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Activation of neutral sphingomyelinase in human neutrophils by polyunsaturated fatty acids

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SUMMARY

Although unesterified polyunsaturated fatty acids (PUFA) have been shown to elicit marked changes in neutrophil function, the associated signal transduction processes require clarification. In this study we examined the effect of PUFA on the sphingomyelin (SM)-signalling cycle in human neutrophils. Treatment of neutrophils with eicosatetraenoic acid [arachidonic acid, 20:4(*n*-6)] caused a decrease in the mass of cellular SM and an increase in the level of ceramide. 20:4(*n*-6)-stimulated neutral sphingomyelinase (SMase) activity of the leucocytes in a time- and concentration-dependent manner. Other unsaturated fatty acids, docosahexaenoic [22:6(*n*-3)], eicosapentaenoic [20:5(*n*-3)], octadecenoic [oleic, 18:1(*n*-9)] and octadecadienoic [linoleic, 18:2(*n*-6)] acids also had the capacity to activate neutral SMase; however, certain 20:4(*n*-6) derivatives {20:4(*n*-6) methyl ester [20:4(*n*-6)ME], 15-hydroperoxyeicosatetraenoic (15-HPETE) and 15-hydroxyeicosatetraenoic (15-HETE) acids}, very-long-chain PUFA {tetracosatetraenoic [24:4(*n*-6)] and octacosatetraenoic [28:4(*n*-6)] acids} and saturated fatty acids [octadecanoic (stearic, 18:0) and eicosanoic (arachidic, 20:0) acids] had no significant effect. Activation of neutral SMase by 20:4(*n*-6) appeared to involve metabolism via 20:4(*n*-6)CoA (arachidonoyl CoA) and was not dependent on prostaglandin and leukotriene synthesis. All of the fatty acids and derivatives tested failed to activate acidic SMase of neutrophils. Ceramide was found to inhibit 20:4(*n*-6)-induced superoxide generation by the cells. It is envisaged that the PUFA-induced ceramide production in neutrophils plays a role in the regulation of biological responses.

INTRODUCTION

Exogenous unesterified polyunsaturated fatty acids (PUFA) such as eicosatetraenoic acid [arachidonic acid, 20:4(*n*-6)] exert a range of effects on neutrophils including stimulation of superoxide production,[1–9] induction of degranulation,[10–12] inhibition of cell migration/chemotaxis,[13] modification of cell adhesion to endothelial cells[14] and enhancement of cell-surface receptor expression.[14] Several agonists can elicit release of endogenous 20:4(*n*-6) from membrane phospholipids by the activation of phospholipase A_2, and 20:4(*n*-6) thus liberated and its eicosanoid metabolites have been proposed to participate in neutrophil stimulation.[15–20] PUFA have been shown to induce several signal transduction processes linked to cellular responses which include activation of various enzymes, modulation of ion channels and interaction with GTP-binding proteins.[21–24]

Recent studies, predominantly those using the human leukaemia cell lines HL-60 and U937, have demonstrated that sphingolipids play a role in signal transduction and cell regulation. A sphingomyelin (SM)-signalling cycle has been identified whereby activation of sphingomyelinase (SMase) by certain extracellular agents (such as tumour necrosis factor-α, interferon-γ, interleukin-1β and 1α,25-dihydroxyvitamin D_3) leads to the hydrolysis of cellular SM to generate ceramide.[25–31] Ceramide has been identified as a key second messenger mediating many of the effects of extracellular agonists, including suppression of cell growth, promotion of cellular differentiation, down-regulation of the *c-myc* proto-oncogene,

