Endotoxic Lipid A Interaction with Human Platelets

STRUCTURE-FUNCTION ANALYSIS OF LIPID A HOMOLOGS OBTAINED FROM SALMONELLA MINNESOTA Res95 LIPOPOLYSACCHARIDE*

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We previously reported that human blood platelets are directly stimulated by endotoxic Lipid A via the protein kinase C pathway (Grabarek, J., Timmons, S., and Hawiger, J. (1988) J. Clin. Invest. 82, 964-971). To study the relationship between the molecular structure of Lipid A and its ability to activate human platelets, we used Lipid A homologs derived from Salmonella minnesota Res95 lipopolysaccharide. Preparations of Lipid A are heterogeneous in regard to the degree of substitution of fatty acids which result in multiple homologs. These were separated by thin-layer chromatography and characterized by fast atom bombardment spectroscopy and related techniques (Johnston R. S., Her, G.-R., Grabarek, J., Hawiger, J., and Reinhold, V. N. (1990) J. Biol. Chem. 265, 8108-8116). The homologs of monophosphoryl Lipid A (MLA) present in fractions TLC-8 (heptaacyl MLA ion, m/z 1953), TLC-7 (three hexaacyl species with predominant MLA ion m/z 1715), and TLC-6 (four pentaacyl homologs with predominant MLA ion, m/z 1505) induced secretion of [14C] serotonin and aggregation of platelets. Lipid A homologs in fractions TLC-5 (three tetraacyl MLA ions, m/z 1323, 1307, and 1279), TLC-4 (one major triacyl MLA ion, m/z 1097), TLC-3 (three triacyl MLA ion, m/z 1276), TLC-2 (a diphasphoryl hexaacyl Lipid A ion, m/z 1795), and several ions of low abundance, and TLC-1 (two ions, m/z 1097 and 666) were not active in regard to human platelet aggregation and [14C] serotonin secretion. The most active homolog was heptaacyl MLA ion, m/z 1953, present in TLC-8, while homologs present in TLC-7 and TLC-6 were 5 and 10 times less active, respectively. Rapid phosphorylation of a human platelet protein of M, 40,000-47,000 (P47), a substrate for protein kinase C activation, preceded secretion of serotonin when platelets were triggered by the most active heptaacyl MLA ion, m/z 1953. These events were time-dependent, with half-maximal response of phosphorylation of P47 at 30 s and [14C] serotonin secretion at 45 s. A marked difference in the degree of phosphorylation of P47 was observed in non-heptaacyl MLA homolog present in TLC-8 inducing complete phosphorylation (97%), whereas less acylated Lipid A homologs present in TLC-1 caused marginal phosphorylation (20%). These results indicate that the degree of acylation of monophosphoryl Lipid A determines its functional properties toward human platelets in regard to secretion of [14C] serotonin, aggregation, and activation of protein kinase C. The key role of fatty acids of endotoxic Lipid A in human platelet activation provides a structural basis for its biological activity and for the potential detoxifying effect of enzymes that remove the fatty acids from Lipid A.

Lipid A is the toxic principle of lipopolysaccharide of Gram-negative bacteria which elicits a wide spectrum of pathophysiological effects in vivo, such as fever, hypotension, thrombocytopenia, disseminated intravascular coagulation, and shock. Many of these effects are produced by stimulation of host cells, including blood platelets, granulocytes, monocytes, lymphocytes, and endothelial cells (1, 2). In recent years the correct structure of the Lipid A molecule (which is highly conserved among species) has been unraveled and significant progress has been made in the elucidation of the structural elements and physicochemical properties determining the endotoxic activity of Lipid A (3-12). Lipid A of Salmonella minnesota is composed of a glucosamine disaccharide with phosphates and two amide and two ester-linked hydroxymyristic acids. Nonhydroxylated, palmitic, lauric, and myristic fatty acids are esterified to the hydroxyl groups of hydroxymyristic acid in the positions 2, 2’, and 3’, respectively (6). Fatty acids are essential for most of the toxic reactions of Lipid A as judged by chemical or enzymatic cleavage of certain functional groups in the Lipid A molecule (1-3, 10-19). Some reports indicate that the phosphates attached to Lipid A may also play an important role in determining its bioactivity (1, 3, 11, 23-26). The discoveries of monosaccharide precursors of Lipid A provided additional information defining the minimal molecular requirements for mitogenic and certain endotoxic functions of lipopolysaccharides (1, 27-37). Fractionation and structural analysis of the Lipid A preparations, which are heterogeneous after acid hydrolysis of lipopolysaccharide, and development of synthetic analogs of Lipid A advanced the current understanding of the structure-function relationship of endotoxic Lipid A (2, 10, 38).

