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EVIDENCE FOR 5,12-diHYDROXY-6,8,10,14-EICOSATETRAENOATE AS A MEDIATOR OF HUMAN NEUTROPHIL AGGREGATION

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SUMMARY

The human polymorphonuclear neutrophil (PMN) aggregation responses to 5(S),12(R)-dihydroxy-cis-6,14-trans-8,10-eicosatetraenoate (diHETE), C5a, N-formyl-methionyl-leucyl-phenylalanine (FMLP), and 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine (AAGPC) were desensitized by preincubating the cells with small amounts of diHETE. Desensitization developed rapidly, persisted in washed cells, and was not due to stimulus inactivation. The desensitized cells exhibited normal aggregation responses to ionophore A23187 and phorbol myristate acetate (PMA). Thus, responsiveness to diHETE appears necessary for the aggregation response to C5a, FMLP, and AAGPC. Endogenous diHETE, which forms rapidly in cells challenged with these latter stimuli, may mediate their aggregating actions.

INTRODUCTION

PMNs rapidly form diHETE when stimulated (1-5). Since this compound is itself a potent stimulator of PMN aggregation (2) and degranulation (6-8), it may mediate these responses. We studied PMN desensitization in order to supply functional evidence supporting this concept. Under certain conditions PMN incubated with a stimulus rapidly lose their ability to aggregate and degranulate in response to that stimulus yet retain full responsiveness to structurally unrelated stimuli (3,8-13). We reasoned that, in contrast to this, PMNs desensitized to a true endogenous mediator should be desensitized to all stimuli acting through the mediator (8,10). In previous studies (8) we found that PMNs totally desensitized to (and, therefore, unable to degranulate when challenged with) diHETE degranulated normally in response to other stimuli. The data suggested that diHETE is not a universal mediator of degranulation. Here, PMN aggregation was similarly studied. In direct

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contrast to degranulation, diHETE effectively desensitized PMN aggregation responses to C5a, FMLP, and AAGPC. Endogenoulsy diHETE, which forms in PMNs exposed to these agents (3-6), may mediate their aggregating actions.

MATERIALS AND METHODS

Reagents and buffer. diHETE (8,14), human C5a (10), AAGPC (10), FMLP (10), A23187 (8), PMA (15), cytochalasin B (CB) (9), and modified Hanks' balanced salt solution free of bivalent cations (9) were obtained and used as described.

Bioassay. Human leukocytes (> 95% PMN, no erythrocytes, and < 5 platelets/100 PMN) were suspended in Hanks' medium at 37°C, stirred continuously, and exposed to three sets of reagents: a) diHETE or equal volume of buffer; b) calcium chloride and magnesium chloride (respective concentration in suspension: 1.4 and 0.7 mM); and c) a challenging stimulus or equal volume of medium used to carry the stimulus. The time and sequence of addition of these reagents are given with each experiment. Where indicated, CB was added to the buffer to highten the magnitude of response to diHETE and AAGPC (10). Just before and at 0.25, 0.5, 1, 2, 4, 8, 11, and 15 min after initiation of aggregation (i.e., when suspensions contained both a challenging stimulus and bivalent cations) samples of the suspension were taken and scored for aggregates with a Coulter Counter (9,10). Results are reported as the large particle percentage (i.e., percentage of all particles that are aggregated) or the maximal change in the large particle percentage (greatest large particle percentage found after initiation of aggregation minus the large particle percentage found just before this initiation). These methods are described in detail (9,10). As determined by release of cytosolic enzymes, none of the reagents or reaction conditions employed here were toxic to PMN (8).