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; C_2-ceramide, *N*-acetylsphingosine; DMSO, dimethyl sulphoxide; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; GLC, gas–liquid chromatography; HBSS, Hanks' balanced salt solution; 15-HETE, 15-hydroxyeicosatetraenoic acid; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; PUFA, polyunsaturated fatty acid(s); 20:4(*n*-6), eicosatetraenoic acid (arachidonic acid); 24:4(*n*-6), tetracosatetraenoic acid; 28:4(*n*-6), octacosatetraenoic acid; 20:5(*n*-3), eicosapentaenoic acid; 22:6(*n*-3), docosahexaenoic acid; 16:0, hexadecanoic acid (palmitic acid); 18:0, octadecanoic acid (stearic acid); 19:0, nonadecanoic acid (nonadecylic acid); 20:0, eicosanoic acid (arachidic acid); 22:0, docosanoic acid (behenic acid); 24:0, tetracosanoic acid (lignoceric acid); 18:1(*n*-9), octadecenoic acid (oleic acid); 22:1, docosenoic acid (erucic acid); 24:1, tetracosenoic acid (nervonic acid); 18:2(*n*-6), octadecadienoic acid (linoleic acid); 20:4(*n*-6)ME, arachidonic acid methyl ester; 20:4(*n*-6)CoA, arachidonoyl CoA; SM, sphingomyelin; SMase, sphingomyelinase; TLC, thin-layer chromatography.

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modulation of cellular protein phosphorylation and induction of programmed cell death (apoptosis).[25–31] In the present paper we have investigated the effect of PUFA on the SM-cycle in human neutrophils. We report that these fatty acids promote the release of ceramide from SM in these cells by the activation of a neutral SMase. Ceramide production in neutrophils elicited by PUFA may down-regulate biological responses.

MATERIALS AND METHODS

Materials

[Methyl-^{3}H]choline chloride (80 Ci/mmol) was purchased from Amersham Australia Pty. Ltd (North Ryde, NSW, Australia). 20:4(*n*-6), eicosapentaenoic acid [20:5(*n*-3)], docosahexaenoic acid [22:6(*n*-3)], octadecenoic acid [oleic acid, 18:1(*n*-9)], octadecadienoic acid [linoleic acid, 18:2(*n*-6)], octadecanoic acid (stearic acid, 18:0), eicosanoic acid (arachidic acid, 20:0), arachidonic acid methyl ester [20:4(*n*-6)ME], bovine serum albumin (BSA, essentially fatty acid free), 2′,7′-dichlorofluorescein, indomethacin, nordihydroguaiaretic acid (NDGA), *p*-nitrophenyl phosphate, β-glycerophosphate, phenylmethylsulphonyl fluoride, Na_3VO_4, Na_2MoO_4, ATP, leupeptin, aprotinin, benzamidine and butylated hydroxytoluene were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-acetylsphingosine (C_2-ceramide) and triacsin C were from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA) and dithiothreitol was from Eastman Kodak Co. (Rochester, NY). Silica gel 60 thin-layer chromatography (TLC) plates (200 × 200 × 0·25 mm) were obtained from E. Merck (Darmstadt, Germany) and scintillation cocktail (Opti Phase 'Hi Safe' 3) was supplied by LKB Wallac (Turku, Finland). To prepare [choline-methyl-^{3}H]SM (6 mCi/mmol), HeLa cells (seeded at $0·5 \times 10^6$ cells/25 ml Dulbecco's Modified Eagle's Medium/dish; 10 × 15 cm dishes) were incubated with [methyl-^{3}H]choline chloride (25 μCi/dish) at 37° in air/CO_2 (19:1, v/v) for 8 days. After harvesting the cells, lipids were extracted by the method of Bligh and Dyer[32] and radiolabelled SM purified by TLC (solvent system chloroform/methanol/acetic acid/water: 50:30:8:3·5, by vol.). Tetracosatetraenoic acid [24:4 (*n*-6)] was prepared as described by Street *et al.*[33] and octacosatetraenoic acid [28:4(*n*-6)] was synthesized by the procedures of Johnson[34] and Johnson and Poulos.[35] 15-hydroperoxyeicosatetraenoic acid (15-HPETE) and 15-hydroxyeicosatetraenoic acid (15-HETE) were prepared as previously described.[36] All other chemicals were of reagent grade. Solvents were distilled prior to use and contained the antioxidant butylated hydroxytoluene (0·005%, w/v).