It is noteworthy that a Lipid A-like substance reappears in the circulation 24 h after experimental injection of endotoxin, and significant amounts of material cross-reacting with Lipid A antiserum appear in the urine (39). An endotoxin-like material reactive with the Limulus amebocyte lysate has been detected in the serum of patients with Gram-negative bacterial urinary tract infections whose blood cultures were nega-
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We observed that the mutant Re595 of S. minnesota, bearing Lipid A and the sugar 2-keto-3-deoxyoctonate but deficient in oligosaccharides, interacts with human platelets (41). More recently, we have reported that human platelets are directly stimulated by endotox Lipid A of S. minnesota Re595 through activation of protein kinase C without apparent involvement of phospholipase C and cyclooxygenase (42). Also, in a cell free system, affinity-purified protein kinase C from human platelets is activated by endotox Lipid A (43). However, the molecular features of Lipid A that are responsible for its interaction with human platelets were not defined. Therefore, we conducted a study of the structure-function relationship of the fractionated Lipid A species in terms of platelet ["C"]serotonin secretion, aggregation, and protein kinase C activation. The fractionated species were characterized by mass spectrometry and related techniques (44). Results of these combined studies allowed us to establish the structural requirements for the interaction of endotox Lipid A with human platelets.

EXPERIMENTAL PROCEDURES

Materials and Reagents—["C"]Hydroxytrypamine binoxalate (serotonin) was purchased from Du Pont-New England Nuclear and 32P as orthophosphate was obtained from Amersham Corp. S. minnesota Re595 Lipid A was from Calbiochem, diphasophyl Lipid A from Escherichia coli was from List Biological Laboratories, Inc. (Campbell, CA), and synthetic Lipid A La-15-PF (506) was a gift from Daiichi Seiyaku Co., Ltd. (Japan). Preparative TLC plates PLK5 were obtained from Whatman. All other reagents were reagent grade or electrophoresis grade.

Preparation of Human Platelets—Blood was drawn from healthy volunteers who had not taken aspirin or any other medication for the preceding 3 days. Platelets were separated from plasma proteins by stepwise albumin gradient centrifugation and Sepharose 2B gel filtration of platelet-rich plasma using modified Tyrode’s buffer, pH 7.35 (45). All experiments were performed with platelets suspended in modified Tyrode’s buffer without phosphate and calcium, pH 7.35.

Fractionation of the Lipid A Mixture—It was done by thin-layer chromatography using preparative TLC plates PLK5 as described in the preceding manuscript (44). In all experiments, Lipid A fractions were suspended in distilled water. Triethylamine was added to a final concentration 0.1%. The concentration of triethylamine in the platelet test sample was from 0.001 to 0.005%. Parallel controls of diluent were run in the platelet incubation mixture for all experiments.

Ultramicro-phosphorus Determination Method—The phosphorus content was used for quantifying the amount of Lipid A. The phosphorus in Lipid A fractions was assayed by modified Fiske and Subbarow procedure (46-48). After evaporation of the chloroform, 0.4 ml of 70% perchloric acid was added. Digestion was carried out for 15 min at 180°C in an electric digestor (Dri-block DB-3H, Technie), with marbles covering the tubes. After the tubes had cooled, 0.5 ml of H2O, 0.2 ml of 5% ammonium molybdate, and 0.05 ml of Fiske-Subbarow reagent were added (1.2 ml final volume). The tubes were placed in boiling water for 10 min, removed, and left at room temperature for 20 min. The absorbance was determined at 830 nm with appropriate blanks and standards. The calibration curve was run in the platelet incubation mixture for all experiments.

Phosphorylation of Human Platelet Proteins—Measurement of protein phosphorylation in intact human platelets was done as described previously (42). In experiments with time course of phosphorylation of platelet proteins, the reaction was stopped by addition of 2 volumes (usually 1 ml) of 15% trichloroacetic acid (52). The experimental samples were spun down in a microcentrifuge at 15,000 X g for 5 min. The precipitated proteins were washed twice with 2 volumes of water to remove residual acid and solubilized in Laemmli electrophoresis sample buffer containing 2-mercaptoethanol (10%). Samples were boiled for 2 min and applied to sodium dodecyl sulfate-polyacrylamide gradient (5-15%) slab gels for electrophoresis according to the method of Laemmli (53).

