Platelet-neutrophil interactions. 12S,20- and 5S,12S-dihydroxyeicosapentaenoic acids: two novel neutrophil metabolites from platelet-derived 12S-hydroxyeicosapentaenoic acid

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Abstract. Dietary marine n-3 polyunsaturated fatty acids have demonstrated an antiinflammatory potential in epidemiologic and intervention studies in humans. Proposed mechanisms, involving only leukocytes, fall short of explaining this potential completely. Enriched by dietary means with eicosapentaenoic acid (EPA), stimulated human platelets release substantial amounts of eicosapentaenoic acid and 12S-hydroxyeicosapentaenoic acid (12S-HEPE) in addition to 12S-hydroxyeicosatetraenoic acid (12S-HETE) derived from arachidonic acid. Human neutrophils metabolize 12S-HETE to 5S,12S-DiHETE when stimulated, whereas unstimulated neutrophils produce 12S,20-DiHETE. This study was undertaken to characterize metabolism of 12S-HEPE in human neutrophils. We demonstrate herein for the first time that 12S-HEPE is metabolized by human neutrophils. In unstimulated neutrophils 20-hydroxylation to 12S,20-DiHEPE occurs, whereas in stimulated neutrophils 5-lipoxygenation to 5S,12S-DiHEPE takes place. The structures of these metabolites were characterized by their relative retention times on reversed-phase high pressure liquid chromatography, by their UV absorbance spectra, and by gas-liquid chromatography-mass spectrometry. With increasing amounts of 12S-HEPE, stimulated neutrophils produced increasing amounts of 5S,12S-DiHEPE, which is virtually inactive biologically. Concomitantly, production of the potent chemokinetic and chemoattractant arachidonic acid derivative leukotriene B_4 decreased. Thus, 12S-HEPE can compete with endogenous arachidonic acid for 5-lipoxygenation in stimulated human neutrophils. 12,20-DiHEPE, LTB_5, and 5S,12S-DiHEPE were detectable after coincubating EPA-enriched platelets with unenriched neutrophils, and arachidonic acid-derived 5-lipoxygenase products were decreased. We conclude that 12S-HEPE can participate in platelet-neutrophil interactions in a manner similar to 12S-HETE. By providing competing substrates for neutrophil 5-lipoxygenase, platelets might contribute to the antiinflammatory potential of dietary n-3 fatty acids through platelet-neutrophil interaction. —**von Schacky, C., A. J. Marcus, L. B. Safier, H. L. Ullman, N. Islam, M. J. Broekman, and S. Fischer.** Platelet-neutrophil interactions. 12S,20- and 5S,12S-dihydroxyeicosapentaenoic acids: two novel neutrophil metabolites from platelet-derived 12S-hydroxyeicosapentaenoic acid. *J. Lipid Res.* 1990. **31:** 801-810.

Supplementary key words 5-lipoxygenase • dietary n-3 fatty acids

Epidemiologic observations and studies in animals and humans document an antiinflammatory potential of fish and fish oils, rich in the two polyunsaturated n-3 fatty acids eicosapentaenoic (EPA) and docosahexaenoic acid (1). This potential is thought to be mediated by alterations in leukotriene formation by leukocytes found as a result of dietary n-3 fatty acids (1). Neutrophil 5-lipoxygenase gives rise to LTB_5, which is one order of magnitude less active than the potent chemotactic and chemokinetic compound LTB_4 derived from arachidonic acid (2-9). The amount of LTB_4 formed was found reduced after 4-6 weeks of fish oil diet in the majority of studies (6-9);

Abbreviations: EPA, eicosapentaenoic acid; HPLC, high performance liquid chromatography; EI, electron impact; 12S-HETE, 12S-hydroxy-5,8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid; 5S,12S-DiHETE, 5S,12S-dihydroxy-6-*trans*-8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid; 12S,20-DiHETE, 12S,20-dihydroxy-5,8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid; 20-OH-leukotriene (LT) B_4, 5S,12R,20-trihydroxy-6-*cis*-8,10-*trans*-14-*cis*-eicosatetraenoic acid; 20-COOH-LTB_4, 5S,12R-20-carboxy-6-*cis*-8,10-*trans*-14-*cis*-eicosatetraenoic acid; 12S-HEPE, 12S-hydroxy-5,8-*cis*-10-*trans*-14,17-*cis*-eicosapentaenoic acid; LTB_5, 5S,12R-dihydroxy-6-*cis*-8,10-*trans*-14,17-*cis*-eicosapentaenoic acid; LTB_4, 5S,12R-dihydroxy-6-*cis*-8,10-*trans*-14-*cis*-eicosatetraenoic acid; 12S,20-DiHEPE, 12S,20-dihydroxy-5,8-*cis*-10-*trans*-14,17-*cis*-eicosapentaenoic acid; 5S,12S-DiHEPE, 5S,12S-dihydroxy-6-*trans*-8-*cis*-10-*trans*-14,17-*cis*-eicosapentaenoic acid; 5S-HETE, 5S-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid.

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parameters of leukocyte function, however, were not consistently altered (1). Since the concentrations of EPA necessary for inhibition of LTB_4 formation in vitro are not attained in vivo (3, 5, 6, 8), competition between both endogenous arachidonic acid and EPA for neutrophil 5-lipoxygenase cannot be invoked as the sole mechanism for reduction of LTB_4 formation.

