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Stereospecific bioactions of 5-hydroxyicosatetraenoate

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5(*S*)-Hydroxyicosatetraenoate stimulates human polymorphonuclear neutrophils (PMNs) to raise their cytosolic calcium. It also potentiates the cells' degranulation responses to platelet-activating factor and diacylglycerols. We synthesized 5(*R*)-hydroxyicosatetraenoate and found it to be 20–100-fold weaker than the natural isomer in these assays. Thus, the arachidonic acid metabolite activates PMNs by a stereospecific possibly receptor-mediated mechanism.

5-Hydroxyicosatetraenoate; cellular Ca^{2+}; Degranulation; Polymorphonuclear neutrophil

1. INTRODUCTION

Stimulated human polymorphonuclear neutrophils (PMNs) rapidly metabolize their resident phospholipids into a series of signaling molecules [1]. They release arachidonate from phospholipids and oxygenate it to 5(*S*)-HETE and LTB_4; deacylate 1-*O*-alkyl-2-acyl-GPC and acetylate the lyso intermediate forming PAF; and cleave phosphatidylinositol and phosphatidylcholine at the *sn*-3 position to yield diacylglycerol [2–4]. LTB_4, PAF and diacylglycerol directly stimulate a number of PMN responses such as degradulation and superoxide anion generation by binding to specific receptors (the receptor for diacylglycerol is protein kinase C). 5(*S*)-HETE, contrastingly, lacks appreciable intrinsic-activity in these assays. However, it does enhance the potency and power of PAF and diacylglycerol in stimulating function [5–8]. Thus, 5(*S*)-HETE may play a unique role in stimulus-response coupling by regulating the efficacy of its companion (i.e., concurrently formed) mediators. The mechanisms involved in these bioactions of 5(*S*)-HETE, therefore, are of particular interest. Recently, 5(*S*)-HETE has been shown to stimulate calcium mobilization in PMNs [8,9]. This effect, which does not require the presence of a second mediator, appears at least partly responsible for 5(*S*)-HETE-induced potentiation of PMN responses to PAF and diacylglycerol. Nevertheless, important questions about the bioactions of 5-HETE still remain unanswered. Among these are uncertainties about structural specificity. It is known that 15-hydroxyicosatetraenoate is bioinactive whereas 5,20-dihydroxyicosatetraenoate and 5,15-dihydroxyicosatetraenoate are 30-fold and 100-fold, respectively, weaker than 5(*S*)-HETE [6,8–10]. However, these compounds differ from 5(*S*)-HETE not only in hydroxy residues but also in the positions and geometries of their double bonds, their 3-dimensional foldings, and their aqueous solubilities. Unlike 5(*S*)-HETE, they may not enter PMNs to any appreciable extent [10]. Ideal structural comparisons should be made with compounds more closely matched in physicochemical characteristics. Accordingly, we have prepared and assessed the bioactivity of 5(*R*)-HETE.

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Abbreviations: 5(*S*)-HETE, 5(*S*)-hydroxy-6,8,11,14-*E,Z,Z,Z*-eicosatetraenoate; 5(*R*)-HETE, (5(*R*)-hydroxy-6,8,11,14-*E,Z,Z,Z*-eicosatetraenoate; LTB_4, leukotriene B_4; PAF, platelet-activating factor; PMA, 4β-phorbol-12-myristate-13-acetate; diC_8, 1,2-dioctanoylglycerol; BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration

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2. MATERIALS AND METHODS

2.1. *Reagents and buffers*

Arachidonic acid (Nu-Chek, Elysian, MN); trichloromethylchloroformate (Fluka, Buchs, Switzerland); cytochalasin B and fatty acid-free BSA (Sigma, St. Louis, MO); Fura-2 pentapotassium salt and Fura-2 acetoxymethyl ester and diC_8 (Molecular Probes, Junction City, OR); and 1,5-diazabicyclo[5.4.0]undecene, dehydroabietylamine, trichlorosilane, triethylamine and 4-dimethylaminopyridine (Aldrich, Milwauke, WS) were purchased from the indicated vendors; PAF was obtained as previously described [5]. All dilutions of 5(*S*)-HETE, 5(*R*)-HETE, PAF and diC_8 were made in a modified [5] Hanks' buffer containing fatty acid-free BSA (2.5 mg/ml). Dilutions of PMA were made in dimethylsulphoxide (DMSO). The final concentration of DMSO in samples was 0.5%.

