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A role for endogenous mono-hydroxy-eicosatetraenoic acids (HETEs) in the regulation of human neutrophil migration

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Summary. The possibility that endogenous mono-hydroxy-eicosatetraenoic acids (HETEs) derived from the lipoxygenation of arachidonic acid might serve a role in human neutrophil migration was examined by studying the effects of depletion of the intracellular HETEs on random migration and chemotaxis. The intracellular contents of approximately 2000 ng of 11-HETE and 500 ng of 5-HETE per 10^8 neutrophils are distributed preferentially in the cellular membranes and are increased by specific chemotactic factors. The depletion of intracellular HETEs that resulted from pre-incubating, washing and resuspending neutrophils in 3–20 μM 5,8,11,14-eicosatetraynoic acid (TYA), an inhibitor of lipoxygenase and cyclooxygenase activity, or in 5–10 μM nordihydroguaiaretic acid (NDGA), a selective inhibitor of lipoxygenase activity, was associated with suppression of neutrophil random migration and chemotaxis to several stimuli without evidence of cytotoxicity. Maximal suppression of migration was achieved by a 30–60 min pre-incubation with the inhibitors, a time-course analogous to that required for optimal depletion of the endogenous HETEs. In contrast, inhibitors of cyclooxygenase activity enhanced random migration and, to a lesser extent, chemotaxis. The inhibition of migration achieved by pre-incubating and maintaining the neutrophils in TYA or NDGA was fully reversed either by washing and resuspending the neutrophils in buffer or by the addition of purified neutrophil 5-HETE in quantities as small as 20 ng/2 × 10^6 neutrophils for random migration and 0·8 ng/2 × 10^6 neutrophils for chemotaxis, while the addition of 11-HETE was less effective. The relationship of the intracellular concentrations of endogenous HETEs to neutrophil migration is consistent with a potential role of the HETEs as cellular mediators.

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INTRODUCTION

The lipoxygenation of arachidonic acid in mammalian cells generates one or more specific mono-hydroxy-eicosatetraenoic acids (HETEs) and the HETEs that predominate in each instance are a distinctive characteristic of the cellular source (Borgeat, Hamberg & Samuelsson, 1976, 1977; Goetzl & Sun, 1979; Hamberg & Samuelsson, 1974a,b; Hubbard & Hough, 1978; Nugteren, 1975; Roberts, Lewis, Hansbrough, Austen & Oates, 1978; Valone, Franklin & Goetzl, 1980). The biological effects of the HETEs have been defined solely in terms of an extracellular role in the regulation of leucocyte function. Purified platelet-derived 12-L-HETE stimulates the directed migration or chemotaxis of human neutrophils and eosinophils *in vitro* at concentrations of 0·7–25 μg/ml, while 0·2–2

μg/ml of 12-L-HETE both stimulates the migration of the leucocytes in the absence of a concentration gradient, a phenomenon termed chemokinesis, and facilitates the responses of the leucocytes to other chemotactic factors (Goetzl, Woods & Gorman, 1977; Turner, Cambell & Lynn, 1975; Turner, Tainer & Lynn, 1977; Goetzl, 1978; Goetzl & Gorman, 1978). With HETEs purified from human neutrophils, eosinophils, and other sources, peak neutrophil and eosinophil chemotactic responses of comparable magnitude were achieved at concentrations of 1–2 μg/ml of 5-HETE, 5–10 μg/ml of 8-HETE or 9-HETE and 10–20 μg/ml of 11-HETE or 12-L-HETE (Goetzl, Brash, Tauber, Oates & Hubbard, 1980a; Goetzl, Weller & Sun, 1980b). A rank order of potency of the HETEs was not apparent for other functions as both the stimulation of chemokinesis and the enhancement of the expression of neutrophil and eosinophil receptors for C3b were optimal at concentrations of 0·4–2 μg/ml for all of the HETEs (Goetzl *et al.*, 1980a; Goetzl *et al.*, 1980b). The functional selectivity of the HETEs was demonstrated by their failure to influence other neutrophil activities including the generation of superoxide, the expression of IgG-Fc receptors and the release of lysosomal enzymes (Goetzl *et al.*, 1980a).

The present studies demonstrate that the suppression of neutrophil random migration and chemotaxis by lipoxygenase inhibitors is associated with a concurrent reduction in HETE content in the membranes of the neutrophils and is specifically reversed by the addition of purified neutrophil 5-HETE.