In the course of cell–cell interactions, the arachidonic acid lipoxygenase product, 12S-HETE, is released from stimulated platelets, and is metabolized by human neutrophils (10). In stimulated human neutrophils, 5-lipoxygenation of 12S-HETE to 5S,12S-DiHETE occurs (11–13). In unstimulated neutrophils, however, a cytochrome P450 enzyme system catalyzes formation of 12S,20-DiHETE (14–16), which is further metabolized to 12S-HETE-1,20-dioic acid, probably by a neutrophil dehydrogenase also responsible for conversion of 20-OH-LTB_4 to 20-COOH-LTB_4 (17, 18).

Dietary EPA is also incorporated into human platelet phospholipids at the sole expense of arachidonic acid (1, 19). Upon platelet stimulation, arachidonic acid and EPA, but not docosahexaenoic acid, are released in substantial amounts, in a ratio of 10:1 (20). The respective 12-lipoxygenation products 12S-HETE and 12S-HEPE, however, are formed in a ratio of 4:1, indicating preferential 12-lipoxygenation of EPA in stimulated platelets (20).

Metabolism of the platelet 12-lipoxygenation product 12S-HEPE, derived from eicosapentaenoic acid, by unstimulated and stimulated human neutrophils was heretofore unknown. If 12S-HEPE is metabolized by stimulated human neutrophils, it is likely to compete for 5-lipoxygenation with endogenous arachidonic acid, which could result in reduced LTB_4 formation. Therefore, we studied metabolism of 12S-HEPE in unstimulated and stimulated neutrophils. We report that, in unstimulated neutrophils, 12S-HEPE is metabolized to 12S,20-DiHEPE, whereas when human neutrophils are stimulated in the presence of 12S-HEPE, 5S,12S-DiHEPE is formed, and simultaneous formation of LTB_4 is depressed in a dose-dependent manner.

MATERIALS AND METHODS

Preparation of 12-monohydroxy fatty acids

Outdated platelet concentrates were obtained from the New York Blood Center or the Munich Red Cross Blood Bank, and were centrifuged (200 *g*, 10 min, 4°C) in the presence of 1 mM EDTA. Platelets were isolated by centrifugation (2000 *g*, 10 min, 4°C), and washed twice in Tris-citrate (63 mM Tris base, 95 mM NaCl, 5 mM KCl, 12 mM citric acid, pH 6.4). Platelets were resuspended in Tris-buffer (pH 7.8, 50 mM), and lysed by freeze-thawing. The lysate was then ultracentrifuged (100,000 *g*, 60 min, 4°C). The supernatant was incubated with eicosapentaenoic acid (Sigma, 90% pure) or arachidonic acid (Sigma, 90% pure) for 60 min at 37°C. The reaction was stopped by adjusting the pH to 3 with 6 N HCl. Extraction was carried out with 1 volume of methanol followed by 2 volumes of ether. After centrifugation (2000 *g*, 5 min, 4°C), the supernatant was washed with water, centrifuged again (2000 *g*, 5 min, 4°C), and taken to dryness under nitrogen. The sample was purified by preparative open silica gel column chromatography (4.2 g Unisil, 200–325 mesh, Clarkson Chemical Co., Williamsport, PA). The first solvent was 100 ml hexane–ether 9:1 (eluate discarded); the second solvent was 100 ml hexane–ether 7:3 with collection of 5-ml fractions. Aliquots of fractions were applied to a channeled Uniplate (Silica-Gel GHL .25 mm, Analtech, Newark, DE), which was developed with hexane–ether–acetic acid 60:40:1. Fractions cochromatographing with external standard monohydroxy fatty acids were further purified on a Waters μBondapak C18 reversed-phase HPLC column. Methanol–water–acetic acid 70:30:0.01, adjusted to pH 5.7 with ammonia was used as solvent (6). The peak containing 12-HEPE (as confirmed by gas–liquid chromatography–mass spectrometry) was quantified; the eluate was collected, extracted (2), and stored under nitrogen (−70°C). The sodium salt was freshly prepared in sodium carbonate for neutrophil incubations.

Preparation of neutrophil suspensions

Venous blood was obtained by a free-flow technique through a 17-G needle from healthy donors after an overnight fast. Neutrophil suspensions were prepared as described previously (14, 16, 17). Briefly, 240 ml blood, anticoagulated with acid citrate dextrose, was centrifuged at 200 *g* (15 min, 20°C). After removal of platelet-rich plasma and dilution to original volume with isotonic saline, the remaining leukocytes and erythrocytes were suspended in 1.22% Dextran T500 (Pharmacia). Leukocyte-rich plasma was centrifuged at 280 *g*, (10 min, 4°C), and the pellets were suspended in cold saline, and layered on Ficoll-Hypaque (density 1.077). After centrifugation at 350 *g* (30 min, 4°C), the interface was removed and remaining erythrocytes in the neutrophil pellet were lysed with distilled water (25 sec). Isotonicity was reestablished with hypertonic saline, followed by addition of phosphate-buffered saline (pH 7.4). The suspension was centrifuged at 280 *g* (5 min, 4°C), and the neutrophils were finally suspended in HEPES buffer containing calcium and magnesium (12). The average yield of one donation was $12 \times 3 \times 10^7$ neutrophils. Trypan blue exclusion averaged 94%, and on stained smears no platelets were observed.

Incubations of unstimulated neutrophils

Three $\times 10^7$ neutrophils or buffer were incubated with 1.4 or 2.8 μg 12S-HETE, 12S-HEPE, or vehicle (control) for 0, 1, 2, 5, 10, and 20 min.

Incubations with stimulated neutrophils

Three × 10^7 neutrophils were stimulated with 2 μM ionophore (Sigma). Two sets of experiments were carried out. *a*) Time course: neutrophils were incubated for 0, 1, 2, 5, 10, and 20 min in the presence or absence of 15 μM 12S-HEPE. *b*) Concentration curve of 12S-HEPE: neutrophils were incubated for 10 min in the presence or absence of 0, 0.3, 0.6, 1.5, 3.0, 6.0, 15, and 30 μM 12S-HEPE.