RESULTS

Effect of Lipid A Fractions on Human Platelet Aggregation and ["C"]Serotonin Secretion—In total eight TLC-purified fractions containing monophosphorylipid A (MLA)1 homologs, structurally characterized in the preceding paper (44), were tested for their activity toward human platelets. On addition of fractionated Lipid A homologs at 5 μM (expressed as Lipid A phosphorus concentration) to a suspension of human platelets separated from plasma proteins, different responses were observed. The most active, fully acylated homolog (heptaacyl MLA ion, m/z 1953) present in TLC-8 gave maximum aggregation of human platelets at 2 min and 82% serotonin secretion at 10 min. The next fraction (TLC-7), with a major hexaacyl MLA ion m/z 1715, was less active, with maximum aggregation at 3 min and 60% serotonin secretion at 10 min. TLC-6, with the most abundant pentaacyl MLA ion, m/z 1505, was even less active, with maximum aggregation at 6 min and 40% serotonin secretion. Lipid A homologs present in the remaining fractions (TLC-5 to TLC-1) evoked very little or no response from platelets in terms of aggregation and serotonin secretion (Fig. 1).

Secretion of ["C"]Serotonin from human platelets stimulated by the active homologs of Lipid A was concentration-dependent. Platelets stimulated with heptaacyl MLA ion, m/z 1953 present in TLC-8 reached half-maximal stimulation at 0.6 μM. The fractions containing predominantly hexa- and pentaacyl homologs were 5 and 10 times less active with half-

1 The abbreviation used is: MLA, monophosphoryl Lipid A.
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FIG. 2. Concentration-dependent secretion of [14C]serotonin in human platelets (2 x 10^9/ml) induced by Lipid A fractions (details as in Fig. 1). Data represent mean values of five independent experiments. Bars indicate the S.E.

maximal response from platelets at concentration 3.0 and 6.0 μM, respectively (Fig. 2).

Effect of Lipid A Homologs on Phosphorylation of P47 Protein—Stimulation of platelets involves the protein kinase C pathway manifested by phosphorylation of protein(s) of M, ranging from 40,000 to 47,000 (P47) (54–56). When 32P04-labeled platelets were incubated with the fully acylated MLA homolog (ion m/z 1953) (10 μM), time-dependent (15 s to 5 min) phosphorylation of the platelet P47 protein was noted (Fig. 3). The time course of phosphorylation was examined quantitatively, and the extent of radioactivity in P47 was expressed as percent of maximal integrated peak area representing the radioactive band. The values observed in diluent controls were subtracted. The time course of phosphorylation of P47 protein in human platelets and secretion of [3H]serotonin from platelets stimulated with heptaacyl MLA homolog present in TLC-8 were compared. Whereas half-maximal phosphorylation was reached in 30 s, secretion of [3H]serotonin reached the half-maximal value at 45 s and maximal response at 2 min (Fig. 4).

In order to examine the relationship of Lipid A structure to the activation of protein kinase C, 32P04-labeled human platelets were stimulated with different fractions of Lipid A (10 μM) (Fig. 5). The completely acylated MLA homolog present in TLC-8 was most active and caused 97% phosphorylation of P47. The preparations containing less acylated hexa- and pentaacyl MLA homologs (TLC-7 and TLC-B) caused 81 and 82% phosphorylation. The TLC-5 and TLC-3 containing tetraacyl homologs induced 70 and 47% phosphorylation of P47, respectively. The major difference between these two fractions is the MLA homolog ion, m/z 1323 (in TLC-5) with two amide and two ester-linked hydroxymyristic acids that suggests the importance of these acids in protein kinase C activation and subsequent higher phos-
phorylation of P47. The importance of hydroxymyristic acid at the position 3 was evident when the two fractions of Lipid A (TLC-4 and TLC-1), with homologs corresponding to ion m/z 1097, were compared. The homologs predominantly present in these fractions contain hydroxymyristic acid at the glucosamine position 3 or 3' and they caused 64 and 20% phosphorylation of P47, respectively.

