Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 1-7

5-L-HYDROXY-6,8,11,14-EICOSATETRAENOATE POTENTIATES THE HUMAN NEUTROPHIL DEGRANULATING ACTION OF PLATELET-ACTIVATING FACTOR

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Received December 3, 1982

Platelet-activating factor (AAGPC) and two of its structural analogues degranulated human neutrophils with respective potencies that were increased up to 100 to 1000-fold by 16 nM to 5 μM of 5-L-hydroxyeicosatetraenoate (5-L-HETE). 5-rac-HETE had similar actions but 8-rac-HETE was without effect. Furthermore, 5-L-HETE did not influence the degranulating actions of C5a, A23187 or a formalated oligopeptide chemotactic factor and none of the HETEs, by themselves, caused degranulation. Thus, 5-L-HETE and AAGPC selectively interact to induce degranulation. Since these products rapidly form in stimulated PMNs, they may serve as potentiator and agonist, respectively, to transduce biological signals into cell function.

AAGPC[1] causes PMN to metabolize their stores of arachidonate via a 5-lipoxygenase into two major products, 5-L-HETE and LTB_4 (1,2). Concomittantly, it stimulates these cells to degranulate and aggregate through reaction sequences inhibited by blockers of 5-lipoxygenase (1-4). Hence, the metabolites may mediate cell function. Of the two products, only LTB_4 reproduces the degranulating actions of AAGPC (3,5,6,7) suggesting that it, rather than 5-L-HETE, is the crucial mediator of this response. Other observations dispute this. First, AAGPC produces a greater degranulation response than LTB_4 (3). Second, the degranulating action of AAGPC and LTB_4 are similarly susceptible to anti-lipoxygenases (3,4). Third, PMN desensitized with LTB_4, and therefore totally unresponsive to it, degranulate

[1]Abbreviations used: AAPGC, platelet-activating factor or 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine; AEGPC, 1-O-alkyl-2-O-ethyl-sn-glycero-3-phosphocholine; APPC, rac-1-O-hexadecyl-2-n-propyl-3-O-phosphorylcholine-propane; 5-L-HETE, 5-L-hydroxy-cis-8,11,14-trans-6-eicosatetraenoate; 5-rac-HETE, the racemate of 5-L-HETE; 8-rac-HETE, 8-rac-hydroxy-cis-5,11,14-trans-9-eicosatetraenoate; LTB_4, leukotriene B_4; FMLP, N-formyl-(S)-methionyl-(S)leucyl-(S)-phenylalanine; BSA, bovine serum albumin; PMN, polymorphonuclear neutrophil(s)

0006-291X/83/040001-07$01.50/0

normally when challenged with AAGPC (7). Such findings do not apply to PMN aggregation, wherein AAGPC and LTB_4 have more comparable potencies (2); LTB_4 has actions relatively insensitive to anti-lipoxygenases (2); and LTB_4-desensitzed cells are hyporesponsive to AAGPC (2,8). Endogenously formed LTB_4, therefore, may mediate AAGPC-induced aggregate formation, but its role in the degranulation response to this same stimulus is more tenuous. This prompted us to re-evaluate the effects of 5-L-HETE on AAGPC-induced degranulation.

MATERIALS AND METHODS

Reagents and buffers. Cytochalasin B and BSA (Sigma Chemical Co., St Louis, MO); FMLP (Peninsula Labs, San Carlos, CA); and A23187 (Calbiochem, La Jolla, Ca) were purchased. AAGPC (derived from beef heart plasmologens); synthetic AEGPC and APPC; human C5a; 5-L-HETE (isolated from rabbit peritoneal PMN challenged with arachidonic acid plus A23187); and 5-rac-HETE and 8-rac-HETE (isolated from autooxidized arachidonate) were prepared, purified, stored, and used as detailed elsewhere (3,9-12). Structural confirmation was based upon: characteristic migration on zonal electrophoresis as a single band for C5a (9); typical elution times as single peaks on high performance liquid chromatography for the HETEs (12); characteristic migration on thin layer chromatography as a single band for the phospholipids (9,10); and definitive fragmentation patterns on mass spectroscopy for the HETEs and phospholipids (9-12). The three phospholipds, FMLP, and C5a were diluted in BSA-containing buffer and added to cell suspensions at a final dilution that made BSA 125 µg/ml of suspension. Varying the BSA concentration from 12.5 to 250 µg/ml in assays of AAGPC or omitting BSA from the assays of C5a or FMLP had no influence on the effects of the HETEs reported here. The buffer was a modified Hanks' balanced salt solution (9).

