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5-OXO-EICOSATETRAENOATE, A POTENT HUMAN NEUTROPHIL STIMULUS

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SUMMARY: 5-Oxo-eicosatetraenoate (5-oxoETE), a newly defined arachidonate metabolite, resembled 5-hydroxyicosatetraenoate (5-HETE) in stimulating neutrophils to mobilize Ca^{2+} and in promoting PMN degranulation responses to other agents. It was, however, 10-fold stronger than 5-HETE and, like leukotriene (LT) B_4, had intrinsic PMN degranulating effects. Nonetheless, 5-oxoETE and 5-HETE desensitized PMN to themselves or each other but not to LTB_4; LTB_4 desensitized to itself but not to 5-oxoETE or 5-HETE; and an antagonist blocked LTB_4 but not 5-oxoETE or 5-HETE. 5-OxoETE and 5-HETE thus induce diverse PMN responses using a shared, down-regulatable, and receptor-like mechanism that does not involve LTB_4 receptors; 5-oxoETE is the preferred natural agonist for this mechanism.

The AA metabolites which form and function in cells seem well-established. PMN, for example, convert AA to various products. Of these, LTB_4 has the most potent and broad bioactions. It thus appears to be the central AA-derived mediator (1). However, the LTB_4 synthetic enzyme, 5-lipoxygenase, also makes 5-HETE. 5-HETE induces little or no PMN degranulation or superoxide anion production but, unlike LTB_4, does enhance the actions of certain stimuli in eliciting these responses. 5-HETE thus seems to mediate PMN function as a stimulus potentiator (2-4). We here study 5-oxoETE, a recently defined AA metabolite (5). 5-oxoETE had the bioactivity spectra of, but was 10-fold more potent than, 5-HETE. It also degranulated PMN. In all respects, 5-oxoETE and 5-HETE acted by a shared, receptor-like mechanism that did not involve LTB_4 receptors. 5-OxoETE is the preferred analog in a novel class of AA metabolites with unexpectedly potent and broad bioactions.

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ABBREVIATIONS: 5-HETE, 5(S)-hydroxy-6,8,11,14-E,Z,Z,Z-eicosatetraenoate; 5-oxoETE, 5-oxo-6,8,11,14-E,Z,Z,Z-eicosatetraenoate; AA, arachidonic acid; BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytosolic Ca^{2+}; Di-C8, dioctanoylglycerol; LTB_4, leukotriene B_4 (5(S),12(R)-dihydroxy-6,8,10,14-Z,E,E,Z-eicosatetraenoate); PAF, platelet-activating factor; fMLP, N-formyl-MET-LEU-PHE.