2.2. *Preparation of 5(S)- and 5(R)-HETEs*

The stereoisomers of 5-HETE were prepared by the procedure of Corey and Hashimoto [11] with the following changes: (i) trichloromethylchloroformate was used as a substitute for phosgene gas to prepare isocyanates of the 5-HETEs and (ii) the diastereomeric urethane derivatives of 5-HETE methyl ester were separated by normal phase HPLC [hexane/isopropanol/glacial acetic acid (995:4:1, v/v), 3 ml/min; 0.75 × 30 cm μ-Porasil column]. Products were identified, analyzed and checked for purity by TLC; normal and reverse-phase HPLC [10]; ultraviolet, infrared and nuclear magnetic resonance spectroscopy; and polarimetry (i.e., the more polar carbamate, which yielded the 5(*S*)-HETE enantiomer, had a rotation of $[\alpha]_D^{23}$ + 42.7° [c = 1.4, benzene] and the less polar carbamate, which yielded the 5(*R*)-HETE, had a rotation of $[\alpha]_D^{23}$ + 6.6° [c = 2.4, benzene]).

2.3. *Degranulation assay*

Cells (1.3 × 10^6) were incubated in 0.5 ml buffer (37°C; 1.4 mM $CaCl_2$) for 20 min; treated with 2.5 μg cytochalasin B for 2–4 min; and challenged simultaneously with varying concentrations of the stimuli (PAF, PMA or diC_8) plus either 5(*S*)-HETE or 5(*R*)-HETE for 5 or 15 min. The samples were placed on ice, centrifuged and the supernatant fluid assayed for lysozyme (EC 3.2.1.17) and β-glucuronidase (EC 3.2.1.21) as described [5]. Results are reported as net enzyme release (± SE), i.e., the percentage of total enzyme released by stimulated cells minus that released by unstimulated, but otherwise identically treated, cells.

2.4. *Cytosolic calcium measurements*

PMNs were loaded with Fura-2 acetoxymethyl ester, washed, and then stimulated [8]. Results are reported as the mean ± SE of the maximal fluorescence emission ratio (340/380 nm).

3. RESULTS AND DISCUSSION

In agreement with previous observations [5], 5(*S*)-HETE (5–500 nM) did not induce PMNs to