Degranulation. Leukocytes (> 96% PMN, fewer than 5 platelets per 100 PMN, and no detectable erythrocytes) were isolated from normal human donor blood (9). 1.3 million PMN were incubated in 0.5 ml buffer (37°C; 1.4 mM calcium chloride; 0.7 mM magnesium chloride) for 20 mins, treated with 2.5 µg cytochalasin B for 2-4 min; and challenged for 5 mins. In studies employing A23187 as stimulus this protocol was altered: cytochalasin B was omitted and the challenge period lengthened to 10 mins. The extracellular fluid of these suspensions was isolated and assayed for lysozyme (E.C. 3.2.1.17), β-glucuronidase (E.C. 3.2.1.21), and lactic acid dehydrogenase (E.C. 1.1.1.27), as described (9). Results are reported as net enzyme release, i.e., percentage of total cellular enzyme released by stimulated, minus that occurring in unstimulated (but otherwise identically treated), cell suspensions. None of the reaction conditions or reagents used here caused net release of the cytosolic enzyme, lactic acid dehydrogenase, and therefore were not cytolytic.

RESULTS AND DISCUSSION

10 µM to 10 nM of 5-L-HETE, 5-rac-HETE, or 8-rac-HETE did not degranulate PMN during the 5 min challenge period. This is in substantial agreement with previous reports (5,6,13). However, as shown in the two left panels of Figure 1, 16-500 nM 5-L-HETE enhanced the degranulating action of AAGPC. This

