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HYDROXYEICOSATETRAENOIC ACIDS AND OTHER UNSATURATED FATTY ACIDS INHIBIT ENDOTOXIN-INDUCED THROMBOPLASTIN ACTIVITY IN HUMAN MONOCYTES

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SUMMARY Human peripheral blood monocytes possessed increased thromboplastin activity when exposed to 10 μg/ml of bacterial endotoxin for 2 h. The effects of endotoxin were strongly inhibited by 10-20 μM of several unsaturated fatty acids: arachidonic, dihomo-gamma-linolenic, linoleic, linolenic and oleic acids. Two saturated fatty acids, arachidic and stearic acids, produced minimal inhibition at 20 μM. Three lipoxygenase-derived hydroxyfatty acids were also inhibitory, with IC_{50} values of 10 μM (5- and 12-hydroxyeicosatetraenoic acids) and 20 μM (15-hydroxyeicosatetraenoic acid). Leukotriene B_4, 1 nM to 1 μM, was inactive. None of the fatty acids affected the ability of standard thromboplastin to shorten the clotting time of normal plasma or affected the activity of endotoxin as measured by a Limulus clotting assay.

Human peripheral blood monocytes show increased levels of the potent procoagulant, thromboplastin (tissue factor; factor III), when stimulated by bacterial endotoxin [1-3]. Thromboplastin activates both the extrinsic and intrinsic blood coagulation cascades, by accelerating the factor VIIa-dependent activation of factors X [4] and IX [5], respectively. Human monocytes also produce a procoagulant factor which functions as a prothrombinase, but its levels do not appear to be influenced by endotoxin [6]. Enhanced monocyte procoagulant activity and subsequent fibrin deposition may play an important role in the pathogenesis of disseminated intravascular coagulation under a variety of conditions [7].

Endotoxin-treated human monocytes metabolize arachidonic acid via the cyclooxygenase pathway to prostaglandins and thromboxanes [8-10], but these metabolites do not appear to mediate the induction of thromboplastin activity [11-13]. However, the stimulation of monocyte thromboplastin activity by

Abbreviation: HETE: hydroxyeicosatetraenoic acid.

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endotoxin can be inhibited by non-specific inhibitors of lipoxygenase [13], suggesting that lipoxygenase metabolites of arachidonic acid may mediate the induction of thromboplastin activity. Human peripheral blood monocytes possess a full complement of the enzymes necessary to metabolize arachidonic acid via the 5-lipoxygenase pathway to 5-HETE and leukotrienes B_4 and C_4 [8,14,15].

The present study was therefore undertaken to investigate whether arachidonic acid and some of its lipoxygenase metabolites could substitute for endotoxin and directly stimulate monocyte thromboplastin activity. Surprisingly, it was found that both arachidonic acid and hydroxyeicosatetraenoic acids (HETEs) did not significantly affect thromboplastin activity in untreated monocytes, and inhibited the development of procoagulant activity in cells treated with endotoxin. Inhibitory effects were also observed with other unsaturated, but not saturated, fatty acids.

MATERIALS AND METHODS

Materials The following materials were used: endotoxin-free RPMI 1640 medium (American Biorganics, North Tonawanda, NY); fetal bovine serum (Hyclone, Logan, UT); endotoxin (E. coli 0111:B4; Difco, Detroit, MI); human plasma deficient in factors V, VII, VIII, X, or XII (Helena, Beaumont, TX); rabbit brain thromboplastin (Ortho, Raritan, NJ); indomethacin and cephalin (Sigma, St. Louis, MO); fatty acids (Nu-Chek-Prep., Elysian, MN); HETEs (Seragen, Cambridge, MA). A portion of the leukotriene B_4 used in this study was generously provided by Dr. Robert Gorman of the Upjohn Company, Kalamazoo, MI, and by Dr. Joe Rokach of Merck-Frosst, Dorval, Quebec, Canada. The remainder was obtained from Seragen.

Cell culture and incubation Blood was drawn by venipuncture from healthy volunteers who had not taken aspirin products for at least 7 days prior to donation. Mononuclear cells were isolated on Ficoll-Hypaque gradients [16]. Cells were washed twice with RPMI medium containing 0.3 mM EDTA, once with RPMI medium containing EDTA plus 20% fetal bovine serum, and then suspended in RPMI medium containing 20% fetal bovine serum only. Approximately 5 x 10^6 cells were plated on 35 mm plastic dishes and allowed to adhere by incubation for 2 h at 37°C in a humidified atmosphere of 5% CO_2. Non-adherent cells were removed by washing with RPMI medium containing 20% fetal bovine serum followed by serum-free medium. Cells were incubated for 15 min with 1 ml serum-free medium containing fatty acids or vehicle (0.1% ethanol). Fifty microliters of a solution of endotoxin in saline (0.9% NaCl), sufficient to give a final endotoxin concentration of 10 µg/ml, was then added to some of the culture dishes. The remainder received saline alone. After further incubation for 2 h, cells were scraped with a rubber policeman and collected by centrifugation (200 x g, 10 min). Cells were then resuspended in 0.2 ml saline, disrupted by three cycles of freeze-thawing, and briefly sonicated.