Preparation of agonists

Fresh dilutions of fatty acids and derivatives (concentrated stocks in chloroform) were prepared daily by taking an aliquot of the stock to dryness under N_2, redissolving the residue in ethanol to make 20 mM, adding water to make 3·3 mM and then Hanks' balanced salt solution (HBSS) to make 20 μM and more dilute solutions. Indomethacin, NDGA, C_2-ceramide (concentrated stocks in ethanol) and triacsin C [concentrated stock in dimethyl sulphoxide (DMSO)] were diluted with HBSS immediately prior to use. Ethanol or DMSO alone appropriately diluted in HBSS were used as controls. The final concentration of ethanol or DMSO in neutrophil incubations was less than 0·2% (v/v). TLC and gas–liquid chromatography (GLC)–mass spectrometry indicated that the lipids were of high purity. Agonists and buffer were shown to be free of endotoxin contamination using the Limulus Amoebocyte Lysate assay.

Isolation of neutrophils

Human neutrophils were isolated from the peripheral blood of healthy volunteers by the rapid single-step method of Ferrante and Thong.[37] In the majority of cases, the preparation of neutrophils was of >99% purity and >99% viability as judged by morphological examination of cytospin preparations and the ability of viable cells to exclude trypan blue stain. Neutrophils were suspended in HBSS ($3·33 \times 10^6$ cells/ml) and used within 30 min of preparation. Trypan blue exclusion and lack of lactate dehydrogenase release indicated that the cells remained viable throughout subsequent incubations.

Determination of SM and ceramide mass

Neutrophils (10^7) were preincubated in 9 ml of HBSS at 37° for 5 min. The cells were then incubated with and without 20 μM 20:4(*n*-6) in 10 ml of HBSS at 37° for 15 min. The incubate was centrifuged at 600 ***g*** for 5 min at room temperature and the medium was discarded. The cell pellet was resuspended in 1 ml of water and 3·75 ml of chloroform/methanol/acetic acid (1:2:0·02, by vol.) was added[38] The mixture was left at 4° overnight and subsequently partitioned by the addition of 1·25 ml of chloroform and 1·25 ml of water.[32] Concentrated lipid extracts (three pooled samples) were applied to silica gel 60 TLC plates and developed in chloroform/methanol/acetic acid/water (50:30:8:3·5, by vol.) to isolate SM and in diethyl ether/methanol (99:1, by vol.) to isolate ceramide. Identification of the lipids was based on a comparison of their TLC mobility with that of authentic unlabelled standards. The SM and ceramide zones were located under ultraviolet light after spraying the plates with 0·2% (w/v) dichlorofluorescein in 95% (v/v) ethanol. The lipids were eluted from the silica gel with chloroform/methanol/acetic acid/water (50:39:1:10, by vol.)[39] and the extracts were partitioned with 1 M NH_4OH to remove the dye and then washed with methanol/water (1:1,v/v). The lipid samples were transesterified and the resulting fatty acid methyl esters quantified by GLC using nonadecanoic acid (nonadecylic acid, 19:0; 50 nmol) as a reference standard.[40]

Assay of neutral and acidic SMase

The micellar SMase assay using exogenous [choline-methyl-^{3}H]SM was conducted according to Wiegmann *et al.*[41] with some modifications. Neutrophils (3×10^6) were preincubated in the presence and absence of triacsin C (acyl CoA synthetase inhibitor), indomethacin (cyclooxygenase inhibitor) or NDGA (lipoxygenase inhibitor; 5–50 μM) in 2·7 ml of HBSS at 37° for 5 min. The cells were subsequently incubated with and without fatty acids or derivatives (2·5–30 μM) in 3 ml of HBSS at 37° for up to 30 min. The treatment was terminated by immersing the incubate in a methanol/dry ice bath for 5 seconds. After centrifugation at 600 ***g*** for 5 min at 4°, the cell pellet was washed twice with 3 ml of ice-cold HBSS. To measure neutral SMase activity, cell pellets were resuspended in 0·2 ml of lysis buffer containing 20 mM HEPES (pH 7·4), 10 mM $MgCl_2$, 2 mM EDTA, 5 mM dithiothreitol, 38 mM