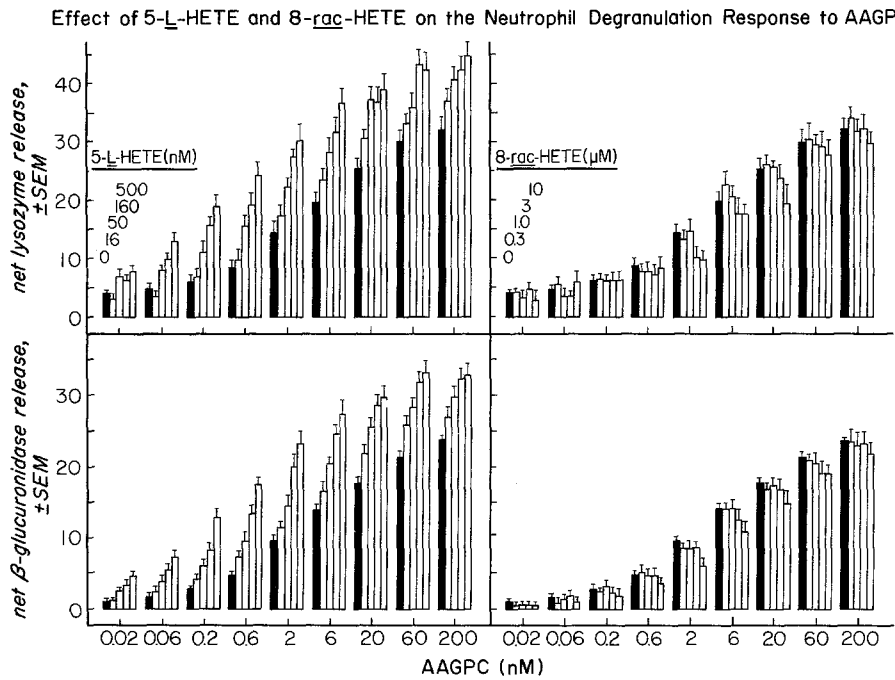

Figure 1 - Net release of lysozyme (upper panels) and β-glucuronidase (lower panels) by neutrophils simultaneously exposed to varying dosages of AAGPC plus 5-L-HETE (panels on left) or 8-rac-HETE (panels on right). Shaded bars give responses induced by AAGPC alone; adjacent unshaded bars give responses induced by AAGPC in the presence of increasing dosages of HETE. All results were first corrected for background release. Each bar gives the mean, ± SEM, for at least seven experiments. For 5-L-HETE studies, all values employing ≥ 50 nM of HETE were significantly ($p < 0.05$, student's unpaired t-test) greater than that found for cells treated with only AAGPC; for 8-rac-HETE, none of these values were significantly different.

effect was dramatic. At 500 nM, for instance, 5-L-HETE increased the potency of AAGPC 40-to-200-fold (e.g., cells challenged with 0.6-2nM AAGPC plus HETE responded like cells challenged with 200 nM AAGPC) and augmented the response to 200 nM AAGPC by 40% (above 200 nM, AAGPC produces decreasing effects [3]). Indeed, at 1.6 or 5μM, the HETE enhanced potency by 100- to 1000-fold and optimal responses by 40-50% (data not shown). 5-rac-HETE had similar actions (Figure 2, lower right panel). Contrastingly, 8-rac-HETE had no effects on AAGPC (Figure 1, panels on right) and 5-L-HETE did not influence the degranulating actions of FMLP, C5a, or A23187 (Figure 2). Hence, 5-L-HETE is a selective potentiator of the bioaction of certain stimuli rather than a non-specific promotor of degranulation per se.

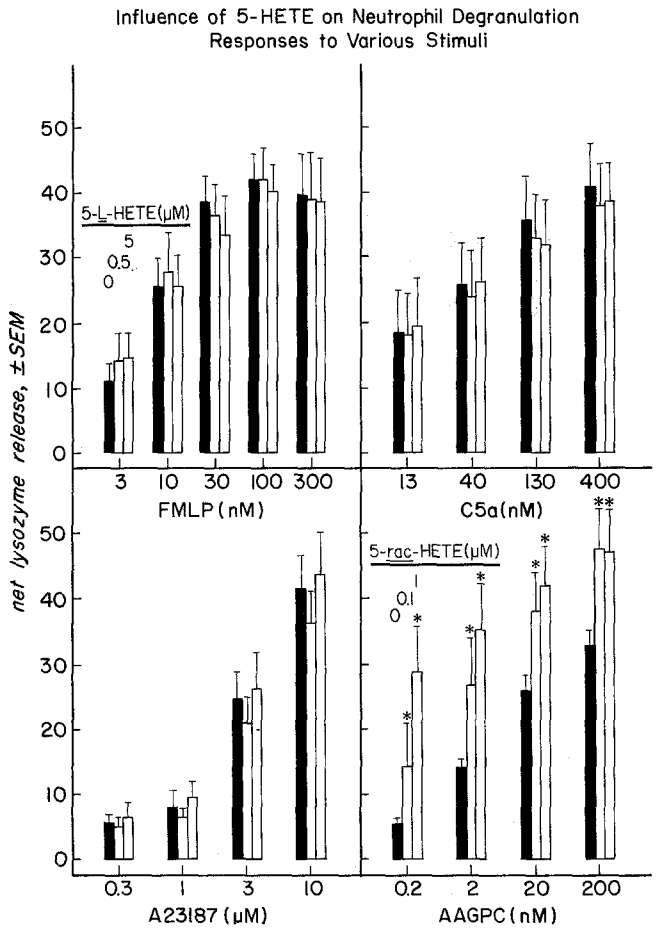

FIGURE 2 - Net release of lysozyme by neutrophils simultaneously exposed to 5-L-HETE plus FMLP (upper left panel), C5a (upper right panel), or A23187 (lower left panel), or exposed to 5-rac-HETE plus AAGPC (lower right panel). Shaded bars give responses induced by the stimulus in the absence of the HETE; adjacent unshaded bars give response induced by stimulus in the presence of increasing dosages of HETE. All responses were corrected for background release. Each bar gives the mean, ± SEM, for at least six experiments. Astericks indicate values significantly ($p < 0.05$ student's unpaired t-test) greater than in identically stimulated cells not exposed to HETE. Results similar to those recorded were found for β-glucuronidase release.