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MATERIALS AND METHODS

Reagents Reagents were obtained and used as in (2-4). LY 255283 was a generous gift of Lilly Research, Indianapolis, IN. To make 5-oxoETE, 29 μmol of *rac*-5-HETE methyl ester (prepared from AA [3]) were reacted (22° C) with dichlorodicyanobenzoquinone (46 μmol, Aldrich Chem. Co.) in 500 μl of diethyl ether and monitored at 210-320 nm. Reaction UV spectra changed from that of 5-HETE (λ_{max} = 236 nm) to that of 5-oxoETE (λ_{max} = 278 nm)(5) over 30 min by which time O.D. at 236 nm had fallen to ~0 and O.D. at 278 nm indicated 68% conversion of 5-HETE to 5-oxoETE methyl ester, based on absorbance coefficients of 26,000/M cm for both compounds (5). Material was redissolved in hexane and applied to a Water's SEP-PAK cartridge. The cartridge was flushed with 2 ml of hexane and 5-oxoETE methyl ester was eluted with 4 ml of hexane:ethyl acetate (93:7, v/v) to obtain 85% recovery of applied compound. Eluants were dried; suspended in dimethoxyethylene (1.5 ml); treated with 0.2 M LiOH (1.5 ml) for 30 min at 22° C; extracted once with hexane to remove hydrophobic impurities; made pH 2; and extracted x3 with hexane to obtain 90% recovery of 5-oxoETE free acid. We purified 5-oxoETE by μ-Bondapak reversed-phase HPLC (1.5x30 cm column; methanol:water:glacial acetic acid, 750:250:0.1, v/v; 3 ml/min). 5-HETE and 5-oxoETE eluted respectively at 25.2 and 25.4 min. 5-HETE/5-oxoETE mixtures eluted in single peaks enriched with 5-HETE in leading and 5-oxoETE in trailing edges as defined by UV spectral analyses with an on-line diode array spectrophotometer (4). The system detected 5-HETE mixed with 5-oxoETE in 1:20 ratios but found no 5-HETE in our 5-oxoETE preparations. Thin-layer chromatography (silica gel G plates pre-activated at 180° C for 3 hr; developed to 15 cm with diethyl ether:hexane:glacial acetic acid [60:40:1, v/v]) resolved 5-oxoETE (R_f = 0.58) from 5-HETE (R_f = 0.43) and detected no contamination of 5-oxoETE preparations by 5-HETE. MS confirmed the identity and purity of 5-oxoETE. Chemical ionization GC/MS of 5-oxoETE methyl ester gave a single peak containing ions m/z = 333 (MH^+) and m/z = 350 ($MH{\bullet}NH_4^+$): 7.5 m DB-1 column, 0.25 mm dia; splitless injection; oven program = 50-250° C at 25° C/min, inj = 250° C, int = 250° C, source = 150° C; He pressure = 7 psi; flow = 1 cm/min; NH_3 at 0.14 Torr. Electron impact spectra showed weak ion signals at m/z = 332 (M^+), 300 ($[M-32]^+$), 231 ($M-(-CH_2)_3CO(OCH_3)$), and 129: Conditions were as for chemical ionization GC/MS. Desorption/chemical ionization MS of 5-oxoETE showed ions at m/z = 319 (MH^+) and 336 ($M{\bullet}NH_4^+$): NH_3 at 0.14 Torr; source = 120° C; desorption current = 450 mA.

Bioassays PMN (>95% neutrophils) were isolated from human blood and suspended in Hanks' buffer (Ca^{2+} = 1.4 mM [3]). For degranulation, $3x10^6$ PMN/ml were incubated at 37° C for 20 min, treated with 0 or 5 μg cytochalasin B for 2-4 min, and challenged. Suspensions were iced and centrifuged to obtain supernates which were assayed for lysozyme, β-glucuronidase, and lactic acid dehydrogenase (4). Results are given as net enzyme release (percentage of total enzyme released by stimulated PMN less that released by unstimulated but otherwise identically treated PMN). The reagents and incubations used did not release cytosolic lactate dehydrogenase. Lysozyme and β-glucuronidase release thus reflected degranulation rather than cytolysis. For $[Ca^{2+}]_i$, PMN were loaded with Fura-2 acetoxymethyl ester; washed; suspended at $1x10^7$ cells/ml; incubated at 37° C for 20 min; and challenged while being excited alternately at 340 and 380 and monitored at 510 nm with a spectrophotofluorometer. Results are given in nM $[Ca^{2+}]_i$ (2).

RESULTS

5-OxoETE stimulated PMN to release secondary (lysozyme) and primary (β-glucuronidase) granule enzymes. Its effect, however, was small compared to LTB_4 which readily induced 5-fold greater responses. 5-HETE also degranulated PMN but its actions were not only small but also occurred only at ≥500 nM (Fig 1, left panel). Nonetheless, TNF-α greatly increased responses to 5-oxoETE and 5-HETE. In PMN treated with the cytokine for 15