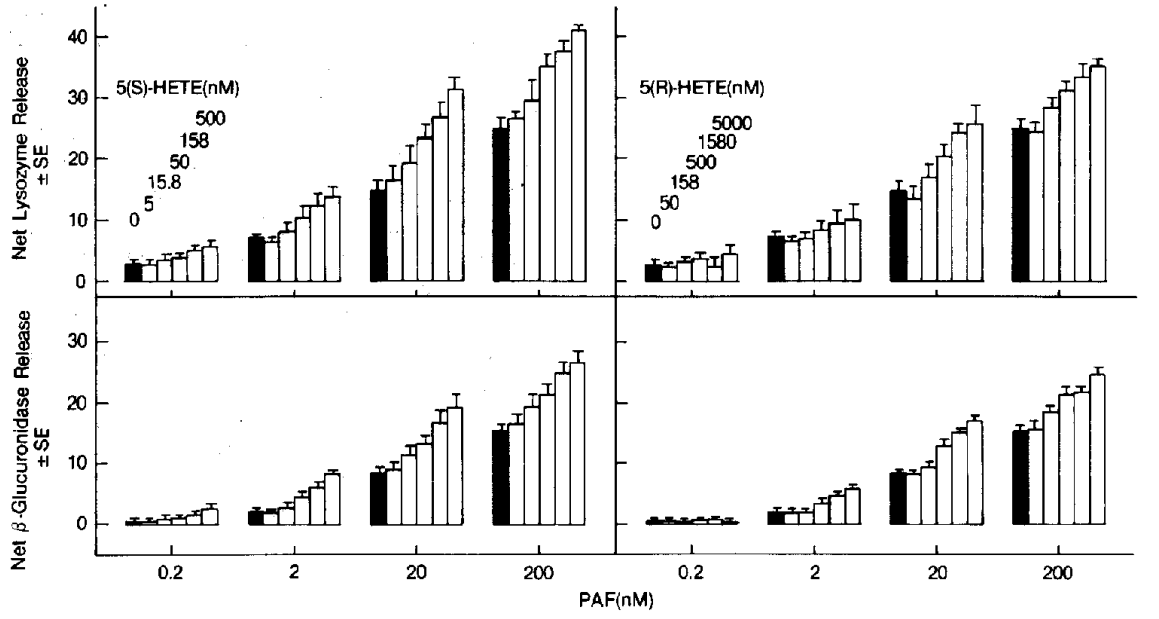

Fig.1. The effect of 5-HETE stereoisomers on PAF-induced net release of lysozyme (upper panels) and β-glucuronidase (lower panels) by PMNs. Shaded bars represent responses elicited by PAF alone; adjacent unshaded bars give the responses induced by PAF in the presence of increasing concentrations of 5(*S*)-HETE (left panels) and 5(*R*)-HETE (right panels). Cells (1.3 × 10^6/0.5 ml) were preincubated with 1.4 mM $CaCl_2$ for 20 min, treated with 2.5 μg cytochalasin B, and then challenged simultaneously with PAF and 5(*S*)-HETE or 5(*R*)-HETE. Results are reported as the mean ± SE for 10–13 separate experiments. Note that the concentrations for 5(*S*)-HETE (left panels) are 10-fold higher than those of 5(*R*)-HETE (right panels).

Table 1

Effect of 5-HETE stereoisomers on the PMN degranulation response to PMA and diC_8[a]

Stimulus (nM)	0	5(S)-HETE (μM) 0.05	5(S)-HETE (μM) 5.0	5(R)-HETE (μM) 0.5	5(R)-HETE (μM) 5.0
PMA (1)	18.8 ± 0.9[b]	24.1 ± 2.2[c]	28.3 ± 2.2[c]	21.9 ± 1.8	22.6 ± 2.4
PMA (3.2)	22.7 ± 2.3	27.7 ± 1.0[c]	31.9 ± 0.7[c]	25.1 ± 0.8	25.9 ± 3.3
diC_8 (3200)	2.5 ± 1.8	2.1 ± 1.3	9.8 ± 2.4[c]	2.8 ± 1.8	1.7 ± 0.9
diC_8 (10000)	7.6 ± 1.5	7.6 ± 1.2	14.6 ± 1.3[c]	6.0 ± 1.6	11.3 ± 1.0[c]

[a] PMNs (1.3×10^6/0.5 ml of buffer) were incubated with cytochalasin B (2.5 μg) for 2–4 min and then challenged for 15 min with the indicated concentrations of stimulus and 5-HETE stereoisomer

[b] Net lysozyme release ± SE for 3–4 separate experiments. The two 5-HETE preparations similarly influenced release of β-glucuronidase

[c] Indicates values that are significantly ($p < 0.05$, Student's paired t-test) higher than those found in cells treated with stimulus alone

release their granule-bound enzymes. 5(*R*)-HETE (50–5000 nM) was likewise without effect. However, as depicted in the two left panels of fig.1, both compounds produced a substantial enhancement of PAF-induced degranulation. The potentiating effects of the unnatural stereoisomer, 5(*R*)-HETE, however, were >20–100-fold weaker than those elicited by 5(*S*)-HETE. The 5-HETE stereoisomers demonstrated similar potency differences in enhancing the PMN-degranulating actions of two protein kinase C activators, PMA and diC_8 (table 1).