Platelet-neutrophil coincubations

Platelet-rich plasma of one donation was partitioned into 12 parts, and labeled with 0 and 1000 μM EPA in the presence of PGD_2 for 2 h, as described previously (21). Platelets were washed twice in the presence of prostaglandin I_2 (21), and their phospholipid fatty acid composition was analyzed by gas-liquid chromatography of the respective methyl esters (22). Three × 10^7 neutrophils were stimulated for 20 min with vehicle (control) or thrombin (2 U/ml) or ionophore (2 μM) in the presence or absence of one part of platelets.

All experiments were carried out at least four times with blood from different donors.

Incubations were stopped by addition of 1.5 volumes acetone. Samples were extracted as previously described (14). Analyses were carried out using a Waters μBondapak C18 reversed-phase HPLC column. Unstimulated or thrombin-stimulated samples were analyzed using the solvent methanol-water-acetic acid 70:30:0.01, adjusted to pH 5.7 with ammonia (6). To the stimulated samples, 500 ng PGB_2 was added as internal standard prior to extraction. Ionophore-stimulated samples were analyzed using a 45-min gradient from 60:40:0.01 to 80:20:0.01 methanol-water-acetic acid (adjusted to pH 5.7 with ammonia). Compounds were identified by comparison with retention times of standards (Paesel, Frankfurt, FRG). Absorbance was recorded at both 229 and 280 nm. Analyses were quantified with a Waters data module, using 280 nm for leukotrienes, 5S,12S-DiHETE, and 5S,12S-DiHEPE. The 5S-HETE was quantified manually in arbitrary units in the same runs, using the internal standard as a correction factor.

For UV spectra and gas-liquid chromatography-mass spectrometry, HPLC eluates thought to contain 12S,20-DiHEPE or 5S,12S-DiHEPE were each collected, pooled, and acidified to pH 3-3.5 with H_3PO_4, followed by extraction (23). After resuspension in methanol, UV spectra were measured in a Cary 118 double-beam spectrophotometer (Varian Associates, Palo Alto, CA). Gas-liquid chromatography-mass spectrometry was performed on a Finnigan MAT44s equipped with a DB 1 fused silica column (30 m; .25 mm internal diameter, J + W Scientific, Inc., distributed by ICT, Frankfurt, West Germany). A portion of the metabolites was hydrogenated in methanol with PtO_2 as catalyst. Samples were esterified in methanol with ethereal diazomethane. Trimethylsilyl-ethers were generated with BSTFA (1 h, 20°C).

RESULTS

Unstimulated neutrophils

When added to unstimulated human neutrophils, 12S-HEPE is metabolized to a new compound, which we have now identified as 12S,20-DiHEPE. The new compound appeared closer to the initial solvent peak than 12S-HEPE in our reversed-phase HPLC system, and is therefore

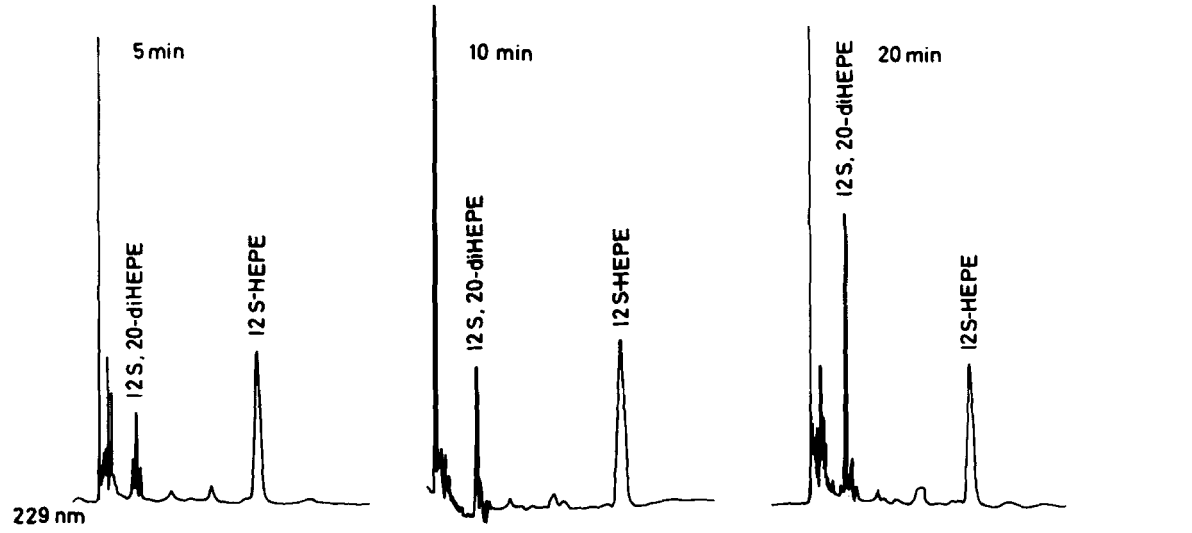

Fig. 1. Human neutrophils were exposed to 12S-HEPE for 5, 10, or 20 min. The reaction was terminated by extraction and products were analyzed by reversed-phase HPLC, as described in Methods. The runs start on the left. There is time-dependent formation of a new compound, now identified as 12S,20-DiHEPE.

more polar (**Fig. 1**). Moreover, it had a retention time as anticipated in comparison with the known retention times of 12S-HETE, 12S-HEPE, and 12S,20-DiHETE. 12S,20-DiHEPE increased in a time-dependent fashion (Fig. 1). When 12S-HEPE was incubated for 1 or 2 min, little or no 12S,20-DiHEPE was detected. In the absence of neutrophils, the quantity of 12S-HEPE remained unaltered, and no other products were detected at any time point. No products were detected when neutrophils were incubated with carrier. When 12S-HETE was used in the time course studies, metabolic conversion to 12S,20-DiHETE was highly comparable. The UV spectrum of 12S,20-DiHEPE displayed an absorption maximum at 237 nm, characteristic for two conjugated double bonds (**Fig. 2**).