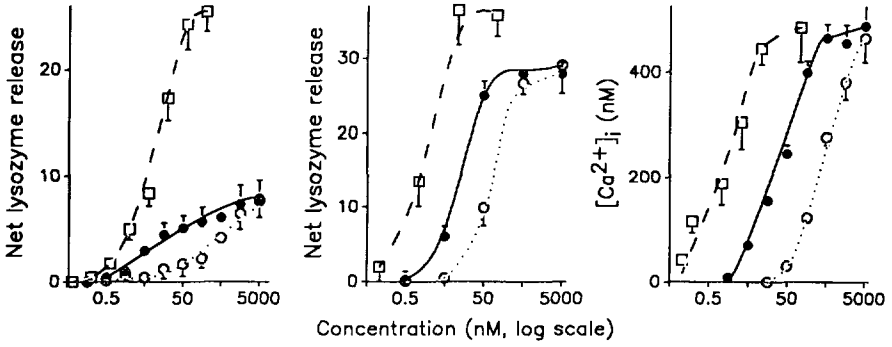

Fig. 1. PMN responses to AA metabolites. PMN were challenged with LTB_4 (dashed lines), 5-oxoETE (solid lines), or 5-HETE (dotted lines). Left panel: net lysozyme release in PMN pretreated with 5 μg/ml cytochalasin B for 2-4 min. β-Glucuronidase was similarly effected by the stimuli. In the absence of cytochalasin B, no net enzyme release occurred. Center panel: lysozyme release by PMN pretreated with 1000 U/ml TNF-α for 12.5 min and 5 μg/ml cytochalasin B for 2.5 min. Results are corrected for lysozyme release induced by TNF-α, cytochalasin B, and 62.5 μg/ml BSA in the absence of 5-oxoETE or 5-HETE. β-Glucuronidase release gave similar results. Without cytochalasin B, LTB_4, 5-oxoETE, and 5-HETE induced small ($\leq$ 12%) but significant net enzyme release at $\geq$ 1, 5, or 50 nM, respectively. Right panel: rises in $[Ca^{2+}]_i$ 15 s after challenge. All stimuli caused $[Ca^{2+}]_i$ rises that peaked by 15 sec and returned to baseline within 1 min. Data are means $\pm$ SEMs for 7-10 studies.

min, LTB_4, 5-oxoETE, and 5-HETE induced large degranulation responses with respective relative potencies of 100, 10, and 1 (Fig 1, center panel). With these PMN, 5-oxoETE and 5-HETE desensitized to themselves or each other but not to LTB_4; LTB_4 desensitized itself but not 5-oxoETE or 5-HETE; and a LTB_4 antagonist, LY 255283 (6), blocked LTB_4 without blocking 5-oxoETE or 5-HETE (Table 1). Evidently, then, 5-oxoETE and 5-HETE act by a common, down-regulatable mechanism which differs from that used by LTB_4.

Table 1

Effects of desensitization and LY 255283 on the degranulation responses of TNF-α-primed PMN[1]

	Inhibitor				
Stimulus (nM)	BSA	5-oxoETE	5(S)-HETE	LTB_4	LY 255283
5-oxoETE, 50	21 ± 2.2[2]	4 ± 2*	2 ± 2*	21 ± 1.5	21 ± 2.9
5-oxoETE, 16	18 ± 2.6	1 ± 3*	1 ± 3*	18 ± 1.9	20 ± 3.5
5-HETE, 500	24 ± 3.6	5 ± 2*	1 ± 1*	26 ± 2.6	24 ± 3.4
5-HETE, 160	18 ± 2.8	4 ± 1*	2 ± 3*	19 ± 2.4	20 ± 2.7
LTB_4, 3	31 ± 3.0	28 ± 2.0	26 ± 3.2	4 ± 2*	15 ± 2.4*
LTB_4, 1	19 ± 3.4	18 ± 3.0	15 ± 1.7	3 ± 2*	5 ± 2*

[1] PMN ($6x10^6$/ml) were treated with 1000 U/ml TNF-α for 10 min; diluted into an equal volume of buffer containing 125 μg/ml BSA; incubated for 2.5 min with $\pm$ 50 nM 5-oxoETE, 500 nM 5(S)-HETE, 3 nM LTB_4, or 2 μM LY 255283; exposed to 5 μg/ml cytochalasin B for 2.5 min; and challenged with the indicated stimulus for 10 min.

[2] Net lysozyme release ($\pm$ SEM, N = 6). Values are corrected for lysozyme released by PMN incubated with TNF-α and BSA $\pm$ an inhibitor but no stimulus. Assay of ß-glucuronidase gave similar results.

* indicates values significantly ($p<0.01$, Students paired t-test) lower than identically stimulated PMN that were pretreated with BSA but no inhibitor.