MATERIALS AND METHODS

Blind-end acrylic chemotactic chambers with a 0·2 ml stimulus compartment and a 0·5 ml leucocyte well (Neuroprobe, Inc., Bethesda, MD), 3 μm pore filters (Sartorius, distributed by Science Essentials Division of Beckman Instruments, Inc., Wakefield, MA), Hanks's balanced salt solution with or without phenol red (Microbiological Associates, Bethesda, MD), ovalbumin recrystallized five times (Miles Laboratories, Inc., Elkhart, IN), Sephadex G-25 and G-75, dextran and Ficoll–Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, NJ), arachidonic acid (Supelco, Inc., Bellefonte, PA), silica gel H 250 μm layer plates (Analtech, Inc., Newark, DE), 39 mm × 30 cm μPorasil columns (Waters Associates, Inc., Milford, MA), indomethacin, zymosan A, nordihydroguaiaretic acid (NDGA), *Micrococcus lysodeikticus*, reagent kits for assaying lactic acid dehydrogenase and β-glucuronidase, bis-*p*-nitrophenyl phosphate and adenosine 5′-triphosphate (ATP) (Sigma Chemical Co., St Louis, MO), DNase (Calbiochem, San Diego, CA), egg white lysozyme (Worthington Biochemical Co., Freehold, NJ), sucrose, ammonium molybdate and tris-(hydroxymethyl)-aminomethane (Fisher Scientific Co., Medford, MA), [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid (specific activity ≥ 60 Ci/mmol) and [γ-^{32}P]-ATP (New England Nuclear Corp., Boston, MA), Eagle's basal medium and heat-inactivated foetal calf serum (Grand Island Biological Co., Grand Island, NY), penicillin G and streptomycin (Eli Lilly & Co., Indianapolis, IN) and organic solvents that had been distilled from glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI), were obtained as noted. 5,8,11,14-eicosatetraynoic acid (TYA) and 9,12-octadecadiynoic acid (DYA) were supplied by Dr James G. Hamilton (Hoffman-La Roche, Inc., Nutley, NJ). Purified synthetic formyl-methionyl (f-Met) peptides were a gift from Dr R. J. Freer (Medical College of Virginia, Richmond, VA). Chemotactic fragments of the fifth component of human complement (C5fr) were generated by incubating 20 ml of fresh serum with 30 mg of zymosan particles for 20 min at 37° as described (Goetzl & Hoe, 1979) and were partially purified by gel filtration on a Sephadex G-75 column that was equilibrated and developed in 0·13 M NaCl–0·12 M Na acetate (pH 5·0). The predominant peak of neutrophil chemotactic activity, which exhibited a mol. wt of 16,800, was concentrated to 10 ml and served as the standard preparation of C5fr.

Assessment of human neutrophil random migration and chemotaxis

Human neutrophils were harvested from venous blood of normal subjects by a previously described method (Goetzl & Austen, 1972) and were centrifuged on Ficoll–Hypaque cushions to achieve a purity of greater than 96% (Böyum, 1968). The neutrophils were washed and resuspended at a concentration of $4{\cdot}2 \pm 0{\cdot}2 \times 10^{6}$ ml in Hanks's balanced salt solution made 0·005 M in Tris–HCl, pH 7·4, and containing 0·4 g/100 ml of ovalbumin (HBSS-OA). The incubation of migration chambers, the processing of filters, and the enumeration of neutrophils in stained filters were carried out as described (Goetzl & Gorman, 1978; Goetzl & Hoe, 1979). In filters from chambers lacking a stimulus, neutrophils were counted at two different levels: one was between 60 and 80 μm from the cell source where the magnitude of control random mig-

ration ranged from 6 to 12 neutrophils/high power field (hpf) and the second level was between 90 and 110 μm from the cell source where counts of 3–5 neutrophils/hpf served as the background for chemotactic responses. In filters from chambers with a chemotactic stimulus, the neutrophils were counted at the same level as had been selected for the background counts in that set of filters. Random migration was expressed as neutrophils/hpf and chemotaxis as net neutrophils/hpf after subtraction of the corresponding background migration in chambers lacking a stimulus. The alterations in neutrophil random migration or chemotaxis induced by the addition of a chemokinetic factor or an inhibitor of the oxygenation of arachidonic acid were expressed by presenting the altered levels of migration as a percentage of the respective control levels. Checkerboard analyses were performed to distinguish chemotaxis from chemokinesis (Wilkinson & Allan, 1978).

Production and purification of HETEs

Human neutrophil HETEs were generated as described (Goetzl & Sun, 1979) by incubating dialysed homogenates of $4–8 \times 10^8$ neutrophils with 2 mg of arachidonic acid and 2×10^6 d.p.m. of [^{3}H]-arachidonic acid at 37° for 4 h in the presence of 100 μM indomethacin. The HETEs were extracted, freed of arachidonic acid by ascending chromatography on a silica gel H thin-layer plate, and resolved and purified by gradient high pressure liquid chromatography (HPLC) (Model No. 322, Altex Scientific, Inc., Berkeley, CA) on a μPorasil column system as described (Goetzl & Sun, 1979).

[^{3}H]-12-L-HETE, that was employed as a tracer to assess the recovery of endogenous neutrophil HETEs, was generated as described for non-radioactive 12-L-HETE (Goetzl *et al.*, 1977; McGuire, Kelley, Gorman & Sun, 1978), except that homogenates of 5×10^{10} human platelets were dialysed for 48 h and filtered on a Sephadex G-25 column in 0·1 M KH_2PO_4–0·05 M Tris–HCl (pH 7·4) to remove endogenous arachidonic acid prior to the incubation with 2·32 μg of [^{3}H]-arachidonic acid (specific activity = 135 Ci/mmol). The [^{3}H]-12-L-HETE (specific activity = 14·3 Ci/mmol) was extracted and purified by sequential silicic acid column chromatography (Goetzl *et al.*, 1977) and HPLC (Goetzl & Sun, 1979). Purified HETEs were quantified by determining the optical density of methanolic solutions at 235 nm (Goetzl & Sun, 1979; McGuire *et al.*, 1978).