p-nitrophenyl phosphate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulphonyl fluoride, 100 μM Na_3VO_4, 100 μM Na_2MoO_4, 750 μM ATP, 20 μM leupeptin, 5 μM aprotinin, 64 μM benzamidine and 0·2% (v/v) Triton-X-100. After 15 min at 4°, the cells were homogenized by repeated aspiration using a 0·2 ml pipette and then centrifuged at 800 *g* for 7 min at 4°. Aliquots of the supernatants (10 Gμl, containing approximately 10 μg of protein) were incubated with [choline-methyl-^{3}H]SM (0·02 μCi, 67 μM) in 50 Gμl final vol. of 20 mM HEPES, 1 mM $MgCl_2$ buffer (pH 7·4) at 37° for 120 min. The amount of [^{3}H]phosphocholine liberated from the [^{3}H]SM substrate was assessed by partitioning the reaction mixture with 0·8 ml of chloroform/methanol (2:1, v/v) and 0·25 ml of water and counting the radioactivity in the aqueous phase (0·4 ml). Radioactivity was measured using 8 ml of scintillation cocktail and a liquid scintillation counter set with external standardization and automatic efficiency control to correct for quenching (Model 1409; Wallac, Turku, Finland). In some cases the aqueous phase was subjected to TLC [solvent system methanol/1·2% (w/v)NaCl/28% (w/w) ammonia: 10:10:1, by vol.] and [^{3}H]phosphocholine was found to account for >95% of the total recovered radioactivity. To measure acidic SMase activity, washed cell pellets were resuspended in 0·2 ml of 0·2% (v/v) Triton-X-100. After 15 min at 4°, the cells were homogenized and centrifuged at 14 000 *g* for 2 min at 4°. Supernatant aliquots were incubated with radiolabelled SM in 50 Gμl final vol. of 250 mM sodium acetate, 1 mM EDTA buffer (pH 5·0) and the amount of [^{3}H]phosphocholine produced was determined as described for the neutral SMase assay.

Measurement of superoxide production

Superoxide release was measured by monitoring the chemiluminescence resulting from the oxidation of lucigenin, a reaction specific for superoxide.[42] Neutrophils (10^6) were preincubated in the presence and absence of C_2-ceramide or triacsin C (1·25–20 μM) in 0·4 ml of HBSS at 37° in an atmosphere of air/CO_2 (19:1, v/v) for 5 min. After preincubation, 20:4(*n*-6) (20 μM final concentration) or the corresponding control was added together with lucigenin (250 μM final concentration) in sufficient HBSS to bring the final assay volume to 1 ml. The mixture was immediately placed in a water-jacketed (37°) luminometer chamber (Model 1251 with MultiUse software; Bio Orbit Oy, Turku, Finland) and the resulting chemiluminescence was recorded over time immediately after addition of the lucigenin. The results were expressed as the maximum rate of superoxide production achieved (in mV) during the assay period (usually 10 min). Superoxide release was not measured by cytochrome *c* reduction because we observed previously that cytochrome *c* selectively inhibits fatty acid-induced superoxide production without significantly affecting responses to other agonists, possibly by binding the fatty acids and reducing their effective concentration.[8]

Statistical analyses

Results are expressed as mean ± SEM. Statistical analyses were performed by a two-tailed Student's *t*-test for unpaired data or by one-way analysis of variance (ANOVA) followed by the Dunnett test for multiple comparisons. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Table 1 shows that treatment of human neutrophils with 20 μM 20:4(*n*-6) for 15 min caused a significant decrease in the mass of SM and a corresponding increase in the level of ceramide. The fatty acid composition of the cellular SM and ceramide was similar, consisting mainly of hexadecanoic acid (palmitic acid, 16:0) and 18:0 with smaller amounts of 18:1, 20:0, docosanoic acid (behenic acid, 22:0), docosenoic acid (erucic acid, 22:1), tetracosanoic acid (lignoceric acid, 24:0) and tetracosenoic acid (nervonic acid, 24:1). Incubation of the cells with 20:4(*n*-6) did not alter the fatty acid composition of the sphingolipids and 20:4(*n*-6) was not incorporated into either compound (data not shown). These results provide indirect evidence that 20:4(*n*-6) induces the hydrolysis of SM to generate ceramide in neutrophils by the activation of SMase and was further investigated.