Coagulation assays Procoagulant activity of the sonicates was measured by a one-stage clotting assay. Assay mixtures contained 0.1 ml sonicate, standard thromboplastin, or saline; 0.1 ml of 25 mM $CaCl_2$; and 0.1 ml of pooled human plasma containing 4% (v/v) cephalin. Clotting times at 37°C were determined. Standard thromboplastin was reconstituted in distilled water, 12 ml/vial, and

assigned a concentration of 100,000 units/ml. Monocyte procoagulant activity was calculated by reference to a standard curve to 3-1,000 units of thromboplastin, which was obtained by plotting log (thromboplastin units) vs log (clotting time).

RESULTS AND DISCUSSION

Human peripheral blood monocytes possessed low levels of procoagulant activity which were stimulated 10-50 fold by a 2 h exposure to 10 μg/ml endotoxin. Monocyte procoagulant and standard thromboplastin activities were expressed in normal plasma and plasma deficient in factors XII or VIII, but not in plasma deficient in factors X, VII or V (Table I). Monocyte procoagulant activity was therefore functionally identical to thromboplastin. When results were pooled from 17 experiments performed on blood obtained from 14 donors (8 male, 6 female), the following data were obtained: untreated cells, 28 $\pm$ 9 units/0.1 ml cell sonicate; endotoxin-treated cells, 377 $\pm$ 58 units/0.1 ml; means $\pm$ SEM.

The effects of several unsaturated fatty acids were examined (Table II). At 10 μM, arachidonic, dihomo-gamma-linolenic, and linoleic acids strongly inhibited the generation of thromboplastin activity in response to endotoxin. At 20 μM, oleic and linolenic acids were also inhibitory. In contrast, the saturated fatty acids stearic and arachidic acids were minimally active. The fatty acids did not appear to affect resting levels of thromboplastin.

TABLE I. Characterization of procoagulant activity in endotoxin-treated human peripheral blood monocytes

	Clotting time (sec)					
Addition	Normal plasma	-XII	-X	-VIII	-VII	-V
Saline	91	>180	>180	>180	92	>180
Rabbit thromboplastin	28	35	>180	30	95	141
Endotoxin-treated monocytes	27	40	>180	37	89	174

Monocytes were incubated for 2 h with 10 μg/ml endotoxin. The clotting times of 0.1 ml of cellular sonicate in normal and factor-deficient plasmas were compared with those of 0.1 ml saline or saline containing 333 units of standard rabbit brain thromboplastin.

TABLE II. Suppression of endotoxin-induced thromboplastin activity in human monocytes by fatty acids

Fatty acid		μM	% Inhibition
Arachidonic acid	20:4	10	57 ± 7 (10)
Dihomo-gamma-linolenic acid	20:3	10	64 ± 7 (10)
Arachidic acid	20:0	20	15 ± 9 (8)
Linolenic acid	18:3	20	49 ± 12 (8)
Linoleic acid	18:2	10	48 ± 13 (9)
Oleic acid	18:1	20	48 ± 7 (8)
Stearic acid	18:0	20	18 ± 4 (8)

Values shown are means ± SEM. Figures in parentheses indicate number of experiments performed. Monocytes were prepared from blood obtained from a total of 11 donors (6 male, 5 female).

The inhibitory effects of arachidonic, dihomo-gamma-linolenic and linoleic acids were unrelated to monocyte cyclooxygenase activity, since they were unchanged by a prior incubation of cells for 15 min with 1 μM indomethacin (Table III). This treatment abolishes monocyte thromboxane production [13]. Attempts to reverse the fatty acid effects with the lipoxygenase inhibitors eicosatetraynoic acid and nordihydroguaiaretic acid were not made since these compounds themselves suppress endotoxin-induced monocyte thromboplastin activity [13].

The effects of authentic HETEs and leukotriene B_4 were also examined (Fig. 1). All of the HETEs tested were inhibitory, with IC_{50} values of 10 μM (5-HETE and 12-HETE), and 20 μM (15-HETE). In contrast, leukotriene B_4 was inactive over the range 1 nM to 1 μM. None of the HETEs or leukotriene B_4 significantly affected resting levels of monocyte thromboplastin.