Fig. 3 shows the mass spectrum of 12S,20-DiHEPE, as methyl ester–trimethylsilyl ether in the electron impact (EI) mode. Prominent ions were at *m/z* 477 = $(M-15(CH_3))^+$; *m/z* 461 = $(M-31(OCH_3))^+$; *m/z* 295 = $(C_{13}-C_{20})^+$; *m/z* 205 = $((C_{13}-C_{20})-90)^+$; *m/z* 197 = $(M-(C_1-C_{12}))^+$; *m/z* 193 = $(M-(C_{12}-C_{20}))^+$. When 12S,20-DiHEPE was hydrogenated, derivatized to a methyl ester–trimethylsilyl ether, and subjected to gas–liquid chromatography–mass spectrometry, selective ion monitoring showed the following ions in the electron impact mode: *m/z* 487 = $(M-15(CH_3))^+$; *m/z* 471 = $(M-31(OCH_3))^+$, *m/z* 455 = $(M-(15+32)(CH_3+CH_3OH))^+$; *m/z* 301 = $(M-(C_{13}-C_{20}))^+$, as we described earlier for hydrogenated 12S,20-DiHETE (14).

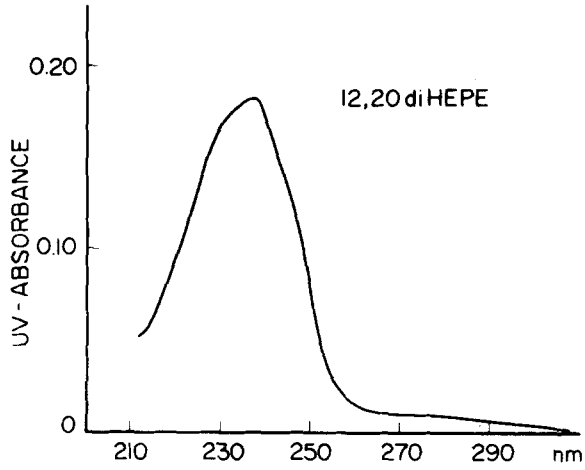

Fig. 2. Ultraviolet absorbance of 12S,20-DiHEPE.

Stimulated neutrophils

12S-HEPE, added to human neutrophils stimulated with ionophore, gave rise to what we have identified as 5S,12S-DiHEPE. 12S-HEPE decreased, whereas 5S,12S-DiHEPE increased in a time-dependent fashion, as depicted in **Fig. 4.** In control incubations, in the absence of 12S-HEPE, a very small peak was detected occasionally at the retention time of 5S,12S-DiHEPE. In our reversed phase HPLC system, 5S,12S-DiHEPE and 5S,12R-DiHEPE, i.e., LTB_5, cochromatograph. In these cases, the small peak

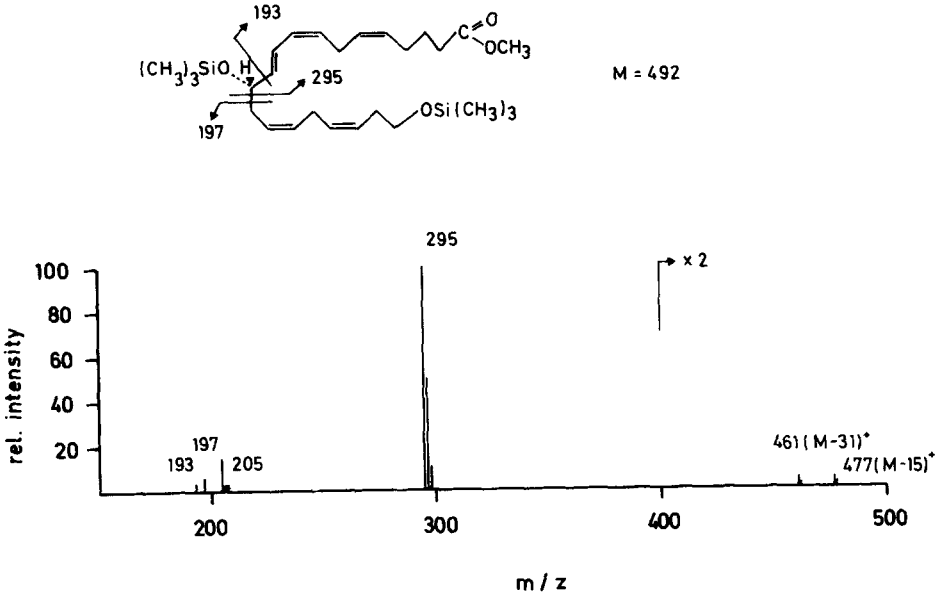

Fig. 3. Mass spectrum of the reversed-phase HPLC-purified metabolite produced by unstimulated human neutrophils from 12S-HEPE. The sample was converted to the methyl ester–trimethylsilyl ether derivative and a spectrum was taken in the EI mode. The ion at *m/z* 295 indicates that the hydroxyl group at C-12 is also present in the new product.