FMLP, C5a, and A23187 appear to elicit degranulation responses by activating arachidonate metabolism in a fashion analogous to AAGPC (3,6,7, 11,14,15). The selective action of 5-HETEs, therefore, is surprising and suggests that at least certain features of the interaction between AAGPC and arachidonate metabolism are unique. One possible explanation for this lies in the way PMN handle AAGPC. These cells rapidly deacetylate AAGPC and then

Table 1 Effect of 5-L-HETE on the Neutrophil Degranulation Response to Two Analogues of AAGPC[1]

Analogue	(μM)	5-L-HETE (nM) 0	50	500
APPC	3	5.3 ± 1.0[2]	13.6 ± 1.6*	20.8 ± 1.7*
APPC	0.3	5.9 ± 0.5	13.2 ± 0.8*	21.7 ± 1.3*
APPC	0.03	1.4 ± 0.7	7.8 ± 1.1*	13.0 ± 10.1*
AEGPC	0.5	16.4 ± 2.1	24.0 ± 1.8*	28.0 ± 1.2*
AEGPC	0.05	10.0 ± 2.6	17.4 ± 2.7*	25.7 ± 1.6*
AEGPC	0.005	3.6 ± 1.8	9.9 ± 2.0*	16.9 ± 2.9*

[1]Neutrophils were exposed to the indicated dosage of analogue and 5-L-HETE simultaneously.
[2]Net release of β-glucuronidase, ± SEM, for four experiments. Similar results were found for net lysozyme release.
*Indicates values significantly ($p < 0.05$, student's paired t-test) greater than the response of cells challenged in the absence of 5-L-HETE (i.e., first column of values).

insert long chain unsaturated fatty acids at the vacated 2-position (16 and personal observations of F.H. Chilton, J.T. O'Flaherty, and R.L. Wykle). This reaction could link 5-L-HETE with deacetylated AAGPC to form a novel phospholipid that influences cell membrane properties and the degranulation response. Stimulated PMN do, in fact, esterfy 5-L-HETE to the 2-position of phosphocholines (6). However, 5-L-HETE also potentiated the actions of AEGPC and APPC (Table 1), two analogues containing non-labile ethyl ether and n-propyl groups, respectively, directly linked to the carbon at position 2. Thus, chemical interaction between 5-L-HETE and AAGPC as described above is probably not required for potentiation. AAGPC, AEGPC, and APPC activate PMN by a common step that is not involved in the responses to FMLP or C5a (9,10). The 5-HETEs may act at this step.

In studies not shown, we found that 500 nM 5-L-HETE augmented lysozyme release when added 1 or 8 min before, simultaneously with, or 1 to 8 min after, 6 nM AAGPC (cells were preincubated with cytochalasin B for 2 min before AAGPC challenge and incubated for 5 min following addition of the last agent, i.e., 5-L-HETE and/or AAGPC). However, cell washing completely reversed potentiation (PMNs were incubated with 500nM 5-L-HETE for 8 min, washed for 2 min [9], treated with cytochalasin B for 2 min, and then

challenged with 2, 6, or 60 nM AAGPC; 5-L-HETE was fully capable of potentiating AAGPC if added after cell washing). Thus, potentiation requires the presence of 5-L-HETE and, surprisingly, is demonstrable even up to 8 min following cell exposure to AAGPC. Since the degranulation response to AAGPC is completed within 1-2 min and cannot be augmented further with a second AAGPC challenge (9), the latter finding makes it unlikely that 5-L-HETE acts extracellularly to increase the bioavailability of AAGPC.