RESULTS AND DISCUSSION

Confirming an earlier report (2), diHETE aggregated human PMN (Figure 1, solid line), inducing increasing effects over 3-100 nM. This response had three characteristics: it reversed rapidly, it was potentiated by CB, and it required Ca^{2+} and Mg^{2+} (data not shown). Furthermore, PMNs incubated with diHETE and then exposed to Ca^{2+} and Mg^{2+} exhibited little or no response (Table 1). Nevertheless, the extracellular fluid of cell suspensions incubated with diHETE for 4 min (but not of suspensions incubated without diHETE) aggregated fresh PMNs (data not shown). Therefore, the time-dependent loss of the aggregation response seen in Table 1 was not due to stimulus inactivation or formation of a rapidly acting inhibitor of diHETE. These conclusions were further supported by restimulating the cells. Following 4 mins of incubation

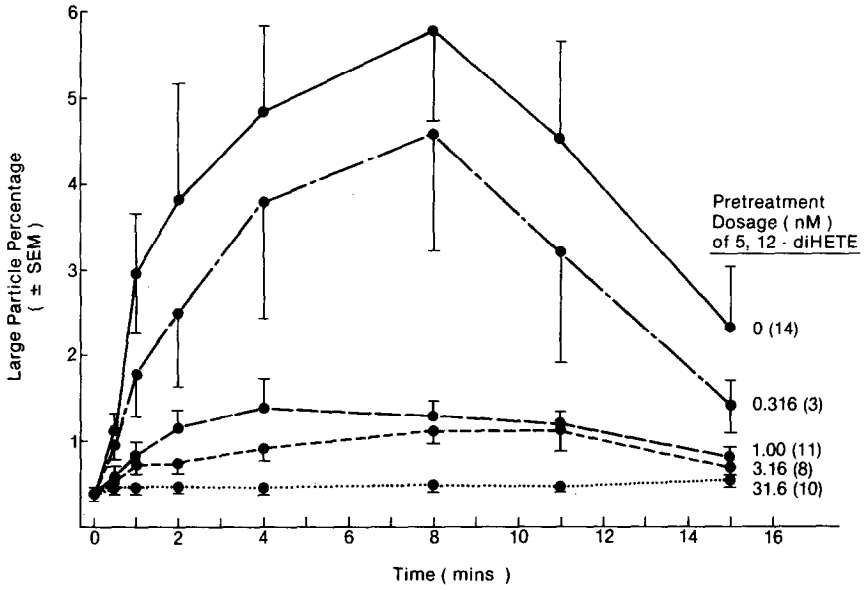

Figure 1 Desensitization of the neutrophil aggregation response to diHETE. Cells were pretreated with 0.5 μg/ml cytochalasin B plus the indicated diHETE dose for 4 min, exposed to calcium and magnesium for 1 min, and at t=0 challenged with 31.6 nM diHETE. The number of experiments for each curve are parenthesized.

TABLE 1
Effect Of Time Of Adding
Bivalent Cations On diHETE-Induced Neutrophil Aggregation[1]

	Time (mins) Between Adding Stimulus and Cations					
Dose (nM)	-1	0	0.5	1	4	8
31.6	6.7 ± 1.3[2]	2.5 ± 0.6	1.0 ± 0.2	0.8 ± 0.2	0.5 ± 0.2	0.3 ± 0.1
10	6.2 ± 1.3	-	-	-	0.6 ± 0.2	-
3.16	3.0 ± 0.9	-	-	-	0.3 ± 0.1	-
1.00	1.0 ± 0.5	-	-	-	0.5 ± 0.3	-

[1] Cells were treated with 1.4 mM calcium and 0.7 mM magnesium for 1 min before exposure to diHETE (t = -1), treated with the bivalent cations simultaneously with diHETE (t = 0), or treated with the cations at various times after exposure to diHETE (t > 0). These studies used cytochalasin B (0.5 μg/ml) to enhance the response but qualitatively similar results were found in the absence of cytochalasin B.

[2] Maximal change in large particle percentage, ± SEM (N > 4), following the onset of aggregation (i.e., from the time suspensions contained both stimulus and cations). Under all the various conditions, unstimulated cells or stimulated cells not treated with the cations had maximal changes in the large particle percentages < 0.6.