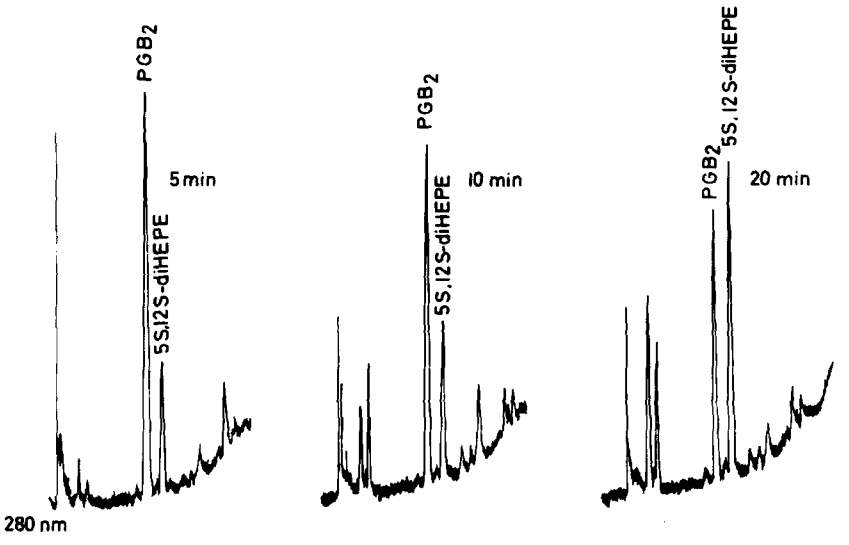

Fig. 4. Human neutrophils were stimulated with ionophore for 5, 10, or 20 min in the presence 15 μM of 12S-HEPE. Reactions were terminated by extraction and products were analyzed by HPLC, as described in Methods. The runs start on the left. There is a time-dependent formation of a compound, later identified as 5S,12S-DiHEPE.

probably represents LTB_5 derived from endogenous eicosapentaenoic acid. 5S,12S-DiHETE and 5S,12R-DiHETE, i.e., LTB_4, also cochromatograph in our HPLC system; however, analyses of incubations in the presence of 12S-HETE revealed no additional peak. After incubations of 1 or 2 min, little or no 5S,12S-DiHEPE was detected. In parallel incubations, in the absence of cells, the amount of 12S-HEPE remained unaltered at all timepoints. UV absorbance of 5S,12S-DiHEPE revealed a pattern characteristic for three conjugated double bonds of a triene structure (**Fig. 5**).

The mass spectrum of the thermal decomposition product of 5S,12S-DiHEPE-methyl ester-trimethylsilyl ether obtained in the electron impact mode is shown in **Fig. 6.** There were prominent ions at *m/z* 492 = M^+; *m/z* 477 = $(M-15(CH_3))^+$; *m/z* 402 = $(M-90((CH_3)_3SiOH))^+$; *m/z* 391 = $(M-101(CH_2-(CH_2)_2-COOCH_3))^+$; *m/z* 354; *m/z* 293 = $(383-90)^+$; *m/z* 211 = $(M-(C_1-C_{11}))^+$; *m/z* 203 = $(M-(C_6-C_{20}))^+$. The ion *m/z* 354, detected in the electron impact mode probably represents CH-CH=CH-CH=CH-CH-CH-$(OSiMe_3)$-$(CH_2)_3$-$C(OCH_3)$=O$^+$SiMe$_3$ from a rearrangement (24). Further proof of structure was obtained by selective ion monitoring in the positive chemical ionization mode (isobutane). Ions monitored were *m/z* 403 = $(MH-90((CH_3)_3SiOH))^+$; *m/z* 313 = $(MH-2\cdot 90)^+$. When 5S,12S-DiHEPE was hydrogenated, derivatized to a methyl ester-trimethylsilyl ether, and subjected to gas-liquid chromatography-mass spectrometry, selective ion monitoring showed the following ions in the electron impact mode: *m/z* 502 = M^+; *m/z* 487 = $(M-15(CH_3))^+$; *m/z* 471 = $(M-31(OCH_3))^+$; *m/z* 215 = $(M-(C_1-C_{11}))^+$; *m/z* 203 = $(M-(C_6-C_{20}))^+$.

When human neutrophils were stimulated for 10 min in the presence of incremental concentrations of 12S-HEPE, formation of LTB_4 remained unaltered up to 6 μM 12S-HEPE, whereas it decreased in a dose-dependent fashion at 15 and 30 μM (**Fig. 7**).

Platelet-neutrophil coincubations

Labeling of platelet-rich plasma with 1000 μM EPA increased EPA in the platelet membrane from a control value of 0.86 (± 0.17) to 1.69 (± 0.19) rel. % (± SD); all other fatty acids were not significantly altered.

Neutrophils were not stimulated by addition of thrombin, as judged by the absence of LTB_4. Whether or not

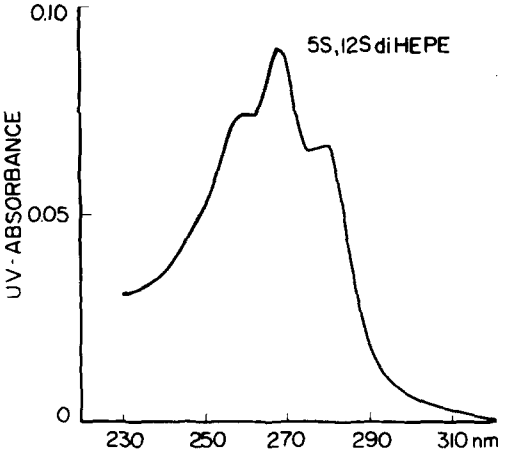

Fig. 5. Ultraviolet absorbance of 5S,12S-DiHEPE.

