THROMBIN-INDUCED CALCIUM-INDEPENDENT DEGRANULATION OF HUMAN NEUTROPHILS

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Abstract—Thrombin, a highly specific coagulation factor, can rapidly trigger lysozyme release from human neutrophils without concomitant activation of the 5-lipoxigenase pathway. This activation was not dependent on the presence of extracellular calcium. Since thrombin also induces the release of hemostatic and inflammatory metabolites from platelets and mast cells, it is proposed that it plays a significant role in amplification of the inflammatory response.

INTRODUCTION

Neutrophils are one of the major cellular components at sites of inflammation. The release of their granule contents, active oxygen species, and arachidonate derivatives is probably responsible for the development and amplification of inflammatory response and its associated tissue destruction (1–4). The initiation of neutrophil activation has been ascribed to many factors, among them are the ligand binding to their surface receptors [Fc, concanavalin A, chemotactic peptide, and phorbol myristate acetate (PMA) receptors] and perturbation of the membrane with the calcium ionophore, A23187 (5).

We have recently reported (6) that thrombin, a highly specific coagulation factor, which has stimulatory effects on platelets, monocytes, endothelial, and smooth muscle cells (7–11), could trigger bone marrow-derived mast cells to degranulate by a Ca2+-dependent mechanism (12). However, in contrast to immunological stimulus, this thrombin-mediated activation did not result in leukotriene synthesis. The role of thrombin in neutrophil activation has not been studied yet, and this study was undertaken to determine if it could mediate human neutrophil activation.
MATERIALS AND METHODS

Chemicals. [14C]Arachidonic acid, 62.2 mCi/mmol (1 Ci = 3.7 \times 10^{10} \text{becquerels}) was from New England Nuclear Corp., Boston, Massachusetts; chloroform, methanol, acetic acid, diethyl ether, petroleum ether, Frutarom, Laboratory Chemicals, Israel; calcium ionophore A23187, Calbiochem-Behring Corp., La Jolla, California; bovine thrombin (600 NIH units/mg protein), human thrombin (3000 units/mg protein), lysozyme, Triton X-100, zymosan, cytochalasin B, and fluorescein isothiocyanate (FITC), Sigma Chemical Co., St. Louis, Missouri; 5-hydroxyeicosatetraenoic acid (5-HETE), Merck, Canada.

Procedures. Neutrophils from healthy human donors were prepared by dextran sedimentation followed by hypotonic lysis of the remaining red cells and centrifugation over Ficoll-Hypaque (13). Approximately 98% of the cells were identified as granulocytes by Gimsa staining; no platelets were identified in the preparation.

Duplicate sets of 1.5 \times 10^6 human neutrophils in 0.5 ml Tyrode's buffer containing 1 mM Ca^{2+}, 0.2 mM Mg^{2+}, and 0.05% gelatin (TG) were stimulated for specified time intervals at 37°C with defined concentrations of thrombin. The incubation was stopped by cooling the samples in melting ice. Release of lysozyme and LDH was expressed as percent of the total activity, which was determined by measuring the enzyme activity in neutrophil preparations treated with 0.02% Triton X-100 (14).

Duplicate sets of 1 \times 10^6 neutrophils in 0.4 ml TG containing 0.1 μCi [14C]arachidonic acid were warmed to 37°C for 5 min, 0.1 ml of TG containing 1 unit thrombin or 0.5 μM calcium ionophore A23187, or TG alone, was added to the samples. After 10 min of incubation, the reactions were stopped by the addition of 1.5 ml methanol, followed by the addition of 3 ml chloroform (15). The chloroform layer was separated, dried, and resuspended in 100 μl chloroform and chromatographed on 20 × 10 thin-layer chromatography plates (analytical silica gel 60 F 254, E. M. Merck Scientific products) in a solvent system of ether-petroleum ether-acetic acid (50:50:1, v/v) (15). The labeled products were identified by autoradiography on XR-5 X-ray film (Eastman Kodak Chemical Co., Rochester, New York) scraped, and quantified in a scintillation counter. Synthetic 5-HETE and [14C]arachidonic acid were used as markers.

The conjugation of FITC to human thrombin (FITC-thrombin) was carried out (16), and the conjugate preserved 85% of the clotting activity which was determined as described (17).

Cytometric analysis was performed using a fluorescence-activated cell sorter (FACS) (Beckton Dickinson, Rutherford, New Jersey) as follows: Duplicate samples of 1 \times 10^6 human neutrophils were preincubated with and without 10 units/ml bovine thrombin in 200 μl TG for 10 min at 4°C. Cells were washed two times with 1.5 ml cold TG, then both sets were incubated with 0.1 unit of FITC-thrombin for 10 min at 4°C, and analyzed by FACS.

RESULTS

Exposure of 1.5 \times 10^6 neutrophils treated with or without cytochalasin B (25 μg), with 0.5–3 units of thrombin in 0.5 ml TG for 10 min at 37°C, resulted in a dose-dependent release of lysozyme (Figure 1). In control experiments, exposure of 1.5 \times 10^6 neutrophils to 3 mg opsonized zymosan (plus 25 μg cytochalasin B) or to 40 ng PMA, resulted in a lysozyme release of 61.1 ± 26.7% and 46.0 ± 3.9% (mean ± SE, N = 3), respectively. Neither one of