LTB_4, 5-oxoETE, and 5-HETE also induced PMN to raise $[Ca^{2+}]_i$. Again, their respective, relative potencies were 100, 10, and 1 (Fig 1, right panel); 5-oxoETE and 5-HETE were able to desensitize themselves and each other but not LTB_4; and LTB_4 desensitized itself but not 5-oxoETE or 5-HETE (Table 2). This confirms conclusions made above, *viz.*, 5-oxoETE and 5-HETE, although not LTB_4, are biological as well as structural analogs. One peculiarity did occur. 5-OxoETE effectively desensitized degranulation (Table 1) yet was relatively weak in desensitizing Ca^{2+} transients (Table 2). 5-HETE desensitized PMN equally well in both assays. We cannot explain this interassay discrepancy. Finally, 5-oxoETE enhanced PMN degranulation responses to PAF (Fig 2) and a diacylglycerol, Di-C8, yet had no effect on LTB_4, fMLP, or ionomycin (Table 3). This stimulus-selectivity is identical to 5-HETE's (4), although 5-oxoETE was 10-fold stronger than 5-HETE in potentiating PAF (Fig 2, right panel) and Di-C8 (not shown). Since LTB_4 does not promote PMN responses to PAF (3), the data provide further evidence that 5-oxoETE is a 5-HETE, rather than a LTB_4, analog.

DISCUSSION

5-OxoETE possesses a 5-HETE-like activity profile (*e.g.*, stimulates Ca^{2+} transients and enhances responses to PAF and Di-C8). It is however, far stronger than 5-HETE and, like LTB_4, degranulates PMN. The last finding prompted retesting 5-HETE. It too stimulated release of granule-bound enzymes. While small and formerly discounted, this action takes on new importance: at nM levels, 5-oxoETE and 5-HETE caused some enzyme release in unprimed PMN and large responses in PMN primed with TNF-α. The eicosanoids seem best regarded as having small intrinsic degranulating activity that translates into much more prominent effects under appropriate conditions of cell stimulation. Both degranulation and Ca^{2+} transient responses to 5-oxoETE and 5-HETE involved a common, down-regulatable,

TABLE 2

Desensitization of PMN Ca^{2+} transient responses by AA metabolites[1]

	Desensitizing agent			
Stimulus (nM)	BSA	5(S)-HETE	5-oxoETE	LTB_4
5(S)-HETE, 500	180 ± 30[2]	34 ± 7*	120 ± 17*	160 ± 15
5-oxoETE, 50	170 ± 22	14 ± 8*	120 ± 14*	160 ± 29
LTB_4, 3	220 ± 42	230 ± 29	300 ± 57	90 ± 32*
PAF, 3	240 ± 73	310 ± 52	300 ± 82	220 ± 45

[1] PMN were exposed to 62.5 μg/ml BSA ± 500 nM 5(S)-HETE, 50 nM 5-oxoETE, or 3 nM LTB_4 and, 2.5 min thereafter, the indicated stimulus.
[2] nM rise in $[Ca^{2+}]_i$ 15 s after exposure to the stimulus. Values are corrected for $[Ca^{2+}]_i$ levels found just before stimulation. N = 5-10, ± SEM.
* values significantly lower ($p < .05$, Students paired-T test) than PMN treated with BSA but no desensitizing agent.

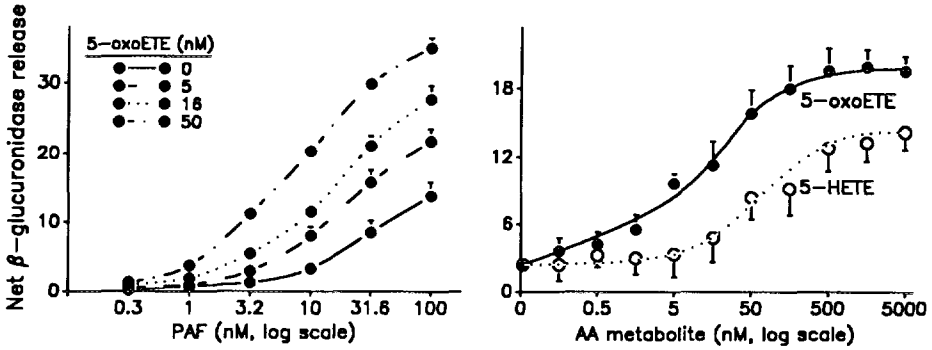

Fig 2. Effects of 5-oxoETE and 5-HETE on PAF-induced degranulation. PMN were treated with 5 μg/ml cytochalasin B for 2-4 min and then simultaneously challenged with indicated concentrations of 5-oxoETE plus PAF (left panel) or challenged with 10 nM PAF plus indicated concentrations of 5-oxoETE or 5-HETE (right panel). Results are corrected for β-glucuronidase released by 5-oxoETE or 5-HETE alone. Data are means ± SEMs for 8 studies.