Analyses of the intracellular and subcellular distribution of HETEs

In order to achieve radiolabelling of newly synthesized HETEs, portions of $2–8 \times 10^8$ neutrophils were incubated with 2–8 μCi of [^{3}H]-arachidonic acid in 2–8 ml of Eagle's basal culture medium containing 100 U/ml of penicillin G, 50 μg/ml of streptomycin and 5% (v:v) foetal calf serum. After 6 h of rocking in a Petri dish at 37°, the labelled neutrophils were recovered, washed twice with the same medium, and resuspended in 4–6 ml of HBSS containing 0·1 g/100 ml of ovalbumin without or with varying concentrations of indomethacin, TYA, or NDGA. The neutrophils were incubated for an additional 15–120 min at 37° without or with C5fr or f-Met-Leu-Ala-Phe and then were centrifuged at 400 ***g*** for 5 min at 4°. The supernatants were transferred to a clean test tube and the neutrophils were resuspended in an equal volume of buffer and sonicated at 200 W for 15 s at 4°. An aliquot of [^{3}H]-12-L-HETE (20,000–30,000 c.p.m.) was added to each supernatant and sonicated neutrophil pellet prior to extraction to recover the endogenous HETEs (Goetzl & Sun, 1979; McGuire *et al.*, 1978).

For analyses of subcellular distribution, washed neutrophils containing radiolabelled endogenous HETEs were suspended in 2 ml of 0·34 M sucrose buffered at pH 7·0 with 0·02 M Tris–HCl and containing 2 μg/ml of DNase. The neutrophils were homogenized and the subcellular fractions resolved and recovered as described (Goetzl & Hoe, 1979). The 400 ***g*** supernatant from the homogenate was layered on a 12 ml 10–60% (g/100 ml) sucrose gradient and centrifuged at 20,000 ***g*** for 45 min at 4°, and then the top 4 ml, including the volume of the original homogenate and the upper region of the gradient, were centrifuged at 100,000 ***g*** for 1 h at 4° to separate the microsomes and other membranes from the cytosol. The 100, 400 and 100,000 ***g*** pellets were resuspended in 1 ml of 0·34 M sucrose and sonicated at 200 W for 2 min at 4° (Model 350, Branson Sonic Power Co., Danbury, CT). Portions of each fraction were assayed for the lysosomal markers (Bainton, Ullyot & Farquhar, 1971; Welsh & Spitznagel, 1971; West, Rosenthal, Gelb & Kimball, 1974) β-glucuronidase (Talay, Fishman & Huggins, 1946) and lysozyme (Smolelis & Hartsell, 1949), the cytoplasmic marker LDH (Kornberg, 1955), and the plasma-membrane markers magnesium-dependent ATPase (DePierre & Karnovsky, 1973; Harlan, DeChatelet, Iverson & McCall, 1977) and alkaline *p*-nitrophenyl phosphatase (Bretz & Baggiolini, 1974). One unit of enzymatic activity was

defined as the quantity of β-glucuronidase which liberated 1 μg of phenolphthalein from phenolphthalein glucuronide per hour, of LDH which generated 1 μmol of NADH per 30 min, of lysozyme which hydrolysed a quantity of *M. lysodeikticus* equivalent to that hydrolysed by 1 μg of egg white lysozyme in 1 h, of *p*-nitrophenyl phosphatase which hydrolysed 1 μmol of *p*-nitrophenyl phosphate per hour, and of ATPase which generated 1 pmol of γ-^{32}P per hour in the presence of a twenty-fold molar excess of *p*-nitrophenyl phosphate.

RESULTS

Effects of inhibitors of arachidonic acid oxygenation on human neutrophil migration

In the initial studies, varying concentrations of a range of inhibitors of the oxygenation of arachidonic acid were added to the neutrophils immediately prior to the assessment of migration (Fig. 1). TYA, which inhibits the activity of both lipoxygenase and cyclo-oxygenase (Samuelsson, 1976; Samuelsson, Granstrom, Green, Hamberg & Hammarstrom, 1975), and nordihydroguaiaretic acid (NDGA), which preferentially inhibits lipoxygenase activity (Tappel, Lundberg & Boyer, 1953), significantly suppressed neutrophil random migration and chemotaxis to C5fr in a dose–response fashion with no evidence of cytotoxicity as assessed by the exclusion of trypan blue dye. In four additional experiments (not shown in Fig. 1), the chemotactic response to 10^{-7} M f-Met-Leu-Ala-Phe was significantly suppressed to a mean (±SD) of 66±12%, 49±16%, and 27±8% of the control levels of 35–48 net neutrophils/hpf by 10, 20, and 40 μM TYA, respectively, and to 53±20% and 29±15% of the control levels by 5 and 10 μM NDGA. In contrast, indomethacin and DYA, which inhibit cyclo-oxygenase but not lipoxygenase activity (Samuelsson, 1976; Samuelsson *et al.*, 1975; Vanderhoek & Lands, 1973), significantly enhanced the random migration of neutrophils in a dose–response manner (Fig. 1). Over the same range of concentrations, indomethacin had no effect on the chemotactic response of neutrophils to C5fr (Fig. 1) or 10^{-7} M f-Met-Leu-Ala-Phe (not shown), while DYA only inhibited the chemotactic response to C5fr (Fig. 1) and to f-Met-Leu-Ala-Phe (not shown) by more than 20% at the highest concentrations of 20 and 40 μM. A time-dependent suppression of random migration and chemotaxis was observed for neutrophils that had been pre-incubated, washed, and resuspended in some concentrations of lipoxygenase inhibitors (Fig. 2) that were inactive or marginally suppressive in the absence of pre-incubation (Fig. 1). Thus, neutrophil chemotaxis was substantially suppressed by 30–60 min of pre-incubation in 3 μM TYA or 2 μM NDGA, while random migration was suppressed by 3 μM TYA but not by 2 μM NDGA. In addition, the suppression of migration that was observed at intermediate concentrations of the lipoxygenase inhibitors (Fig. 1) frequently was increased by pre-incubation (Fig. 2). Checkerboard analyses in two of the experiments confirmed the suppression of chemotaxis by the lipoxygenase inhibitors. In contrast, 20 μM indomethacin enhanced random migration significantly and augmented chemotaxis with peak effects at 30 min (Fig. 2).