TABLE 2

Aggregation Responses of diHETE-Desensitized Neutrophils

Stimulus	Percentage Inhibition	
	Unwashed[1]	Washed[2]
diHETE (31.6 nM)[3]	99.4*[4]	88.6*
FMLP (500 nM)	78.0*	62.3*
AAGPC (400 nM)[3]	68.6*	43.3*
C5a (400 nM)	57.8*	ND

1 These cells were preincubated with 10 nM diHETE for 4 min, treated with calcium and magnesium for 1 min, and then exposed to the indicated stimulus. The maximal change in the large particle percentage (see Materials and Methods) was calculated after addition of stimulus and compared to identically challenged cells that were not preincubated with diHETE in order to obtain the percentage inhibition.

2 These cells were preincubated with 10 nM diHETE for 4 min and rapidly (<3 min) washed by a procedure that removed >99.9% of the extracellular fluid (9) before being suspended in fresh buffer (containing bivalent cations) for 2 min and then challenged. Results were compared to identically challenged but undesensitized cells to obtain percentage inhibition.

3 In these studies buffer contained 0.5 μg CB (qualitatively similar results were found without CB).

4 Percentage inhibition for at least 8 experiments. The SEM was <20% of the mean.

* Indicates values significantly ($p < 0.05$, students t-test) lower than those found in undesensitized cell.

ND - not done

with diHETE, PMNs were treated with Ca^{2+} and Mg^{2+} for 1 min and then challenged with fresh diHETE. Little or no response ensued when cells were initially incubated with > 1 nM diHETE (Figure 1, interrupted lines). Furthermore, unresponsiveness persisted after the cells were washed (Table 2). Thus, the unresponsitivity seen here is a form of cellular desensitization (9,11).

C5a, FMLP, and AAGPC also desensitize PMNs. Cells preincubated with one of these agents neither aggregate nor degranulate when rechallenged with that agent but respond normally when challenged with the other agents (9-13). This desensitization, then, is stimulus-specific and suggests that each stimulus binds to and then inactivates its cell receptor while leaving unrelated

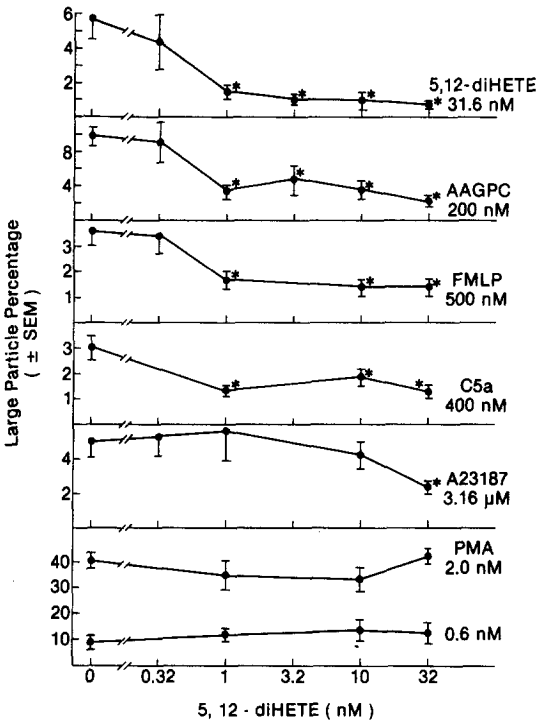

Figure 2 Aggregation response of diHETE-desensitized neutrophils to various stimuli. Cells were desensitized as described in Figure 1. Studies with diHETE or AAGPC as challenging stimuli used 0.5 μg/ml cytochalasin B. Each point is the mean of > 8 experiments. Studies giving qualitatively similar results to those shown were found at various doses of challenging stimuli (diHETE, 3-100 nM; AAGPC, 2-200 nM; FMLP, 20-500 nM; C5a, 20-400 nM; A23187, 0.3-3 μM; and PMA, 0.2 6 nM) and in the absence of cytochalasin B. Astericks indicate those values significantly ($p < 0.05$) lower than identically challenged but undesensitized cells.