Fig.2 illustrates the effect of the 5-HETE stereoisomers on the maximal fluorescence emission ratio (340/380 nm), a direct measurement of $[Ca^{2+}]_i$, in Fura-2-loaded PMN. We previously reported that the biosynthetic 5(*S*)-HETE, at 5 μM, elevated $[Ca^{2+}]_i$ from basal levels (~80 nM) to around 250–350 nM within 15 s. After 15 s, $[Ca^{2+}]_i$ declined and reached pre-stimulatory levels in 2.5–5 min [8,9]. Similar calcium transients were observed with the chemically synthesized 5-HETEs (not shown). 5(*S*)-HETE, however, was again ~20–50-fold more potent than 5(*R*)-HETE in inducing the response.

Our technique for synthesizing 5-HETE stereoisomers required resolving a diastereomeric mixture of carbamylated 5-HETEs into respective precursors of 5(*S*)- and 5(*R*)-HETE by HPLC. This step did not afford perfect resolution: the final products may be cross-contaminated by 1–3%. The bioactions of 5(*R*)-HETE found here, therefore, may reflect this cross-contamination. (A racemic mixture of 5-HETE was almost as potent as 5(*S*)-HETE. Hence, 5(*R*)-HETE did not inhibit 5(*S*)-HETE.) In any event, our results indicate that 5(*S*)-HETE is at least 20–100-fold more potent than 5(*R*)-HETE. This suggests that the various actions of 5(*S*)-HETE may proceed via a common, stereospecific, and possibly receptor-mediated mechanism. We have accumulated other evidence favoring this: the receptor uncoupling agent, pertussis toxin, inhibits the calcium mobilizing actions of 5(*S*)-HETE; and 5(*S*)-HETE, while down-regulating PMNs to a second 5(*S*)-HETE challenge, does not similarly down-regulate

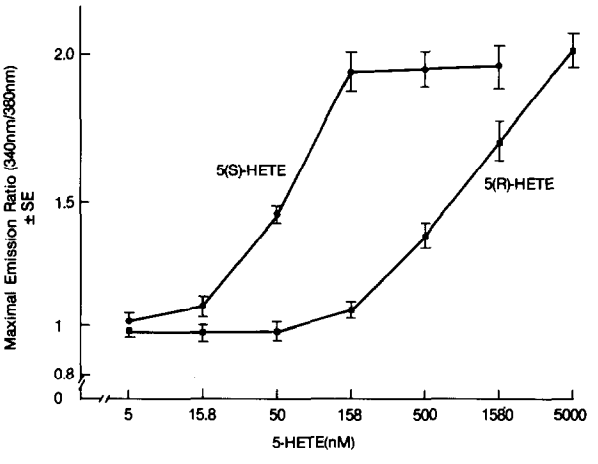

Fig.2. The effect of 5(*S*)-HETE (circles) and 5(*R*)-HETE (squares) on the maximal fluorescence emission ratio (340/380 nm) of Fura-2-loaded PMNs. Each point represents the mean ± SE for 5–8 separate experiments.

responses to LTB_4 or PAF in the calcium mobilizing assay [9]. However, the receptor hypothesis obviously needs more direct support. We have examined PMNs and PMN membranes for the specific binding of [^{3}H]5(*S*)-HETE and [^{3}H]5(*S*)-HETE methyl ester. Unfortunately, the preparations rapidly and quantitatively metabolized both ligands even at 4°C. This has completely interfered with our binding assessment. Nevertheless, the results reported here support further efforts to define the putative 5(*S*)-HETE receptor. Given the unique bioactions expressed by 5(*S*)-HETE, such receptors seem sure to have novel transduction mechanisms and influences upon the PAF- and diacylglycerol-induced intracellular events promoting function.

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