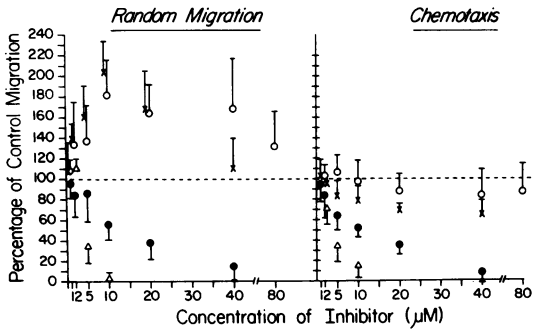

Figure 1. Dose–response relationships of the effects on human neutrophil migration of inhibitors of arachidonic acid oxygenation. Each point and bar represents the mean ±SD of the results of four experiments with neutrophils from separate donors. The control (100%) level of random migration ranged from 8·4 to 10·6 neutrophils/hpf and the control level of chemotaxis to a 1/80 dilution of C5fr ranged from 28·1 to 43·9 neutrophils/hpf. Random migration was significantly different from the control level at $P<0.01$ for 10 and 20 μM indomethacin, 2–20 μM DYA, 10–40 μM TYA, and 5 and 10 μM NDGA. Chemotaxis was significantly different from the control level at $P<0.01$ for 20 and 40 μM TYA and 2·5–10 μM NDGA. •, TYA; ▵, NDGA; ○, indomethacin; ×, DYA.

The intracellular content and subcellular distribution of endogenous HETEs in human neutrophils: effects of chemotactic stimuli and lipoxygenase inhibitors

The time-dependence of the suppression of random migration and chemotaxis by lipoxygenase inhibitors (Fig. 2) suggested that a functionally critical reduction in the neutrophil content of endogenous HETEs occurred within 60 min of the interruption of the synthetic pathway. Further, analyses of the elimination of exogenous [^{3}H]-12-L-HETE from prelabelled

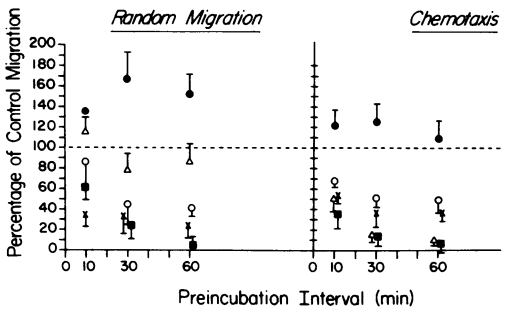

Figure 2. Time course of the effects on human neutrophil migration of inhibitors of arachidonic acid oxygenation. Each point and bar represents the mean ± SD of the results of three experiments with neutrophils from separate donors. The control (100%) level of random migration ranged from 7·3 to 10·8 neutrophils/hpf and the control chemotactic response to a 1/60 dilution of C5fr ranged from 29·8 to 50·7 net neutrophils/hpf. Random migration was significantly different from the control level at $P < 0{\cdot}02$ for 3 μM TYA at 30 and 60 min, 10 μM TYA at 10–60 min, 5 μM NDGA at 10–60 min and 20 μM indomethacin at 10–60 min. Chemotaxis was significantly different from the control level at $P < 0{\cdot}02$ for 3 μM and 10 μM TYA at 10–60 min and 2 μM and 5 μM NDGA at 10–60 min. ●, Indomethacin 20 μM; ○, TYA 3 μM; ×, TYA 10 μM; △, NDGA 2 μM; ■, NGDA 5 μM.

neutrophils indicated that the depletion was maximal after 60 min at 37° and was largely attributable to release rather than to degradation (Goetzl, Derian & Valone, 1980c). Thus, the content and release of the predominant endogenous HETEs were examined in the same time interval for three preparations of unstimulated neutrophils. The mean cellular contents of 5-HETE, 8-HETE, and 11-HETE were 586, 218, and 1824 ng/10^8 neutrophils, respectively, prior to incubation and were maintained at 493, 189, and 1841 ng/10^8 neutrophils after 30 min and at 488, 209, and 2016 ng/10^8 neutrophils after 60 min at 37° in HBSS-OA. The neutrophil intracellular contents accounted for a mean of 56% and 42% of the total 5-HETE, 58% and 40% of the 8-HETE, and 65% and 53% of the 11-HETE in the neutrophil suspensions at 30 min and 60 min of incubation, respectively.