receptors fully capable of triggering aggregation and degranulation (8,9,15). Conversely, mediator-induced desensitization should be response-specific, leaving PMNs fully able to exhibit responses that are not dependent on the mediator. Previously, we showed that diHETE desensitized the PMN degranulation response to itself but not to C5a, FMLP, or AAGPC (8). In contrast to this, we find that diHETE partially cross-desensitizes the PMN aggregation response to these stimuli (Figure 2) and this effect persists in washed cells (Table 2). Thus, cross-desensitization is response-specific, occurring in PMNs capable of degranulating normally to these stimuli. The cells must be inhibited at a step beyond stimulus recognition and in a pathway mediating

aggregation but not degranulation. This unique type of desensitization supports the concept that endogenous diHETE mediates the aggregating (but not degranulating) actions of certain stimuli. Not all aggregating stimuli need be mediated in this way. For instance, PMA does not cause PMN to form diHETE (16). Its aggregating action, unlike those of C5a, FMLP, or AAGPC (5,17), is not blocked by inhibitors of diHETE formation (16). The normal aggregation response to PMA by diHETE-desensitized cells (Figure 2, lowest panel), therefore, supports previous suggestions (16) that the phorbol ester aggregates PMNs by a mechanism quite distinct from that of the more physiological stimuli. On the other hand, we find that diHETE-desensitized PMNs respond almost normally to A23187 (Figure 2). This ionophore causes PMN to form diHETE (1,2) and has aggregating actions that are blocked by inhibitors of diHETE formation (17). Thus, A23187 may activate PMNs by multiple pathways some of which are capable of mediating a near normal aggregation response independently of diHETE.

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REFERENCES

1. Borgeat, P. and Samuelsson, B. (1979) Proc. Natl. Acad. Sci. USA 76:2148-2152.
2. Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E., and Smith, M.J.H. (1980) Nature 286:264-265.
3. Goetzl, E.J. (1980) Immunol. 40:709-719.
4. Bokoch, G.M. and Reed, P.W. (1980) J. Biol. Chem. 255:10223-10226.
5. O'Flaherty, J.T., Stimler, N., Swendsen, L., and Wykle, R.L.: (1981) Fed. Proc. 40:1710 (abstract).
6. Bokoch, G.M. and Reed, P.W. (1981) J. Biol. Chem. 256:5317-5320.
7. Goetzl, E.J. and Pickett, W.C. (1980) J. Immunol. 125:1789-1791.
8. O'Flaherty, J.T., Wykle, R.L., McCall, C.E., Shewmake, T.B., Lees, C.J., Thomas, M. (1981) Biochem. Biophys. Res. Commun. 101:1290-1296.
9. O'Flaherty, J.T., Kreutzer, D.L., Showell, H.L., Vitkauskas, G., Becker, E.L., and Ward, P.A. (1979) J. Cell Biol. 80:564-572.
10. 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.
11. Henson, P.M., Schwartzman, N.A., and Zanolari, B. (1981) J. Immunol. 127:754-759.

12. Shaw, J.O., Pinckard, R.M., Ferrigini, K.S., McManus, L.M., and Hanahan, D.J. (1981) J. Immunol. 127:1250-1255.
13. Skubitz, K.M. and Craddock, P.R. (1981) J. Clin. Invest. 67:1383-1391.
14. O'Flaherty, J.T., Wykle, R.L., Lees, C.J., Shewmake, T., McCall, C.E., Thomas, M.J. (1981) Am. J. Pathol. in press.
15. Niedel, J.E., Kahane, I., and Cuatreacasas, P. (1979) Science 205: 1412-1414.
16. O'Flaherty, J.T., DeChatelet, L.R., McCall, C.E., and Bass, D.A. (1980) Proc. Soc. Exp. Biol. Med. 165:225-232.
17. Waite, M., DeChatelet, L.R., King, L., and Shirley, P.S. (1979) Biochem. Biophys. Res. Commun. 90:984-992.