Ceramide can be generated from SM breakdown by several different types of SMase, including a neutral, Mg^{2+}-dependent SMase which is localized in the outer leaflet of the plasma membrane; a neutral SMase which shows no dependence on divalent cations and is resident in the cytosol and; an acidic SMase which has no dependence on divalent cations and is located in the endosomal/lysosomal compartments of the cell.[29,41,43–47] Each SMase appears to act on a distinct SM pool to release ceramide which plays a particular role in signal transduction.[46,47] Fig. 1 shows that incubation of neutrophils with 20 μM 20:4(*n*-6) caused rapid activation of neutral SMase which peaked at 5 min and then declined to almost the basal level after 10 min which persisted up to 30 min. The fatty acid had no significant effect on the activity of acidic SMase over the 30-min period (Fig. 1). The transient nature of neutral SMase activation by exogenous fatty acid may be due to suppressive feedback mechanisms in the cell. Activation of neutral SMase in the leucocytes was observed with a concentration of 20:4(*n*-6) as low as 2·5 μM. Maximum neutral SMase activity was obtained with 5 μM 20:4(*n*-6) and remained constant up to 30 μM (Fig. 2). Acidic SMase was not activated with any concentration of 20:4(*n*-6) used (Fig. 2). Deby-Dupont *et al.*[48] reported that the concentration of 20:4(*n*-6) in normal human plasma can be as high as 5 μg/ml (16·4 μM) and that this level can increase up to 10 fold in various circumstances related to stress (e.g. cardiovascular surgery). It is therefore likely that the level of 20:4(*n*-6) found to activate

Table 1. Effect of 20:4(*n*-6) on the mass of SM and ceramide in neutrophils

Lipid	Treatment	
	Control	20:4(*n*-6)
	Amount of lipid (nmol/3 × 10^7 cells)	
SM	24·6 ± 1·1	19·7 ± 0·9*
Ceramide	3·4 ± 0·5	7·6 ± 1·3*

Neutrophils (10^7) were preincubated in 9 ml of HBSS at 37° for 5 min. The cells were then incubated with and without 20 μM 20:4(*n*-6) in 10 ml of HBSS at 37° for 15 min. Cellular SM and ceramide levels (for three pooled samples) were determined as described in the Materials and Methods. Each value is the mean ± SEM of three analyses. This experiment was repeated twice with similar results. *$P < 0.05$, for significant differences between treatment with 20:4(*n*-6) and control (two-tailed Student's *t*-test for unpaired data).

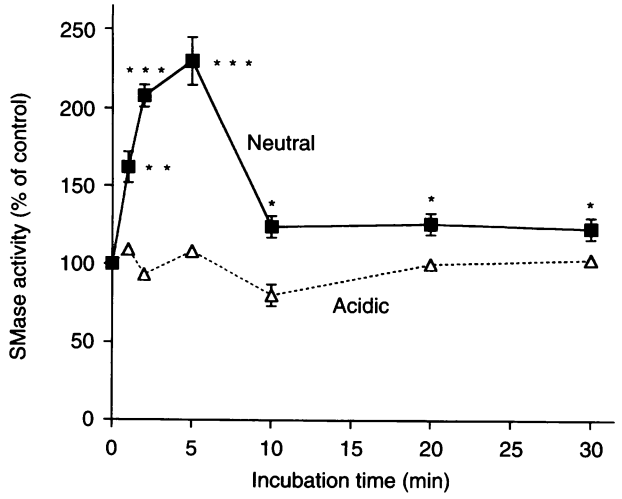

Figure 1. Effect of 20:4(*n*-6) incubation time on neutral and acidic SMase activity in neutrophils. Neutrophils (3×10^6) were preincubated in 2·7 ml of HBSS at 37° for 5 min. The cells were subsequently incubated with and without 20 μM 20:4(*n*-6) in 3 ml of HBSS at 37° for 0–30 min. Neutral and acidic SMase activity was determined as described in the Materials and Methods. Each point represents the mean ± SEM for three determinations. This experiment was performed three times with similar results. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$, for significant differences between treatment with 20:4(*n*-6) and corresponding control at a particular time point (two-tailed Student's *t*-test for unpaired data).

