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EFFECTS OF INHIBITORS OF ARACHIDONIC ACID METABOLISM ON THROMBOPLASTIN ACTIVITY IN HUMAN MONOCYTES

David J. Crutchley
Research Division, Miami Heart Institute,
Miami Beach, Florida 33140

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SUMMARY Human isolated monocytes possess low levels of procoagulant activity, which was stimulated 10-30 fold by brief (2 hr) exposure to 10 μg/ml endotoxin. This activity was expressed in normal or factor XII-deficient plasma, but lost in plasma deficient in factors X or VII, indicating that it was due to thromboplastin. The stimulation of monocyte thromboplastin by endotoxin was inhibited in a dose-dependent manner by two phospholipase A_2 inhibitors, 4-bromophenacyl bromide and quinacrine, and by two lipoxygenase inhibitors, eicosatetraynoic acid and nordihydroguaiaretic acid. Two cyclooxygenase inhibitors, aspirin and indomethacin, prevented endotoxin-induced increases in thromboxane B_2 production but had no effect on thromboplastin production. These results suggest that a component in the sequence of lipid deacylation, arachidonic acid release, and metabolism via lipoxygenase may mediate the stimulation of monocyte thromboplastin activity by endotoxin.

It has been known for some time that leukocytes possess procoagulant activity [1,2]. This activity resides in the monocyte fraction and is due to thromboplastin (tissue factor; factor III), which activates the extrinsic coagulation system [2-4]. Monocyte thromboplastin activity is readily stimulated by exposure of cells to bacterial lipopolysaccharides (endotoxin) [1-4], although the mechanism for this remains unclear. Increased monocyte thromboplastin activity may be important in the pathogenesis of disseminated intravascular coagulation.

Exposure of human monocytes to endotoxin results in an increased production of prostaglandins [5,6], suggesting that these or other metabolites of arachidonic acid may mediate the effects of endotoxin on monocyte thromboplastin. We have therefore investigated the effects of inhibitors of arachidonic acid metabolism on thromboplastin activity in human monocytes.

MATERIALS AND METHODS

Materials The following materials were used for this study: endotoxin-free RPMI 1640 medium (American Biorganics, North Tonawanda, NY); fetal bovine

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serum (Hyclone, Logan, UT); endotoxin (E. Coli 0111:B4; Difco Labs, Detroit, MI); human plasma deficient in factors XII, X, or VII (Helena Labs, Beaumont, TX); Ficoll-Paque (Pharmacia, Piscataway, NJ); rabbit brain thromboplastin (Ortho, Raritan, NJ); tritium-labelled thromboxane B_2 (Amersham, Arlington Heights, IL); unlabelled thromboxane B_2 (Upjohn, Kalamazoo, MI); quinacrine hydrochloride, 4-bromophenacyl bromide, nordihydroguaiaretic acid, indomethacin, aspirin, cephalin (Sigma, St. Louis, MO). Eicosatetraynoic acid was a gift from Dr P. Sorter, Hoffmann- LaRoche, Nutley, NJ, and anti-thromboxane B_2 antiserum was a gift from Dr L. Levine, Brandeis University, Waltham, MA.

Cell culture and incubation Blood was drawn by venipuncture from healthy volunteers who denied taking aspirin or aspirin products for at least 7 days prior to donation. Mononuclear cells were isolated on Ficoll-Hypaque gradients [7]. Cells were washed twice with serum-free RPMI medium and once with RPMI medium containing 20% fetal bovine serum and 0.3 mM EDTA. Cells were then resuspended in RPMI medium containing 20% fetal bovine serum and counted. Approximately 5 x 10^6 cells were plated on 35 mm plastic dishes and allowed to adhere by incubation for 2 hr at 37°C in a humidified atmosphere of 5% CO_2. Non-adherent cells were removed by two washes with RPMI medium containing 20% fetal bovine serum followed by one wash with serum-free medium. The remaining cells were incubated for 15 min with 1 ml serum-free medium containing inhibitors or 0.1% ethanol. Fifty microliters of a solution of endotoxin in isotonic saline, sufficient to give a final endotoxin concentration of 10 µg/ml, was added to half of the culture dishes. The remainder received saline alone. After a further incubation for 2 hr, cells were scraped with a rubber policeman and collected by centrifugation (200 x g, 10 min). The pellets were washed with cold saline A solution, resuspended in 0.2 ml isotonic saline, subjected to three cycles of freeze-thawing, and briefly sonicated.