The ability of chemotactic factors and inhibitors of lipoxygenase activity to influence the concentrations of endogenous 11-HETE and 5-HETE in the neutrophils and the supernatant fluid was analysed at several points during the initial 60 min of incubation at 37° (Fig. 3). The concentrations of 11-HETE and 5-HETE in the neutrophils incubated in buffer alone were maintained at 1870–2126 ng/10^8 neutrophils and 482–564 ng/10^8 neutrophils, respectively, while the levels of 11-HETE and 5-HETE released in the supernatants amounted to approximately 1500-2000 ng/10^8 neutrophils and 700–1000 ng/10^8 neutrophils, respectively, after 60 min. The introduction of a maximally chemotactic concentration of C5fr stimulated increases in the neutrophil content of 11-HETE and 5-HETE that were apparent by 15 min, the earliest time point examined, were maximal at 30 min and had diminished by 60 min (Fig. 3). The concentrations of both HETEs in the supernatant fluids were higher at all time points with C5fr stimulation, and the ratios for C5fr relative to buffer achieved peaks at 60 min of 2·3 for 11-HETE and 2·7 for 5-HETE. In two additional experiments (not shown), the introduction of 10^{-7} M f-Met-Leu-

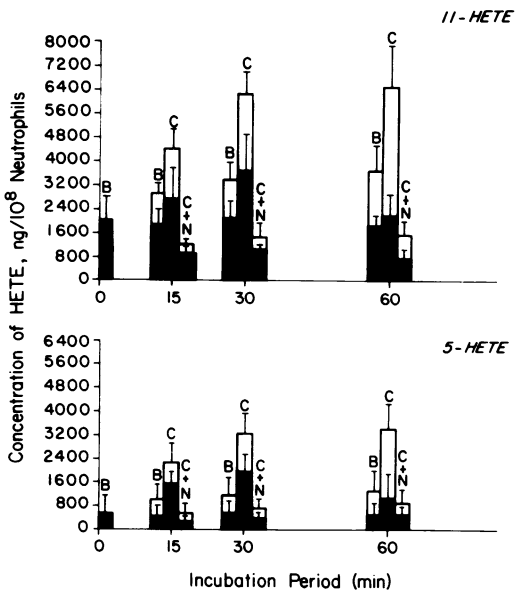

Figure 3. Effects of C5fr and an inhibitor of lipoxygenase activity on the endogenous HETE levels in human neutrophils. In each of three experiments, seven portions of 0·7–0·8 × 10^8 neutrophils were pre-incubated 30 min at 37° in HBSS-OA and three portions were pre-incubated similarly in HBSS-OA containing 5 μM nordihydroguaiaretic acid (N). After being washed twice, three of the portions that had been pre-incubated in HBSS-OA were resuspended in HBSS-OA alone (B), three were resuspended in a 1/80 final dilution of C5fr (C), and one was extracted without further incubation (B, time 0), while the three portions that had been pre-incubated in N were resuspended in the C5fr (N+C). After incubation at 37° for the times indicated, the supernatant fluids (□) and the neutrophil pellets (■) that had been resuspended in HBSS-OA were sonicated, acidified, and extracted to permit the recovery and quantification of the endogenous HETEs. Each bar and bracket represents the mean ± SD of the results of the three experiments.

Ala-Phe increased the mean neutrophil content of 11-HETE by 61% and of 5-HETE by 197% at 30 min and resulted in 1·8-fold and 2·4-fold mean rises in the supernatant concentrations of 11-HETE and 5-HETE, respectively, at 60 min. A 30 min pre-incubation of neutrophils in 5 μM nordihydroguaiaretic acid (NDGA or N), a lipoxygenase inhibitor, not only prevented the C5fr-induced rises in the concentrations of 11-HETE and 5-HETE (Fig. 3), but suppressed the neutrophil content of 11-HETE in the presence of C5fr to 50% or less of the baseline content at all time points and suppressed the corresponding contents of 5-HETE to 62% and 71% of the baseline values at 15 min and 30 min, respectively. In one of the experiments (not shown) pre-incubation of the neutrophils with 5 μM NDGA without the addition of C5fr reduced the baseline concentrations of 11-HETE to 36%, 32% and 27% of the values in buffer alone at 15 min, 30 min, and 60 min, respectively, and reduced the corresponding baseline concentrations of 5-HETE to 59%, 35% and 41% of the control values. NDGA also reduced the extent of release of 11-HETE and 5-HETE in the presence of C5fr to less than one-half of that for neutrophils incubated in buffer alone (Fig. 3).

The subcellular distribution of the predominant endogenous HETEs was assessed employing neutrophils that had been pre-incubated with [^{3}H]-arachidonic acid, washed and incubated in 80 μM indomethacin or 20 μM TYA for 60 min at 37°. Each subcellular fraction was extracted and the constituent HETEs were resolved by sequential thin layer chromatography and HPLC. The predominant components, 11-HETE and 5-HETE (Table 1), were identified by co-chromatography with standards that had been defined by gas chromatography–mass spectrometry. When quantified by optical density at 235 nm, the total yield from 10^8 neutrophils was 1360 ng of 11-HETE and 684 ng of 5-HETE in the presence of indomethacin alone, of which over 85% of each was localized in the membrane-rich 100 *g* pellet and the light membrane fraction from the sucrose gradient. Incubation of the neutrophils in 20 μM TYA, followed by washing, reduced the contents of 11-HETE and 5-HETE by 38% and 43%, respectively, in the 100 *g* pellet, by 33% and 65% in the light membrane fraction, and by 36% and 58% overall (Table 1). The calculation of the contents of HETEs from the levels of radioactivity was based on an estimate of the specific activity of arachi-