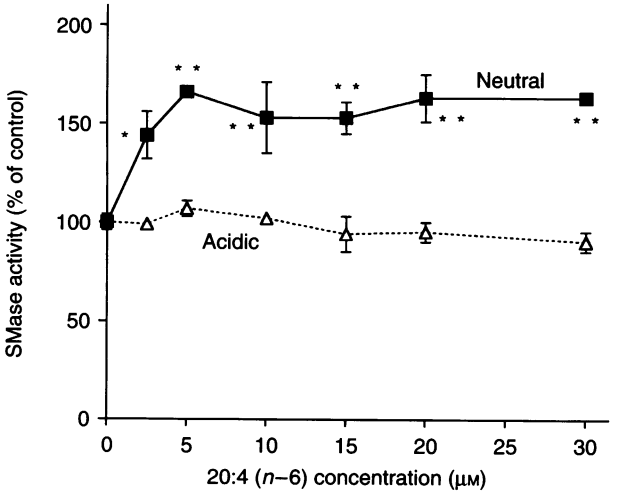

Figure 2. Effect of 20:4(*n*-6) concentration on neutral and acidic SMase activity in neutrophils. Neutrophils (3×10^6) were preincubated in 2·7 ml of HBSS at 37° for 5 min. The cells were then incubated with 0–30 μM 20:4(*n*-6) in 3 ml of HBSS at 37° for 2 min. Neutral and acidic SMase activity was determined as described in the Materials and Methods. Each point represents the mean ± SEM for three determinations. This experiment was conducted three times with similar results. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$, for significant differences between treatment with 20:4(*n*-6) and control (one-way ANOVA followed by the Dunnett test for multiple comparisons).

neutral SMase is physiologically attainable, particularly during inflammation and disease. Table 2 shows the effect of various fatty acids and derivatives on neutral SMase activity in neutrophils. Long-chain PUFA induced substantial activation of

Table 2. Effect of different fatty acids and derivatives on neutral SMase activity in neutrophils

Fatty acid or derivative	Neutral SMase activity (% of control)
Control	100 ± 7
20:4(*n*-6)	154 ± 6**
22:6(*n*-3)	153 ± 9**
20:5(*n*-3)	152 ± 4**
18:1(*n*-9)	130 ± 4*
18:2(*n*-6)	129 ± 2*
24:4(*n*-6)	120 ± 8
28:4(*n*-6)	102 ± 8
20:0	106 ± 9
18:0	101 ± 4
15-HPETE	109 ± 13
15-HETE	108 ± 3
20:4(*n*-6)ME	95 ± 6

Neutrophils (3×10^6) were preincubated in 2·7 ml of HBSS at 37° for 5 min. The cells were then incubated with and without various fatty acids or derivatives (20 μM) in 3 ml of HBSS at 37° for 2 min. Neutral SMase activity was determined as described in the Materials and Methods. Each value is the mean ± SEM of three analyses. This experiment was performed twice with similar results. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$, for significant differences between treatment with fatty acid or derivative and control (one-way ANOVA followed by the Dunnett test for multiple comparisons).

neutral SMase, particularly 20:4(*n*-6), 20:5(*n*-3) and 22:6(*n*-3) and to a lesser extent 18:1(*n*-9) and 18:2(*n*-6). Very-long-chain PUFA [24:4(*n*-6) and 28:4(*n*-6)], saturated fatty acids (18:0 and 20:0) and certain 20:4(*n*-6) derivatives [20:4(*n*-6)ME, 15-HPETE and 15-HETE] had no significant effect on the activity of the enzyme. None of the fatty acids or derivatives were found to activate acidic SMase (data not shown). After our manuscript was submitted for publication, Schissel *et al.*[49] reported that several cell types secrete an acidic Zn^{2+}-stimulated SMase. We could not detect SMase activity in the medium after incubation of neutrophils with 20:4(*n*-6) under our conditions (results not shown).