Coagulation assays Procoagulant activity of the sonicates was measured by a one-stage clotting assay [8]. 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. Monocyte procoagulant activity was calculated by reference to a standard curve to rabbit brain thromboplastin, which was obtained by plotting log (brain standard dilution) vs log (clotting time).

Thromboxane assays Conditioned medium was collected from cells incubated with endotoxin, aspirin or indomethacin, and centrifuged (750 x g, 10 min) to sediment any detached cells. Thromboxane B_2, the stable hydrolysis product of thromboxane A_2, was measured in the supernatant by radioimmunoassay techniques described previously [9].

RESULTS AND DISCUSSION

Human isolated monocytes, incubated for 2 hr in serum-free medium, possessed low but measurable procoagulant activity. This was stimulated 10-30 fold by treatment with 10 µg/ml endotoxin. The procoagulant activity of human monocytes, like that of the standard rabbit brain thromboplastin, was maintained in plasma deficient in Factor XII but lost in plasma deficient in Factor X or Factor VII (Table I). Both basal and stimulated monocyte procoagulant activities were therefore due to thromboplastin. When results were pooled from 18 experiments performed on blood obtained from 14 donors, the

TABLE I

Characterization of human monocyte procoagulant activity

Addition	Clotting time (sec)			
	Normal plasma	-XII	-VII	-X
Saline	125	>300	131	>300
Rabbit thromboplastin, 10 units	85	125	123	>300
Rabbit thromboplastin, 300 units	47	49	127	218
Monocytes, control	97	213	128	225
Monocytes, endotoxin-treated	49	56	125	221

Isolated monocytes were incubated for 2 hr with 10 μg/ml endotoxin or saline. After incubation, cells were collected, subjected to freeze-thawing, and sonicated. Procoagulant activity was measured in assays consisting of 0.1 ml of the additions stated, 0.1 ml of 25 mM $CaCl_2$, and 0.1 ml of normal plasma or plasma deficient in Factors XII, VII, or X. Values shown are means of 2-3 assays of monocytes obtained from a single donor.

following data were obtained: control cells, 14 ± 1 units/ 0.1 ml cell sonicate; endotoxin-stimulated cells, 292 ± 28 units/ 0.1 ml; mean ± SE).

The effects of several inhibitors of arachidonic acid metabolism were then examined (Figure 1). The phospholipase A_2 inhibitors 4-bromophenacyl bromide [10] and quinacrine [11] potently inhibited endotoxin-stimulated monocyte thromboplastin activity (IC_{50} 1 μM and 5 μM, respectively). Eicosatetraynoic acid, which inhibits arachidonic acid metabolism via both lipoxygenase and cyclooxygenase [12], and nordihydroguaiaretic acid, which more selectively inhibits lipoxygenase [13], also blocked the stimulation of monocyte thromboplastin activity (IC_{50} 20 μM and 5 μM, respectively). None of these compounds strongly suppressed resting levels of thromboplastin.

The effects of two inhibitors of arachidonic acid metabolism via cyclooxygenase, aspirin and indomethacin [14], were studied. Since the major cyclooxygenase metabolite in monocytes appears to be thromboxane A_2 [15,16], the effects of these compounds on thromboxane production were also studied. As shown in Table II, exposure of monocytes to 10 μg/ml endotoxin produced a 12-fold increase in cellular thromboplastin activity. This was accompanied by a 5-fold increase in the levels of thromboxane B_2 in the medium; whether the thromboxane was derived from monocytes or from contaminating platelets was not