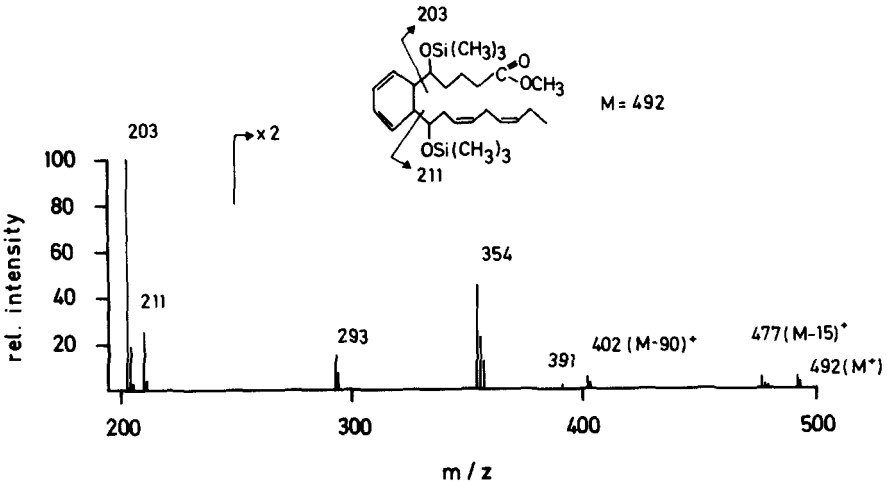

Fig. 6. Mass spectrum of the thermal decomposition product of 5S,12S-DiHEPE-methyl ester-trimethylsilyl ether in the electron impact mode. The exact structure of the ring is hypothetical. Molecular weight is two masses lower than 5S,12S-DiHETE; the ion at *m/z* 203 is highly characteristic for $(M-(C_6-C_{20}))^+$ and the ion at *m/z* 211 is highly characteristic for $(M-(C_1-C_{11}))^+$.

added platelets were prelabeled with EPA, amounts of 12S-HETE and 12S,20-DiHETE detected were unchanged. EPA-prelabeling of platelets increased 12S-HEPE from 216 (± 378) to 324 (± 435) ng, and of 12S,20-DiHEPE from 15 (± 9) to 153 (± 59) ng ($P < 0.05$, paired *t*-test).

Addition of ionophore stimulated both platelets and neutrophils. Prelabeling platelets with EPA increased the combined amounts of LTB_5 and 5S,12S-DiHEPE from 34.7 (± 13.5) to 81.1 (± 34.0) ng ($2P < 0.01$, paired *t*-test), whereas this treatment decreased the combined amounts of LTB_4 and 5S,12S-DiHETE from 500 (± 134) to 389 (± 107) ng ($P < 0.05$, paired *t*-test), as compared to addition of platelets not prelabeled with EPA. The results are summarized in **Table 1.**

DISCUSSION

Previously, we demonstrated that platelets of humans ingesting n-3 fatty acids release large amounts of 12S-HETE and 12S-HEPE, in a ratio of about 4:1, upon stimulation (20). We have also shown that unstimulated neutrophils in proximity to platelets metabolize 12S-HETE to 12S,20-DiHETE (14), whereas, in stimulated neutrophils, 12S-HETE is subject to 5-lipoxygenation to 5S,12S-DiHETE (12). We demonstrate here that 12S-HEPE is also being processed by human neutrophils, and thus can participate in platelet-neutrophil interactions.

Unstimulated human neutrophils metabolize 12S-HEPE in a time-dependent fashion (Fig. 1) to a new compound, now identified as 12S,20-DiHEPE. The new metabolite was detectable only in neutrophil suspensions exposed to 12S-HEPE. Controls and time-course studies both point to enzymatic formation of 12S,20-DiHEPE. The UV-absorbance of 12S,20-DiHEPE (Fig. 2) is identical to that of 12S-HEPE (25), indicating that the intrinsic double bond structure remained intact. Using the same deriva-

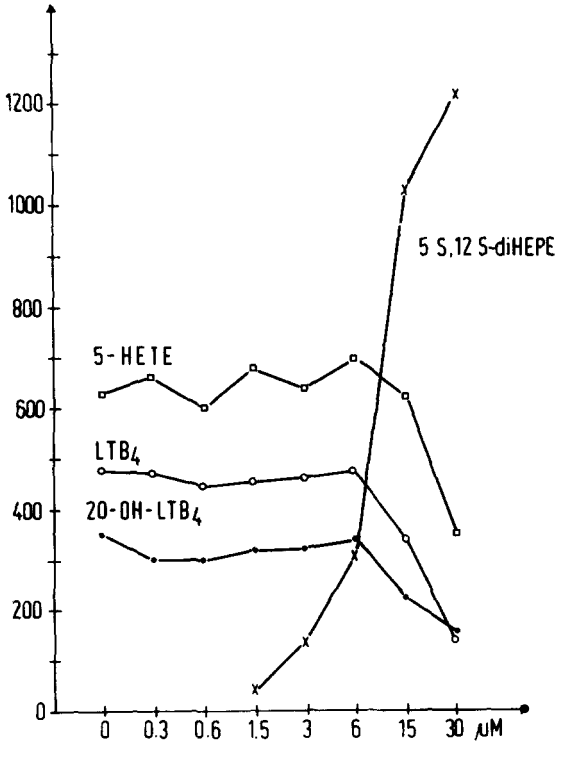

Fig. 7. Stimulation of human neutrophils with ionophore in the presence of incremental concentrations of 12S-HEPE. LTB_4, 20-OH-LTB_4, and 5S,12S-DiHEPE are expressed as ng, whereas 5S-HETE is expressed in arbitrary units, using the internal standard as a correction factor. The mean of four experiments from different donors is depicted. At all points, the standard error was about 20% of the mean.