TABLE III. Failure of indomethacin to reverse fatty acid-induced inhibition of monocyte thromboplastin activity

		% Inhibition	
Fatty acid	μM	Control	1 μM Indomethacin
Arachidonic acid	10	64 ± 5	63 ± 13
Dihomo-gamma-linolenic acid	10	82 ± 4	85 ± 4
Linoleic acid	10	83 ± 5	81 ± 6

Values shown are means ± SEM of 4 experiments. Monocytes were prepared from blood obtained from 4 donors (2 male, 2 female).

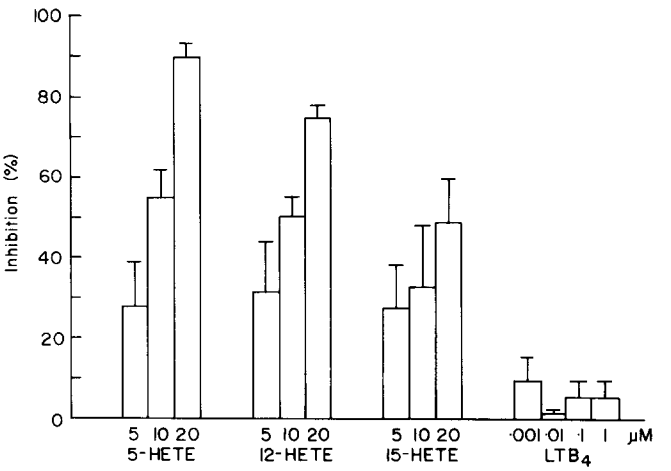

Figure 1. Effects of HETEs and leukotriene B_4 on endotoxin-induced thromboplastin activity in human monocytes. Values shown are means ± SEM of 5-6 experiments. Monocytes were prepared from blood obtained from a total of 8 donors (4 male, 4 female).

The inhibitory effects of unsaturated fatty acids and HETEs were cell dependent since none of these compounds at 20 μM affected the ability of standard thromboplastin to shorten the clotting time of normal plasma, or altered the activity of endotoxin as measured by a Limulus clotting assay.

The mechanism whereby unsaturated fatty acids and HETEs inhibit the induction of monocyte thromboplastin activity by endotoxin is at present unknown. The effects of arachidonic acid were not due to its conversion via the cyclooxygenase pathway. It is also unlikely that they were due exclusively to its conversion by the monocytes to 5-HETE, or by contaminating platelets to 12-HETE [17], since the approximately equal potencies of arachidonic acid and HETEs would require almost quantitative conversion of the added fatty acid. The inhibitory effects of HETEs would appear to be in conflict with previous studies showing that two non-specific inhibitors of HETE formation, eicosatetraynoic acid and nordihydroguaiaretic acid, are also inhibitory [13]. A possible explanation is that some other component of cellular lipid deacylation (for example, lysophospholipids) mediates the stimulation of thromboplastin activity, and the lipoxygenase inhibitors acted primarily as inhibitors of phospholipase A_2 [18]. In keeping with this, other phospholipase A_2 inhibitors such as bromophenacyl bromide, quinacrine and dexamethasone also inhibit endotoxin-

induced monocyte thromboplastin activity [11,13], and unsaturated fatty acids and HETEs can inhibit phospholipase A_2 [19,20]. Clearly, the possible relationship between cellular lipid deacylation and thromboplastin synthesis merits further study.

The concentrations of HETEs required to suppress monocyte thromboplastin activity are pharmacological, and are unlikely to be achieved in vivo following endotoxin stimulation of monocytes. However, it should be noted that similar concentrations are required for the induction of human neutrophil and eosinophil chemotaxis [21-23], and neutrophil degranulation [24]. Furthermore, 5-HETE and 12-HETE can be taken up by cells and esterified into phospholipids [25-27]. A prolonged production of 5-HETE by stimulated monocytes, or prolonged exposure of monocytes to 12-HETE released from platelets, could therefore alter thromboplastin activity over a period of time.

The ability of fatty acids and HETEs to be taken up and incorporated into cellular phospholipids may provide a clue to their mechanism of action. Thromboplastin consists of a 52,000 Dalton protein (apoprotein III) complexed with phospholipid [4, 27]. Apoprotein III is not procoagulant, and the development of procoagulant activity is strongly affected by the nature of the phospholipids employed for relipidation [27]. Unsaturated fatty acids and HETEs may not directly affect cellular levels of thromboplastin but rather alter its procoagulant activity by changing its lipid structure. However, direct evidence for this hypothesis awaits measurement of apoprotein III in endotoxin- and HETE-treated human monocytes, and its correlation with procoagulant activity.

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