Table 1. Subcellular distribution of endogenous HETEs in human neutrophils

Pre-incubation condition*	HETE	100 *g* pellet†	400 *g* pellet†	Sucrose gradient†			
				Primary granules	Secondary granules	Membranes	Cytosol
Indomethacin (80 μM)	11-HETE	720‡/730§	—/51	—/19	—/26	480/365	160/137
	5-HETE	370/99	—/16	—/6	—/11	220/54	94/36
TYA (20 μM)	11-HETE	450/406	—/—	—/—	—/—	320/159	106/95
	5-HETE	210/42	—/—	—/—	—/—	78/28	—/13

* Portions of 3×10^8 neutrophils that had taken up [^{3}H]-arachidonic acid were pre-incubated in buffer with 80 μM indomethacin or 20 μM TYA and washed twice prior to subcellular fractionation; the washed neutrophils retained 279,000 d.p.m. and 240,000 d.p.m., respectively. The HETEs extracted from each subcellular fraction were purified by sequential thin layer and high pressure liquid chromatography.

† The 100 *g* pellet contained 43% of the total Mg^{2+}-ATPase and 36% of the total *p*-nitrophenyl phosphatase recovered; the 400 *g* pellet contained nuclei as assessed by phase-contrast microscopy, but less than 10% of any of the enzymatic markers; the primary granules contained 65% of the β-glucuronidase and 24% of the lysozyme; the secondary granules contained 50% of the lysozyme; the crude light membrane fraction contained 41% of the Mg^{2+}-ATPase and 39% of the *p*-nitrophenyl phosphatase; and the cytosol contained 88% of the LDH, 16% of the β-glucuronidase and 13% of the lysozyme. The mean of the total content of enzyme (100%) per 10^8 neutrophils was: 15·9 units for β-glucuronidase, 284 units for lysozyme, 35·8 units for Mg^{2+}-ATPase, 216 units for *p*-nitrophenyl phosphatase, and 1·3 units for LDH.

‡ The concentration of each HETE on the left of the diagonal line was calculated from the OD at 235 nm; the units are ng/10^8 neutrophils.

§ The concentration of each HETE on the right of the diagonal line was calculated from the radioactivity determined in a β-scintillation counter; the units are ng/10^8 neutrophils.

donic acid and its products. This value was calculated from a content of arachidonic acid of 0·08 mg/10^8 neutrophils and from the total radioactivity of labelled neutrophils that had been incubated in indomethacin or TYA. The arachidonic acid content of human neutrophils was derived from the known quantities of phospholipids (Gottfried, 1967) and the number of moles of arachidonic acid per 100 moles of fatty acids in the phospholipids (Smolen & Shohet, 1974). The contents of 11-HETE in the subcellular fractions of neutrophils incubated in indomethacin or TYA, as determined from the levels of radioactivity, were comparable to those calculated from the measurements of optical density (Table 1). In contrast, the contents of 5-HETE determined from the levels of radioactivity ranged from 20 to 36% of the contents calculated from the measurements of optical density, but did indicate a depletion of 5-HETE by the incubation in TYA.

Reversal of the suppressive effects of lipoxygenase inhibitors on human neutrophil migration

The potential reversibility of the inhibition of human neutrophil migration achieved by TYA and NDGA was studied both by washing and resuspending the neutrophils in buffer without inhibitor and by supplying neutrophil-derived HETEs exogenously in the continued presence of the inhibitors. Neutrophils that had been pre-incubated for 30 min at 37°, washed and resuspended in 10 μM TYA exhibited intracellular concentrations of 11-HETE and 5-HETE that were 58% and 56% of the respective control values, while comparable pre-incubation and maintenance in 5 μM NDGA reduced the concentrations of the HETEs to 43% and 33% of those of control neutrophils (Table 2). The random migration and chemotaxis of the neutrophils depleted of endogenous HETEs by TYA or by NDGA were suppressed to a similar extent. When portions of neutrophils that had been pre-incubated with TYA or NDGA were washed and resuspended in buffer alone, the neutrophil contents of 11-HETE and 5-HETE were restored to 84% or more of the corresponding levels in control neutrophils and the inhibition of random migration and chemotaxis was reversed concomitantly (Table 2).

Purified neutrophil HETEs were added to portions of 2×10^6 neutrophils which had been pre-incubated for 30 min and resuspended in 10 μM TYA, and to control neutrophils, utilizing quantities of HETEs that encompassed the 5–25 ng amounts depleted by the treatment with TYA (Fig. 4). 5-HETE and 11-HETE alone and in combination enhanced the random and chemotactic migration of control neutrophils by 60% or less at the concentrations that were examined. The suppressed random migration of HETE-depleted neutrophils was restored by 5-HETE and 11-HETE with different dose–response relationships. Random migration was returned to over 80% of the corresponding

Table 2. Reversal of the suppression of human neutrophil migration by the removal of lipoxygenase inhibitors