Figure 3 indicates that pretreatment of the neutrophils with triacsin C {to block the production of arachidonoyl CoA [20:4(*n*-6)CoA] from 20:4(*n*-6)} inhibited the activation of neutral SMase by 20:4(*n*-6). Conversely, pretreatment of the leucocytes with indomethacin or NDGA [to block the synthesis of prostaglandins and leukotrienes from 20:4(*n*-6), respectively] did not modulate the 20:4(*n*-6)-stimulation of the enzyme. These results suggest that conversion of PUFA to their CoA derivatives (and possibly subsequent esterification into complex lipids) may be necessary for their activation of neutral SMase. Activation of the enzyme is not dependent on the production of oxygenated fatty acid derivatives. Jayadev *et al.*[50] recently identified 20:4(*n*-6) as a mediator of SM hydrolysis in human leukaemia-derived HL-60 cells in response to tumour necrosis factor-α. They found that 20:4(*n*-6) activated neutral SMase in a cell-free system. Our data extend this previous report by demonstrating that 20:4(*n*-6) and other PUFA activate neutral SMase in intact human neutrophils.

PUFA induce multiple effects on neutrophil function.[1–14] The modes of regulation of these biological effects need to be further elucidated. Ceramide has been reported to accumulate in leucocytes stimulated with *N*-formyl-L-methionyl-L-leucyl-

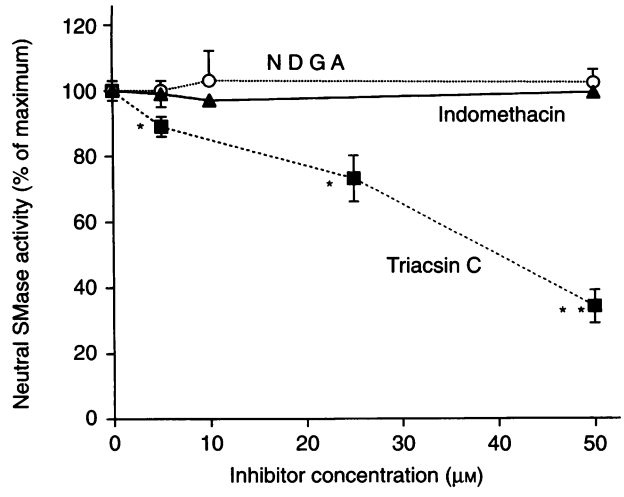

Figure 3. Effect of acyl CoA synthetase, cyclooxygenase and lipoxygenase inhibitors on 20:4(*n*-6) activation of neutral SMase in neutrophils. Neutrophils (3×10^6) were preincubated in the presence and absence of triacsin C, indomethacin or NDGA (5–50 μM) in 2·7 ml of HBSS at 37° for 5 min. The cells were then incubated with and without 20 μM 20:4(*n*-6) in 3 ml of HBSS at 37° for 2 min. Neutral SMase activity was determined as described in the Materials and Methods. Each point represents the mean ± SEM for three determinations. This experiment was performed twice with similar results. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$, for significant differences between pretreatment with and without inhibitor (one-way ANOVA followed by the Dunnett test for multiple comparisons). The inhibitors had no significant effect on the basal neutral SMase activity.

