the extent of phosphatidylcholine (PC) hydrolysis by phospholipase D and the amount of superoxide produced by neutrophils (26, 27). We investigated whether or not phospholipase D–mediated hydrolysis of phospholipids was involved in PUFA-induced migration inhibition. The following experiments were based on the "transphosphatidylation reaction," a unique property of the phospholipase D enzymes (28, 29). In the presence of an alcohol, phospholipase D substitutes the alcohol species for the cleaved basic head group to form a phosphatidylalcohol. In the normal situation where the alcohol is water, the products formed are phosphatidic acid and the base. If, however, ethanol is present then the products are the base and phosphatidylethanol. It is presently believed that this is the only way such phosphatidylalcohol species can be formed, and thereby provides a direct means of specifically measuring phospholipase D activity. Ethanol at 500 μM did not affect the ability of PUFA to inhibit cell migration (data not shown). This indicates that the inhibitory effect of fatty acids is independent of the hydrolysis of phospholipids by phospholipase D.

This was independently confirmed using DL-propranolol, an inhibitor of phosphatidic acid phosphohydrolase that converts phosphatidic acid to diglyceride (30). This also had no effect at 250 μM.

FMLP also induced a rapid increase in the intracellular Ca^{2+} concentration, which is believed to play a crucial role in biological responses. Exposure to $C_{22:6,6n-3}$ at concentrations known to inhibit chemotaxis reduced the magnitude of the subsequent Ca^{2+} mobilization effected by FMLP (Table I).

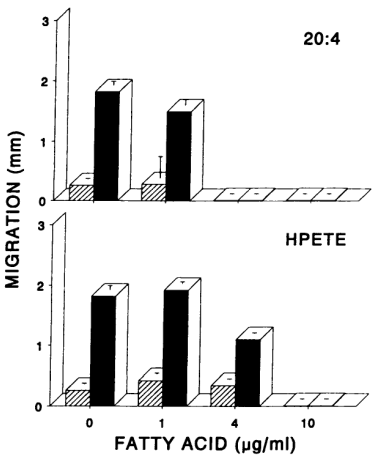

Figure 10. Concentration-related effects of cell migration inhibition induced by $C_{20:4,n-6}$ and $C_{20:4,n-6}$ hydroperoxy. Neutrophils (10^6/ml) were treated with the indicated concentrations of fatty acids and then tested for ability to migrate randomly (▬) or in a chemotactic gradient (▬) generated with FMLP. The results are the mean±SEM of triplicates and are representative of three experimental runs.

Discussion

There is increasing evidence that PUFA are involved in the regulation of the inflammatory response by modulating the activity of phagocytic cells such as neutrophils. When metabolized via the cyclooxygenase and lipoxygenase pathway, PUFA produce a range of cellular inflammatory mediators (12–14). We and others have previously demonstrated that n-3 PUFA, like n-6 PUFA, can activate the neutrophil oxygen-dependent respiratory burst leading to the generation of oxygen-derived reactive species (5–11). Further evidence for a potential role of PUFA in the regulation of inflammatory processes is provided by the data presented in this report, which show that some PUFA have neutrophil migration inhibitory activity. It was determined that the structure of the fatty acid was important in determining its biological activity. Thus, removal of the double bonds, extension of the carbon chain length, esterification or hydroxylation of the fatty acid, and removal of the carboxyl group all reduced the biological activity of the fatty acids. For example, $C_{20:4,n-6}$ was highly active while $C_{32:4,n-6}$ showed no activity. Further structure specificity of the migration inhibitory activity of the PUFA was evident from studies revealing $C_{18:2,n-6}$, $C_{18:3,n-3}$ showed small activity while $C_{18:1,n-9}$ lacked migration inhibitor activity.