	Control	TYA	TYA + washing	NDGA	NDGA + washing
Intracellular concentration of HETE (ng/10^8 neutrophils)*					
11-HETE	1911 ± 326	1116 ± 265	1609 ± 491	824 ± 284	1761 ± 347
5-HETE	608 ± 94	342 ± 108	543 ± 125	202 ± 41	584 ± 115
Random migration (% of control)†	—	17 ± 6	81 ± 14	12 ± 5	106 ± 10
Chemotaxis (% of control)‡	—	39 ± 13	93 ± 22	20 ± 9	123 ± 31

* Portions of $1{\cdot}6 \times 10^8$ neutrophils were pre-incubated at 37° for 30 min in 10 μM TYA, 5 μM NDGA, or HBSS-OA alone, washed twice, and resuspended in TYA, NDGA, or HBSS-OA alone (control, TYA + washing, and NDGA + washing). Samples of neutrophils were removed immediately for assessment of migration and the remainder of each portion was incubated for 30 min at 37°, centrifuged and the neutrophil pellet extracted for purification and quantification of intracellular HETEs. Each value is the mean ± 0·5 the range for the results of two experiments.

† Control random migration (100%) was 10 and 13 neutrophils/hpf, respectively, in the two experiments; each value is the mean ± 0·5 the range.

‡ Control chemotaxis to a 1/80 dilution of C5fr (100%) was 42 and 38 net neutrophils/hpf, respectively in the two experiments; each value is the mean ± 0·5 the range.

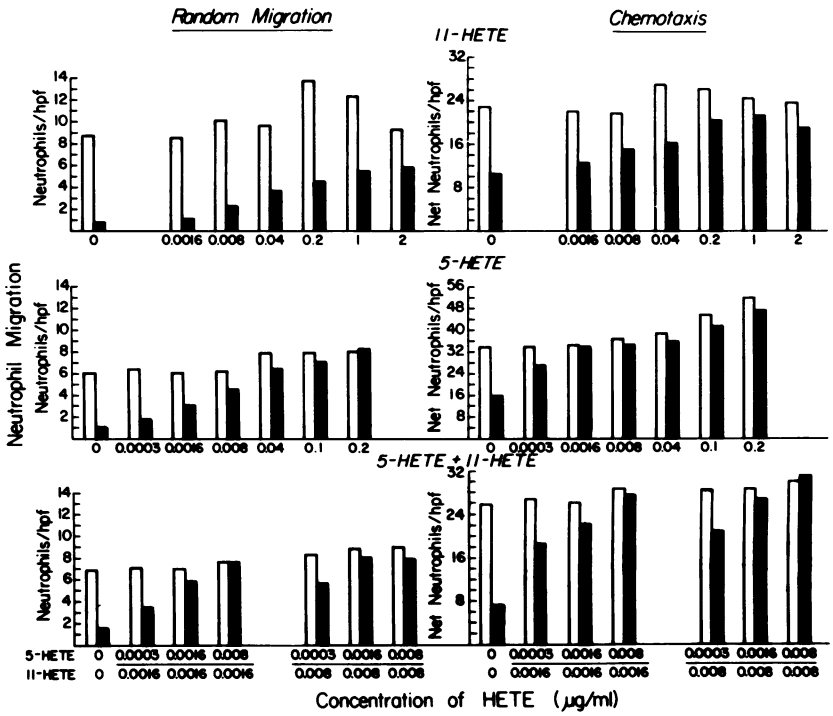

Figure 4. Reversal of TYA inhibition of neutrophil migration by endogenous HETEs. Portions of neutrophils were pre-incubated in buffer with (■) or without (□) 10 μM TYA for 30 min at 37°, washed and resuspended in buffer or 10 μM TYA prior to the addition of the concentration of 5-HETE and/or 11-HETE that are indicated. Each bar is the mean of two experiments for 11-HETE alone, 5-HETE alone, or the combination of 5-HETE and 11-HETE; the control values are shown as the first set of bars on the left in each frame.

control level by 0·04 μg/ml or 20 ng/2 × 10^6 neutrophils of 5-HETE, but was improved only to 64% of the control level with 2 μg/ml of 11-HETE. Similarly, the suppressed chemotaxis of HETE-depleted neutrophils was restored to 80% or higher of the corresponding control levels by 0·0016 μg/ml or 0·8 ng/2 × 10^6 neutrophils of 5-HETE, while 0·2 μg/ml or more of 11-HETE was needed to achieve a comparable degree of restoration. A checkerboard analysis in one of the experiments indicated that the enhanced migration was predominantly chemotaxis. The addition of 0·0016 μg/ml or 0·008 μg/ml of 11-HETE with 5-HETE lowered the concentration of 5-HETE required for restoration of random migration to 0·0016 μg/ml. In contrast, the concentration of 5-HETE which restored chemotaxis was not less in the presence of 0·0016 μg/ml or 0·008 μg/ml of 11-HETE than with 5-HETE alone (Fig. 4).