The results reported here are the first to implicate 5-L-HETE in the PMN degranulation response to a physiological stimulus. They also demonstrate an alternative to the notion that LTB_4 is the exclusive mediator of degranulation formed by 5-lipoxygenase. That is, the endogenous 5-L-HETE formed in AAGPC-challenged cells may interact with exogenous AAGPC to effect degranulation. Indeed, AAGPC rapidly forms in PMNs exposed to a wide range of stimuli (16-18) and would be free to interact with the 5-L-HETE that also forms under most of these conditions (1,2,6,11,14,15). The two products, then, could serve as agonist and poteniator, respectively, to mediate function. As outlined in the Introduction, this mechanism need not be evoked to explain PMN aggregation and, apparantly, does not apply to FMLP-, C5a-, or A23187-induced degranulation (which are not influenced by exogenous 5-L-HETE--Figure 2). However, it is an attractive hypothesis for AAGPC-induced degranulation and has relevancy to other types of cells, stimuli, and/or responses wherein the mediating role of LTB_4 is tenuous.

ACKNOWLEDGEMENTS:

Thank you to Mrs. Anne Winburn for preparing the manuscript. This work was supported in part by NIH grants HL27799, HO 26257, HL 29293, HL 26818, and AI 17287.

REFERENCES

1. Chilton, F.H., O'Flaherty, J.T., Walsh, C.E., Thomas, M. J., Wykle, R.L., DeChatelet, L.R., and Waite, B.M. (1982) J. Biol. Chem. 257, 5402-5407.
2. Lin, A.H., Morton, D.R., and Gorman, R.R. (1982) J. Clin. Invest. 70, 1058-1065.
3. O'Flaherty, J.T., Wykle, R.L., Lees, C.J., Shewmake, T., McCall, C.E., and Thomas, M.J. (1981) Am. J. Pathol. 155, 264-269.
4. Smith, R.J. and Bowman, B.J. (1982) Biochem. Biophys. Res. Commun. 104, 1455-1501.

5. Goetzl, E.J. and Pickett, W.C. (1980) J. Immunol. 125, 1789-1791.
6. Stenson, W.F., and Parker, C.W. (1980) J. Immunol. 124, 2100-2104.
7. O'Flaherty, J.T., Wykle, R.L., McCall, C.E., Shewmake, T.B., Lees, C.J., and Thomas, M. (1981) Biochem Biophys. Res. Commun. 101, 1290-1296.
8. O'Flaherty, J.T., Hammett, M.J., Shewmake, T.B., Wykle, R.L., Love, S.H., McCall, C.E., and Thomas, M.J. (1981) Biochem. Biophys. Res. Commun. 103, 552-555.
9. O'Flaherty, J.T., Lees, C.J., Miller, C.H., McCall, C.E., Lewis, J.C., Love, S.H., and Wykle, R.L. (1981) J. Immunol. 127, 731-737.
10. Wykle, R.L., Surles, J.R., Piantadosi, C., Salzer, W.L., and O'Flaherty, J.T. (1982) FEBS Lett. 141, 29-32.
11. Borgeat, P., and Samuelsson, B. (1979) J. Biol. Chem. 254, 7865-7869.
12. O'Flaherty, J.T., Thomas, M.J., Lees, C.J., and McCall, C.E. (1981) Am. J. Pathol. 104, 55-62.
13. Showell, H.J., Naccache, P.H., Borgeat, P., Pincard, S., Vallerand, P., Becker, E.L., and Sha'afi, R.I. (1982) J. Immunol. 128, 811-816.
14. Bokoch, G.M., and Reed, P.W. (1981) J. Biol. Chem. 256, 4156-4159.
15. Jubiz, W., Radmark, O., Malmsten, C., Hansson, G., Lindgren, J.A., Palmblad, J., Uden, A.M., and Samuelsson, B. (1982) J. Biol. Chem. 257, 6106-6110.
16. Lee, T-c, Malone, B., Wasserman, S.I., Fitzgerald, V., and Snyder, F. (1982) Biochem. Biophys. Res. Commun. 105, 1303-1308.
17. Betz, S.J., and Henson, P.M. (1980) J. Immunol. 125, 2756-2763.
18. Camussi, G., Aglietta, M., Coda, R., Bussolino, F., Piacibello, W., and Tetta, C. (1981) Immunology. 42, 191-199.