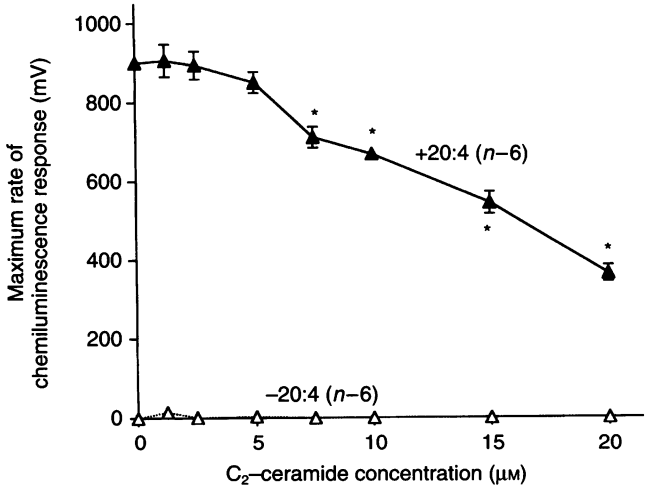

Figure 4. Effect of C_2-ceramide pretreatment on neutrophil superoxide production induced by 20:4(*n*-6). Neutrophils (10^6) were preincubated with and without C_2-ceramide (1·25–20 μM) in 0·4 ml of HBSS at 37° for 5 min. The maximum rate of superoxide production was then measured in the presence and absence of 20 μM 20:4(*n*-6) in 1 ml final volume of HBSS containing 250 μM lucigenin at 37° over 10 min as described in the Materials and Methods. Each point represents the mean ± SEM for three determinations and has been corrected for the response by cells incubated in HBSS alone. This experiment was repeated twice with similar results. $^*P<0{\cdot}01$, for significant differences between pretreatment with C_2-ceramide and control (one-way ANOVA followed by the Dunnett test for multiple comparisons).

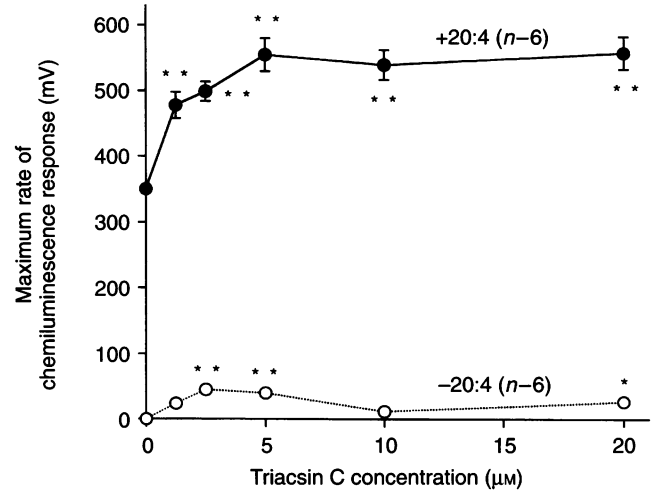

Figure 5. Effect of triacsin C pretreatment on neutrophil superoxide production induced by 20:4(*n*-6). Neutrophils (10^6) were preincubated with and without triacsin C (1·25–20 μM) in 0·4 ml of HBSS at 37° for 5 min. The maximum rate of superoxide production was then measured in the presence and absence of 20 μM 20:4(*n*-6) in 1 ml final volume of HBSS containing 250 μM lucigenin at 37° over 10 min as described in the Materials and Methods. Each point represents the mean ± SEM for three determinations and has been corrected for the response by cells incubated in HBSS alone. This experiment was conducted three times with similar results. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$, for significant differences between pretreatment with triacsin C and control (one-way ANOVA followed by the Dunnett test for multiple comparisons).

L-phenylalanine (FMLP) at a time when superoxide production is terminated[51] and that ceramide analogues are potent inhibitors of the respiratory burst.[51,52] Pretreatment of neutrophils with C_2-ceramide was found to suppress superoxide formation elicited by 20:4(*n*-6) (Fig. 4). When the cells were pretreated with triacsin C (shown above to be an inhibitor of neutral SMase and thus presumably a suppressor of ceramide formation) there was enhancement of 20:4(*n*-6)-induced superoxide production as anticipated (Fig. 5). It is noteworthy that 5 μM triacsin C gave rise to maximum promotion of 20:4(*n*-6)-induced superoxide production (Fig. 5) but this concentration only caused about 10% reduction in neutral SMase activity elicited by 20:4(*n*-6) (Fig. 3). Although we do not know the exact reason for this disparity, a possible explanation is that neutrophil superoxide formation is extremely sensitive to changes in the level of endogenous ceramide. In conclusion, it is likely that generation of ceramide from SM breakdown in neutrophils by the action of PUFA can negatively regulate responses initially induced by these agonists. This phenomenon, which may be applicable to other cell types, is currently being studied.

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