DISCUSSION

The specific subcellular distribution of the HETEs in human neutrophils and the rapid alterations in the intraneutrophil levels of HETEs in response to relevant stimuli and inhibitors suggest a critical functional role for the products of lipoxygenation of arachidonic acid in the neutrophil. Means of approximately 2000 ng of 11-HETE and 500 ng of 5-HETE were recovered from 10^8 unstimulated neutrophils. As the efficiency of extraction of [^{3}H]-12-L-HETE from pre-labelled neutrophils was 55–60%, approximately 90 pmol of 11-HETE and 23 pmol of 5-HETE are contained in 10^6 unstimulated neutrophils. The intracellular contents of the quantitatively predominant 5-HETE and 11-HETE were maintained during periods of incubation of 15–60 min at 37° in buffer alone (Fig. 3). Within 15 min after the addition of C5fr, the intracellular level of HETEs increased significantly and achieved peak levels at 30 min that were 175% and 345%, respectively, of the quantities of 11-HETE and 5-HETE in unstimulated neutrophils. The rates of release of 11-HETE and 5-HETE by neutrophils incubated for 60 min at 37° in buffer alone were approximately

1500–2000 ng/10^8 neutrophils and 700–1000 ng/10^8 neutrophils, respectively, which were increased 2·3-fold and 2·7-fold by C5fr (Fig. 3). The pre-incubation of human neutrophils with radiolabelled arachidonic acid prior to homogenization permitted an analysis of the subcellular distribution of the endogenously produced 5-HETE and 11-HETE (Table 1). Over 85% of the endogenous contents of 1360 ng of 11-HETE and 684 ng of 5-HETE were localized in the membrane-rich fractions which included the plasma membrane, as assessed by the distribution of defined enzymatic markers. The remainder of the endogenous HETEs were recovered in the cytosol, while essentially none were found in the nuclei or lysosomal granules.

Analyses of the time course of depletion of intracellular HETEs by the incubation of neutrophils with lipoxygenase inhibitors suggested that 30–60 min were required for the extracellular release of quantities of HETEs equal to the intracellular contents of unstimulated neutrophils. The pre-incubation for 30 min at 37°, washing and resuspension of neutrophils in 10 μM TYA or 5 μM NDGA reduced the intracellular concentrations of HETEs to approximately 40–65% of the levels in neutrophils processed in buffer alone (Fig. 3, Table 2). HETEs were depleted to a similar extent in the subcellular fractions of neutrophils by a 60 min incubation at 37° in 20 μM TYA prior to washing and homogenizing the neutrophils (Table 1). As assessed by the optical density of the purified HETEs, the pre-incubation in TYA reduced the content of 11-HETE and 5-HETE in the neutrophil membrane fractions by 33–38% and 43–65%, respectively. A similar extent of depletion of 11-HETE was calculated from the determinations of radioactivity (Table 1), but the very low specific radioactivity of the 5-HETE precluded a meaningful parallel calculation.

The non-cytotoxic suppression of neutrophil random migration and chemotaxis to C5fr and f-Met-Leu-Ala-Phe by inhibitors of lipoxygenase activity (Figs 1 and 2) provided the initial evidence that endogenous HETEs are critical for the normal maintenance of neutrophil mobility. NDGA, a selective lipoxygenase inhibitor (Tappel *et al.*, 1953), and TYA, which inhibits both lipoxygenase and cyclo-oxygenase activities (Samuelsson, 1976; Samuelsson *et al.*, 1975), suppressed random and chemotactic migration without altering cellular viability (Fig. 1). In contrast, the cyclo-oxygenase inhibitors that have no effect on lipoxygenase activity, such as indomethacin (Samuelsson *et al.*, 1975) and DYA, a fatty acid with triple bonds that is structurally analogous to TYA (Osbond, Philpott & Wickens, 1961; Vanderhoek & Lands, 1973), enhanced random migration with little or no effect on chemotaxis. When neutrophils were pre-incubated with lipoxygenase inhibitors for varying intervals prior to washing and resuspending the neutrophils with the inhibitors, optimal suppression of neutrophil migration was observed after 30–60 min of pre-incubation which correlates with the period required for the turnover of the rapidly exchanging compartments of endogenous neutrophil HETEs (Fig. 3). Concentrations of TYA and NDGA which reduced the neutrophil content of 11-HETE by 42% and 57%, respectively, and of 5-HETE by 44% and 67% after 30 min of pre-incubation resulted in nearly maximal suppression of random migration and chemotaxis (Table 2). Washing the neutrophils removed sufficient amounts of the inhibitors to restore the intracellular concentrations of endogenous HETEs to 84% or more of the levels in control neutrophils and concomitantly reversed the suppression of random migration and chemotaxis.

The ability to restore the suppressed levels of migration of HETE-depleted neutrophils to normal by the addition of purified neutrophil-derived HETEs established that the effects of the lipoxygenase inhibitors are primarily attributable to a reduction in the normal concentrations of HETEs in neutrophil membranes (Fig. 4). The extent of restoration of the intracellular levels of endogenous HETEs that was required for reversal of the suppression of migration was a function both of the specific HETE and the form of migration. The HETE content of 2×10^6 neutrophils was reduced by approximately 10–25 ng for 11-HETE and 6–8 ng for 5-HETE following pre-incubation with TYA and washing (Fig. 4). The addition of only 0·8 ng of 5-HETE per 2×10^6 neutrophils reversed the chemotactic defect, while 20 ng of 5-HETE were necessary to reverse the suppression of random migration. For 11-HETE, 100 ng per 2×10^6 neutrophils were required to reverse the chemotactic defect and up to 1000 ng failed to reverse the suppression of random migration. The greater potency of 5-HETE than 11-HETE as a neutrophil chemotactic factor thus is maintained in its role as an intracellular mediator of neutrophil migration.

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