TABLE 1. Summary of findings of the present study

	Neutrophils + 12-HEPE	Neutrophils + 12-HEPE	Neutrophils	Neutrophils	Neutrophils + Platelets	Neutrophils + Platelets + EPA	Neutrophils + Platelets	Neutrophils + Platelets + EPA
Stimulus		Ionophore	Thrombin	Ionophore	Thrombin	Thrombin	Ionophore	Ionophore
Detectable compound								
12-HETE	–	–	–	–	+	+	+	+
12,20-DiHETE	–	–	–	–	+	+	–	–
12-HEPE	+	+	–	–	–	+	–	+
12,20-DiHEPE	+	–	–	–	–	+	–	–
LTB_4	–	+	–	+	–	–	+	+
5S,12S-DiHETE	–	–	–	–	–	–	+	+
LTB_5	–	–	–	–	–	–	–	+
5S,12S-DiHEPE	–	+	–	–	–	–	–	+

Not detectable (–); detectable (+). For methodological reasons (see text), LTB_4 was not separated from 5S,12S-DiHETE and LTB_5 was not separated from 5S,12S-DiHEPE on HPLC; the information is based on inference.

tive, and a comparable mass spectrometric technique, hydrogenated 12S,20-DiHEPE displayed the same prominent ions we demonstrated for hydrogenated 12S,20-HETE (14). The mass spectrum of unhydrogenated 12S,20-DiHEPE, as a methyl ester–trimethylsilyl ether derivative (Fig. 3), is definitive evidence for the molecular structure of 12S,20-DiHEPE. Human neutrophils 20-hydroxylate arachidonic acid (26), 12S-HETE (15), and LTB_4 (27), by cytochrome P450 enzyme systems (16, 18, 28, 29). In close proximity to carbon 20, the configuration of these molecules is similar to 12-HEPE, which has, however, an additional double bond at carbon 17. Therefore, a P450 enzyme system might also be involved in formation of 12S,20-DiHEPE.

When human neutrophils are stimulated with the ionophore A23187, however, addition of 12S-HEPE resulted in formation of a compound that we have now identified as 5S,12S-DiHEPE. Interestingly, 12S,20-DiHEPE was not detectable in these experiments. Formation of 5S,12S-DiHEPE was not detectable in control experiments and was time-dependent, indicating enzymatic formation. The compound absorbs UV light with a maximum and in a pattern highly characteristic for leukotrienes (Fig. 5, ref. 30). 5S,12S-DiHEPE is a stereoisomer of 5S,12R-DiHEPE, i.e., LTB_5, and cochromatographs with it in our HPLC systems. When 5S,12S-DiHEPE is hydrogenated, derivatized, and subjected to capillary gas–liquid chromatography–mass spectrometry, prominent ions are detected that also can be detected upon identical analysis of the respective derivatives of LTB_4 and LTB_5 (6, 30). 5S,12S-DiHEPE undergoes thermal decomposition during gas–liquid chromatography of the respective methyl ester–trimethylsilyl ether, as evidenced by "tailing" in the total ion current chromatogram (not shown). 5S,12S-DiHEPE and 5S,12S-DiHETE carry identical *trans-cis-trans* triene units (**Fig. 8,** ref. 24). At this characteristic double bond phase, both compounds undergo cyclization during gas–liquid chromatography, and a disubstituted cyclohexadiene ring is formed (24). Therefore, by gas–liquid chromatography–mass spectrometry in the electron impact mode, the thermal decomposition product of the 5S,12S-DiHEPE methyl ester–trimethylsilyl ether derivative is detected (Fig. 6).

When human neutrophils are stimulated in the presence of incremental exogenous concentrations of 12S-HEPE, production of 5S,12S-DiHEPE increases in a dose-dependent manner, whereas production of other neutrophil 5-lipoxygenase products, LTB_4 and 5-HETE from endogenous arachidonic acid, decreases (Fig. 7). A similar phenomenon can also be observed when 12S-HETE is added to porcine leukocytes (13). The large increase of 5S,12S-DiHEPE makes cell damage as the reason for reduced LTB_4 formation highly unlikely. The concentrations of exogenous EPA or 12S-HEPE necessary for reduction of neutrophil LTB_4 formation are both 15–30 μM (Fig. 7, refs. 3, 5, 6, 8). Thus, when added exogenously, platelet-derived 12S-HEPE competes with neutrophil-derived, endogenous arachidonic acid for 5-lipoxygenation.

Since it is a 5,12-dihydroxyeicosapolyenoic acid, 5S,12S-DiHEPE might be a substrate for the neutrophil 20-hydroxylase that catabolizes LTB_4 and similar mono- and dihydroxy fatty acids (28). Thus, 5S,12S-DiHEPE might, by competition for the enzyme, impede 20-hydroxylation, i.e., inactivation (27, 29, 30) of LTB_4. We found no evidence for delayed catabolism of LTB_4, inasmuch as, in time-course experiments with 15 μM 12S-HEPE, formation of LTB_4 was depressed at 1–20 min (data not shown). Therefore, 5S,12S-DiHEPE does not seem to impair catabolism of LTB_4.