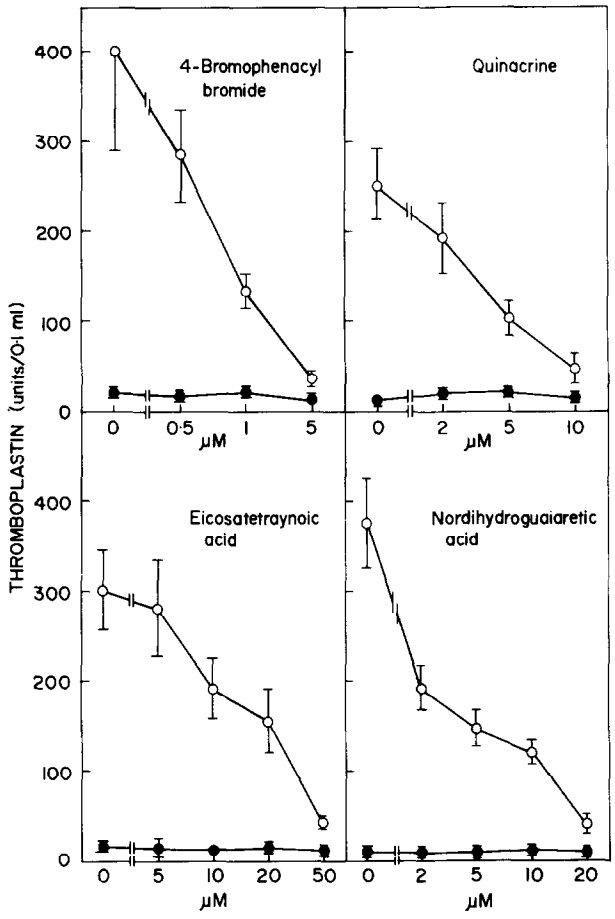

FIGURE 1. Effect of inhibitors of arachidonic acid release or metabolism on human monocyte thromboplastin activity. Isolated monocyte cultures were incubated for 15 min with inhibitors at the concentrations shown. Cultures then received 10 μg/ml endotoxin (O), or saline (●). After a further incubation for 2 hr, monocytes were collected and procoagulant activity was determined. Values are means ± S.E. of experiments performed on monocytes obtained from 3-5 donors.

determined. Indomethacin, 1 μM, and aspirin, 50 μM, abolished the increase in thromboxane but had no effect on thromboplastin activity. Therefore, thromboxane A_2 and other cylooxygenase metabolites of arachidonic acid do not mediate the stimulation of monocyte thromboplastin activity by endotoxin. The failure of indomethacin and aspirin to inhibit endotoxin-induced increases in monocyte thromboplastin activity has also been noted by Prydz and Lyberg [17].

These results would seem to suggest that a metabolite of arachidonic acid derived via lipoxygenase may mediate endotoxin-induced stimulation of thrombo-

Table II

Effects of aspirin and indomethacin on endotoxin-stimulated thromboplastin activity and thromboxane B_2 production

		Thromboplastin (units/ 0.1 ml)		Thromboxane B_2 (ng/ml)	
Inhibitor	μM	Control	+Endotoxin	Control	+Endotoxin
None	-	18 ± 4	227 ± 50	0.7 ± 0.2	3.7 ± 0.9
Aspirin	50	13 ± 3	162 ± 19	0.5 ± 0.2	0.4 ± 0.2
Indomethacin	1	14 ± 3	215 ± 39	0.2 ± 0.1	0.2 ± 0.1

Isolated monocytes were incubated for 15 min with 1 ml serum-free medium containing inhibitors at the concentrations indicated. Endotoxin was then added to a final concentration of 10 μg/ml; control cultures received saline. After incubation for a further 2 hr, conditioned medium was aspirated and thromboxane B_2 was determined by radioimmunoassay. Monocytes were collected and thromboplastin was measured by a one-stage clotting assay. Values shown are means ± S.E. of 6 experiments.

plastin activity in monocytes. However, the exact metabolite involved remains unidentified. Such a metabolite could conceivably arise from any of three sources: from monocytes themselves, which contain a 5-lipoxygenase enzyme [16], from contaminating lymphocytes, which contain 5-, 11-, and 15-lipoxygenases [18,19], or from contaminating platelets, which contain primarily a 12-lipoxygenase [12]. In addition, electron microscopic studies of monocytes prepared by standard gradient techniques have shown significant numbers of activated platelets adhering to the monocyte surfaces [16]. Such close contact could facilitate cellular cooperation leading to the production of 5,12-dihydroxylated derivatives, in a manner analogous to that reported for human platelets and neutrophils [20].

An alternative explanation of these results is suggested by the work of Lanni and Becker [21], who showed that high concentrations of eicosatetraynoic acid and nordihydroguaiaretic acid can inhibit a purified preparation of phospholipase A_2. If similar events occur in isolated monocytes, then these agents, like 4-bromophenacyl bromide and quinacrine, may exert their effects on monocyte thromboplastin activity by inhibiting the deacylation of cellular phospholipids. In this case, some other component of lipid deacylation, for example, lysophospholipid or arachidonic acid itself, may mediate the induc-

tion of monocyte thromboplastin activity. In this context, it is noteworthy that the effects of endotoxin on monocyte thromboplastin activity can be inhibited by dexamethasone [17], an agent which can also induce the synthesis of cellular inhibitors of phospholipase A_2 [22,23].

In summary, these preliminary studies have suggested a direct relationship between arachidonic acid metabolism and the extrinsic coagulation system. Given the importance of arachidonic acid metabolites to cardiovascular biology and inflammation, and the relevance of leukocyte procoagulant activity to pathological intravascular coagulation, such a relationship merits further investigation.

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