Role of Phosphate Groups of Lipid A in Its Reactivity toward Human Platelets—The results of our experiment have indicated that MLA homologs, with the phosphate group at position 4', activate human platelets. To investigate the role of phosphate groups in the Lipid A molecule in the stimulation of human platelets, we separated by TLC E. coli diphosphoryl Lipid A into fractions. The major diphosphoryl hexacyl component of E. coli Lipid A did not aggregate human platelets and did not cause [14C]serotonin secretion above control levels, similarly to S. minnesota fraction TLC-2, the only preparation in which the diphosphoryl homolog was identified (44). However, the minor fraction isolated as E. coli monophosphoryl hexacyl Lipid A produced full activation of human platelets, similar to the effect of its structural analog isolated from S. minnesota Re595. Under the same conditions, the synthetic E. coli-type Lipid A (compound 506), which has two phosphate groups at the position 1 and 4' (57), did not induce human platelet aggregation and [14C]serotonin secretion (data not shown).

DISCUSSION

Although we have recently presented data documenting that natural Lipid A isolated from endotoxic lipopolysaccharide of S. minnesota Re595 stimulates human platelets through modulation of protein kinase C (42), the structural basis for that interaction was not understood. The results presented herein provide experimental evidence for the structural requirement of the Lipid A molecule in the activation of human platelets. Lipid A preparations obtained from hydrolysis of lipopolysaccharide extracted from the mutant Re595 of S. minnesota are composed of 16 discernible homologs. The homologs differed in number, type, and position of fatty acids (44). Our observations, based on structural analysis of Lipid A homologs present in TLC fractions, indicate that the activation of human platelets, as measured by secretion of [14C]serotonin and aggregation, appears to require the presence of at least five fatty acids. The reactivity toward human platelets of these Lipid A homologs was expressed increasingly in parallel to the number of acyl residues present in the molecule. This observation is in agreement with previously described structural requirements of Lipid A in some of its biological activities (13, 18). However, in some studies the synthetic, completely acylated Salmonella-type Lipid A was not as active as less acylated forms (15, 16). The basis for this difference is not yet clear.

Interestingly, complete or near complete acylation is not absolutely necessary for activation of platelet protein kinase C. Even tetraacyl and triacyl monophosphoryl Lipid A homologs present in fractions TLC-5 and -4 induced significant activation of protein kinase C in platelets, although serotonin secretion and aggregation were not evoked. Either protein kinase C pathway is necessary but not solely involved in full secretory and aggregatory response of human platelets to monophosphoryl Lipid A homologs, or homologs differ in their ability to interact with more than one known species of protein kinase C (58). Our results of experiments with two fractions (TLC-4 and TLC-1) containing homologs with the same ion, m/z 1097, but different positions of the hydroxymyristic acid, indicate that hydroxymyristic acid attached to glucosamine at the position 3 is essential for Lipid A activation of platelet protein kinase C. Whether the diphosphoryl Lipid A homolog present in low amounts in fraction TLC-2 contributes to phosphorylation of P47 cannot be discerned due to the presence of other monophosphoryl homologs therein.

In our experiments monophosphoryl, but not diphosphoryl, Lipid A induced activation of human platelets. The phosphate groups attached to glucosamines at the positions 1 and 4' in Lipid A are important for the bioactivity of this molecule in some systems (1, 2, 11). By providing an electrostatic charge, the phosphate groups may modulate the lipophilic properties of Lipid A toward biological membranes. It has been suggested by results from x-ray and Fourier-transform-infrared investigation that in the bacterial membrane fatty acid chains of Lipid A are oriented perpendicularly to the membrane surface (59–61). The hydrophilic region of the Lipid A molecule, i.e., the diphosphorylated glucosamine disaccharide, seems to be oriented approximately 45° to the membrane surface, forming a shed roof-like surface structure. A comparable interaction of the Lipid A molecule with the human platelet membrane is plausible. The second phosphate group probably increases the local negative charge of the platelet membrane and diminishes the platelet-platelet interaction that is necessary for platelet aggregation and serotonin secretion.

Our results indicate that acyloxyacyl groups and the phosphate groups in the Lipid A molecule play important roles in human platelet activation. They provide a sound structural basis for the molecular mechanism of Lipid A activity and its inactivation. The latter is of particular interest in view of previously described enzymatic decylation and dephosphorylation of the Lipid A moiety by mouse macrophages and human neutrophils (62–65). The hitherto unidentified enzyme which will split off hydroxymyristic acid at position 3 in Lipid A will be potentially most effective in detoxifying endotoxic Lipid A. These enzymatic modifications of endotoxic Lipid A can be part of neutrophil and macrophage defense mechanisms, preventing the interaction of endotoxin with different cellular targets such as human platelets, endothelial cells, and macrophages. Whether the same fatty acids in Lipid A are responsible for its functional effect in all cellular targets, not only human platelets, remains to be established.

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