We have speculated that the fatty acid–induced stimulation of neutrophil NADPH oxidase may be mediated via the phospholipase D–catalyzed hydrolysis of phospholipids other than phosphatidylcholine (10), and that the molecular events that initiate the fatty acid–induced stimulation of the respiratory burst differ from those induced by PMA and FMLP (9). From our present studies it is apparent that the signal transduction

Table I. The Effect of $C_{22:6,n-3}$ on the FMLP-induced Neutrophil Ca^{2+} Mobilization

Neutrophil treatment	Ca^{2+} mobilized
	nM
Diluent	158±12.5 (n = 12)
$C_{22:6,n-3}$ + diluent	364±10.9 (n = 8)
Diluent + FMLP	1,013±155.5 (n = 4)
$C_{22:6,n-3}$ + FMLP	524±85.2 (n = 4)

Neutrophils were pretreated with 5 μg/ml of PUFA for 30 min and then stimulated with FMLP. The results are presented as the mean±SD (number of experiments shown in parentheses).

systems used by the fatty acids to inhibit cell migration are different from those by which the fatty acids activate the neutrophil NADPH oxidase (9, 10), and by which they mediate the death of intraerythrocytic malaria parasites (25). We found that the fatty acid–induced activation of the neutrophil NADPH oxidase was sensitive to W-7, an inhibitor of the calmodulin-dependent enzyme system (31). In contrast, W-7 had no effect on the PUFA-induced cell migration inhibition. Furthermore, the migration inhibitory activity of the PUFA was not dependent on the phospholipase D–catalyzed hydrolysis of phospholipids since it could not be reduced by treating cells with ethanol and propranolol. A role for protein kinase C in both the fatty acid–induced NADPH oxidase (9, 10) and now migration inhibition was excluded on the basis of the inability of the protein kinase inhibitors H-7 and staurosporine to affect these activities of the fatty acids.

There are two other major points of interest with respect to the cell migration inhibitory properties of these fatty acids. One is whether the fatty acid needs to be converted to a metabolite for the expression of its activity. Several lines of evidence indicated that it was the intact PUFA, and not a metabolite or metabolites, that were responsible for the migration inhibitory effects. First, lipoxygenase and cyclooxygenase inhibitors did not alter the PUFA-induced depression of cellular locomotion. Second, the antioxidants SOD, catalase, and vitamin E had no effect on the PUFA inhibiting neutrophil migration. Finally, although the hydroperoxy of $C_{20:4,n-6}$ was also effective in inhibiting neutrophil migration, far higher concentrations were required than for the free fatty acid. In contrast, oxidized fatty acids were more effective than were the free fatty acids in effecting the killing of *Plasmodium falciparum*, a response abrogated by SOD, catalase, and vitamin E (25).

The other interest relates to the target upon which the fatty acids act. Our results with FMLP-induced chemokinesis showed that the PUFA had no effect on cell migration (chemokinesis) once the cells had been stimulated with FMLP. This suggests that the effects of these fatty acids are unlikely to be nonspecific. This is further supported by Corey and Rosoff (32), who excluded a detergent action as the mechanism of activation of NADPH oxidase by PUFA. Badwey et al. (11) reported that $C_{18:2,n-6}$ and $C_{20:4,n-6}$ readily partition into membranes affecting the protein–lipid interactions and concluded that the biological effects of the fatty acids were due to changes to the physical properties of the membranes rather than by acting as second messengers. Recently, Steinbeck et al. (33) have provided evidence that $C_{18:3,n-3}$ and $C_{20:4,n-6}$ induce similar decreases in polarization of the membrane lipid structure probe 1, 6-diphenyl-1,3,5-hexatriene, indicating that both fatty acids interact with the surface membrane and alter the structure of the plasma membrane to similar degrees. However, our results show that $C_{18:2,n-6}$ and $C_{18:3,n-3}$ had very little effect on neutrophil migration compared with $C_{20:4,n-6}$, $C_{20:5,n-3}$, and $C_{22:6,n-3}$. In view of these findings it is unlikely that the effect of PUFA on neutrophil migration is solely due to physical alterations of the cells from a membrane–fatty acid interaction.

Ca^{2+} mobilization is important for FMLP-induced chemotactic responses (34). Our studies showed that pretreatment of neutrophils with $C_{22:6,n-3}$ inhibited their chemotactic migration to FMLP and also decreased the amount of Ca^{2+} mobilized by this agonist. Thus, an effect of PUFA on the ability of FMLP to mobilize Ca^{2+} may in part contribute to the depressed cell migration seen. However, this is unlikely to contribute significantly to the migration inhibition induced by the PUFA since random migration in the absence of FMLP was also inhibited despite a rise in intracellular Ca^{2+} concentration induced by the PUFA alone. It is not known if these fatty acids prevent the coupling of the FMLP receptor to its G protein. $C_{20:4,n-6}$ has been reported to promote the binding of [^{35}S]GTPγS to neutrophil plasma membrane in a pertussis toxin–sensitive manner (35), conceivably leading to the activation of G protein–linked signaling systems. It is unlikely that such an effect would be inhibitory to random or chemoattractant-stimulated migration.