and therefore presumably receptor-mediated mechanism. Since PMN interconvert 5-oxoETE and 5-HETE (5), 5-HETE's bioactions might require it first be metabolized to 5-oxoETE. This would explain the relative potencies of 5-oxoETE and 5-HETE as well as their sharing of a common PMN-activating mechanism. Other observations counter such notions. 5-oxoETE and 5-HETE induced kinetically identical $[Ca^{2+}]_i$ rises that began within 5 sec and peaked by 15 sec. Metabolism of exogenously applied 5-HETE might not proceed with the rapidity required of these responses. 5-HETE thus may act by its own, rather than its metabolite's, effects, and catalyzed interconversions of 5-HETE and 5-oxoETE may serve as a means for regulating the potencies of endogenously formed products. In any event, 5-oxoETE and 5-HETE did not cross-desensitize with LTB_4 or show sensitivity to a LTB_4 antagonist. PMN

Table 3

Stimulus selectivity of the potentiating actions of 5-oxoETE in PMN degranulation assays[1]

	5-oxoETE (nM)				
Stimulus (nM)	BSA	5	50	500	5000
Di-C8, 3	7.1 ± 1.7[2]	10.0 ± 2.9*	11.6 ± 2.2*	12.3 ± 1.7*	12.6 ± 1.4*
Di-C8, 10	14.1 ± 2.4	18.3 ± 3.8*	22.3 ± 2.2*	nd	nd
LTB_4, 3	16.1 ± 1.6	nd	nd	14.8 ± 1.7	12.3 ± 1.5
fMLP, 3	12.7 ± 2.5	nd	nd	12.9 ± 1.9	10.8 ± 1.2
ionomycin, 100	20.9 ± 1.5	nd	nd	19.9 ± 2.3	18.7 ± 2.0

[1] PMN ($3x10^6$/ml) were treated with 5μg/ml cytochalasin B for 2-4 min and then challenged simultaneously with LTB_4 or fMLP plus 5-oxoETE for 5 min or, alternatively, challenged for 10 min (in the absence of cytochalasin B) with Di-C8 or ionomycin plus 5-oxoETE.
[2] Net lysozyme release (± SEM, N = 6). Values are corrected for the lysozyme released by cells incubated with BSA ± 5-oxoETE but no stimulus.
* indicates values significantly ($p < 0.01$, Students paired t-test) higher than identically stimulated PMN treated with 62.5 μg/ml BSA but no 5-oxoETE.
nd, not done.

thus evince functionally discrete mechanisms for responding to LTB_4 and 5-oxoETE/HETE. While the latter mechanism resembles the LTB_4 mechanism in linking to Ca^{2+} transients and eliciting cytokine-primed degranulation, it diverges from the LTB_4 mechanism in promoting the actions of PAF and causing only small degranulation responses in unprimed PMN. The two mechanisms thus have overlapping, not identical, influences and may serve different roles in PMN. Agonist specificities of these mechanisms, accordingly, become of special interest. Current work indicates cells make many AA metabolites with oxo and hydroxy residues (5,7,8). We suggest these metabolites be tested for effects on not only the LTB_4 but also the 5-oxoETE/HETE mechanism. 5-OxoETE and 5-HETE are representatives of a novel class of biologically analogous AA metabolites. They apparently operate by a receptor mechanism different from the receptor mechanism used by LTB_4. Some as yet untested AA metabolite might stimulate PMN *via* both mechanisms to prove that LTB_4 and putative 5-oxoETE/HETE receptors, like β_1 and β_2 adrenergic receptors, are closely related.

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