The reversed-phase HPLC-purified product resulting from an incubation of a human platelet sonicate with racemic 5-HEPE, probably 5S,12S-DiHEPE, has been shown to have very little chemotactic and no proaggregatory activity on human neutrophils (4). Binding of 5S,12S-DiHEPE to LTB_4 binding sites was comparable to LTB_4, whereas LTB_5 binds far less (2, 4, 31). Thus, formation of

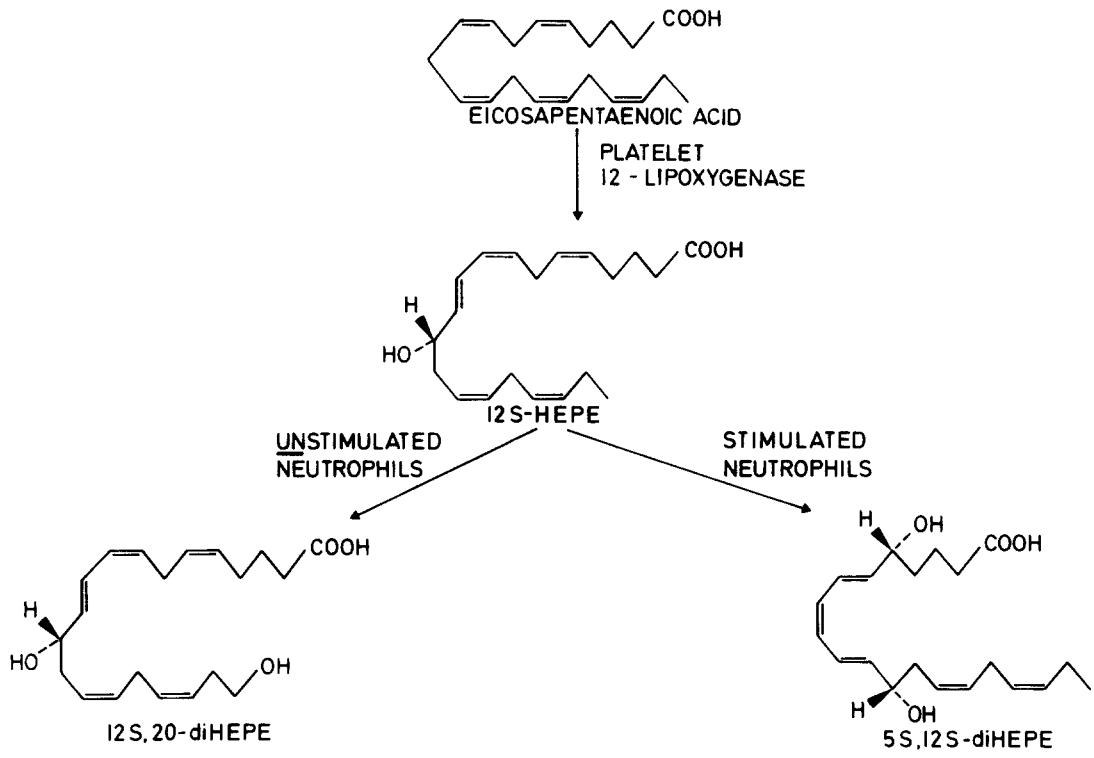

Fig. 8. Metabolism of 12S-HEPE released from stimulated platelets during dietary n-3 fatty acid supplementation by unstimulated (left) and stimulated (right) neutrophils.

5S,12S-DiHEPE during platelet-neutrophil interactions might result in LTB_4-receptors being partially occupied by biologically inactive material.

Interestingly, addition of as much as 1000 μM exogenous EPA to platelet-rich plasma only doubled the amount of EPA in platelet phospholipids. Although the resulting plasma levels are much lower, supplementing a human diet with roughly 2.5/day n-3 fatty acids for 4 weeks also doubled EPA in the platelet membrane (1, 19). Thus, platelets seem to conserve their fatty acid composition in spite of a different plasma fatty acid composition. In keeping with the results of our previous experiments with dietary supplementation (1, 19), our present finding in vitro points toward definition of most of platelet phospholipid fatty acid composition during cell maturation.

The platelet-neutrophil coincubation experiments demonstrated further that platelet-derived 12S-HEPE is a physiologic substrate for neutrophil metabolism. Human neutrophils processed 12S-HEPE identically, whether it was added exogenously or was presented to them by stimulated platelets.

We are unaware of current HPLC technology capable of separating according to double bond (LTB_4 vs LTB_5) as well as according to stereochemistry (S vs R). Therefore, we were unable to demonstrate in platelet-neutrophil coincubations that platelet-derived 12S-HEPE competitively inhibits neutrophil 5-lipoxygenase. The sum of LTB_4 and 5S,12S-DiHETE produced by stimulated neutrophils in the presence of platelets, however, was reduced by prelabeling the platelets with EPA. We, therefore, conclude that, when providing EPA and 12S-HEPE, stimulated platelets reduce the amount of arachidonic acid-derived 5-lipoxygenase products formed by stimulated neutrophils.

In summary, we demonstrate that cell-cell interactions in the eicosanoid system are not limited to arachidonic acid or derivatives. The platelet lipoxygenase product formed during ingestion of dietary n-3 fatty acids, 12S-HEPE, is metabolized by human neutrophils. In unstimulated neutrophils, the 20-hydroxylated product 12S,20-DiHEPE appears. In stimulated neutrophils, however, hydroxylation occurs in the 5S-position to 5S,12S-DiHEPE, a compound virtually biologically inactive. Competition for 5-lipoxygenation between platelet-derived EPA and 12S-HEPE and endogenous arachidonic acid and for receptor occupancy between 5S,12S-DiHEPE and LTB_4 are two possible mechanisms for antiinflammatory action of EPA mediated by cell-cell interactions.

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