Cell migration is dependent on the reorganization of the actin cytoskeletal network (36, 37). Evidence is accumulating suggesting that the various cytoskeletal elements or their regula-regulation could be targets for PUFA action. Hoover et al. (38) reported that $C_{18:2,n-6}$ altered the organization of cytoskeletal elements of lymphocytes and found that the number of amoeboid, presumably motile forms was reduced after treatment with PUFA. In fibroblasts, actin reorganization leading to the accumulation of polymerized actin in membrane ruffles, and the formation of stress fibers and focal adhesions, are reported to be regulated by the small GTP-binding proteins, rac and rho (39, 40). Lysophosphatidic acid (LPA) and $C_{20:4,n-6}$ but not $C_{20:0}$ have been shown to inhibit *n*-chimaerin, a GTPase-activating protein (GAP) for rac (41) and rho-GAP (42). Such actions of the lipids would prevent inactivation of rac and rho, and would be expected to result in enhanced migration. In support of this, our preliminary data with LPA (data not presented), a lipid reported to stimulate the rho-dependent formation of stress fibers and focal adhesions (39), showed this acid to enhance migration of neutrophils in response to FMLP. The inhibition of migration by various fatty acids would be consistent with the suggestion that other sites of action may be possible. Thus, PUFA may affect the activation of rac and/or rho and/or a step downstream of the activation of the small GTP binding proteins such as the physical steps in actin polymerization and depolymerization or the formation of focal adhesions. For example, we have recently shown that PUFA induce increased expression of integrin receptors on neutrophils (43). This upregulation of integrin receptors induced by the fatty acids may result in very tight attachment of neutrophils to the extracellular matrix, rendering them immobile. It is well accepted that migration of neutrophils is influenced by the degree of adherence of the cells to the surface on which they crawl. Indeed our previous findings (43) showed that PUFA increased neutrophil adhesion. This effect was seen over a similar fatty acid concentration range as that causing inhibition of neutrophil migration. It was also evident that $C_{20:4,n-6}$ and $C_{22:6,n-3}$ were more effective than $C_{20:5,n-3}$ in increasing cell adhesion, and this then correlates with their ability to inhibit neutrophil migration.

Some PUFA were found to be effective at inducing migration inhibition at concentrations as low as 1 μg/ml ($\sim$ 3.3 μM) depending on the cell concentration. Although the exact physiological and pathophysiological concentration of these fatty acids are still to be clarified, recent measurements of $C_{20:4,n-6}$ generation after pancreatic islet cell stimulation showed intracellular concentrations of 50–100 μM (44). Furthermore, the free fatty acid concentration in plasma has been reported to be between 0.2 and 2.0 mM (45) and the percentage of $C_{20:4,n-6}$ in plasma free fatty acid can reach 9% (46). Thus, our findings are of physiological relevance.

Here we describe an additional activity of PUFA that is pertinent to cellular inflammation, immunity, and tissue damage. By interacting with neutrophils, the fatty acids render the cells unresponsive to chemotactic agents. It would thus appear from the observation of the weaker effect of $C_{20:5,n-3}$ on cell migration compared with $C_{20:4,n-6}$ and $C_{22:6,n-3}$, that there are advantages to increasing the ratio of $C_{22:6,n-3}/C_{20:4,n-6}$ compared with $C_{20:5,n-3}/C_{20:4,n-6}$ for therapeutic purposes in inflammatory diseases. The phenomenon of PUFA-induced neutrophil migration inhibition may be a mechanism for self-limiting inflammatory responses. When sufficient neutrophils are recruited they kill bacteria and die, releasing quite high concentrations of arachidonic and other unsaturated fatty acids. These could suppress further recruitment of cells. Since diets enriched in dihomogammalinolenic acid are reported to be beneficial in atopic diseases, it would be interesting to extend these studies to eosinophils.

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