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Spider and Bacterial Sphingomyelinases D Target Cellular Lysophosphatidic Acid Receptors by Hydrolyzing Lysophosphatidylincholine

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Bites by Loxosceles spiders can produce severe clinical symptoms, including dermonecrosis, thrombosis, vascular leakage, hemolysis, and persistent inflammation. The causative factor is a sphingomyelinase D (SMaseD) that cleaves sphingomyelin into choline and ceramide 1-phosphate. A similar enzyme, showing comparable bioactivity, is secreted by certain pathogenic corynebacteria and acts as a potent virulence factor. However, the molecular basis for SMaseD toxicity is not well understood, which hampers effective therapy. Here we show that the spider and bacterial SMases D hydrolyze albumin-bound lysophosphatidylcholine (LPC), but not sphingosylphosphorylcholine, with Km values (~20–40 μM) well below the normal LPC levels in blood. Thus, toxic SMases D have intrinsic lysophospholipase D activity toward LPC. LPC hydrolysis yields the lipid mediator lysophosphatidic acid (LPA), a known inducer of platelet aggregation, endothelial hyperpermeability, and pro-inflammatory responses. Introduction of LPA1 receptor cDNA into LPA receptor-negative cells renders non-susceptible cells susceptible to SmaseD, but only in LPC-containing media. Degradation of circulating LPC to LPA with consequent activation of LPA receptors may have a previously unappreciated role in the pathophysiology of secreted SMases D.

Envenomation by Loxosceles spiders, endemic to temperate and (sub)tropical regions of the Americas, Africa, and Europe, can lead to local skin injury as well as to serious systemic toxicity, including thrombus formation, vascular leakage, hemolysis, and persistent inflammation (1–3). In severe cases, the hemolytic complications can lead to renal failure and death, especially in children (2, 3). Treatment is difficult; antivenoms are not very effective, and the use of corticosteroids or anti-inflammatory medication is controversial (3). The toxin responsible for the local and systemic effects of Loxosceles venom is an unusual sphingomyelinase D (SMaseD) that converts sphingomyelin (SM) in the outer leaflet of the plasma membrane to ceramide 1-phosphate (N-acylsphingosine 1-phosphate) (4–7). Strikingly, while SMaseD is not found elsewhere in the animal kingdom, a similar enzyme is produced as an exotoxin by some pathogenic bacteria, notably Corynebacterium pseudotuberculosis, Corynebacterium ulcerans, and Arcanobacterium (formerly Corynebacterium) hemolyticum (8–10). C. pseudotuberculosis causes lymphadenitis in animals but is also pathogenic for humans, while C. ulcerans and A. hemolyticum are pathogens of pharyngitis and other human infections (11); in no case is the molecular basis for virulence known (12). The SMaseD from C. pseudotuberculosis, also named SM-specific phospholipase D (PLD), is an essential virulence determinant that contributes to the persistence and spread of the bacteria within the host (13). The Loxosceles and C. pseudotuberculosis SMases D have the same molecular mass (31–32 kDa) and share about 30% sequence similarity (see “Results”). In model systems, the spider and bacterial enzymes provoke remarkably similar pathophysiological effects, including platelet aggregation, endothelial hyperpermeability, complement-dependent hemolysis, and neutrophil-dependent skin necrosis (4–7, 9, 14–16).

Despite decades of study it remains unclear how SMaseD can elicit such a wide variety of biological effects, particularly, since ceramide 1-phosphate is not known as a signaling molecule. In contrast to ceramide, which may reorganize lipid microdomains and associated signaling complexes (17, 18), ceramide 1-phosphate is a bivalent-prefering phospholipid that is unlikely to significantly perturb membrane structure. Furthermore, mammalian cells treated with SMaseD from either Loxosceles deserta or C. pseudotuberculosis do not convert newly formed ceramide 1-phosphate to ceramide nor does SMaseD treatment affect membrane permeability or cell viability (19, 20).

Given the lack of understanding of SMaseD bioactivity, we set out to re-examine the substrate specificity and cellular effects of the enzyme. Our interest was stimulated by a report of more than 30 years ago, showing that partially purified SMaseD from C. pseudotuberculosis (ovis) can catalyze the release of choline from lysophosphatidylcholine (LPC) but not from phosphatidylcholine (PC) (21). LPC is an abundant plasma component and removal of its choline headgroup yields lysophosphatidic acid (LPA), now known as a pleiotropic lipid mediator acting on specific G protein-coupled receptors in numerous cell types (22, 23). Yet, the possibility that degradation of plasma LPC might contribute to SMaseD toxicity has re-

The abbreviations used are: SMaseD, sphingomyelinase D; LPC, lysophosphatidylcholine; LPA, lysophosphatidic acid; PC, phosphatidylcholine; SM, sphingomyelin; SPC, sphingosylphosphorylcholine; PLD, phospholipase D; MAP, mitogen-activated protein; GFF, green fluorescent protein; Lox-SMaseD, SMaseD from L. laeta; Cp-SMaseD, SMaseD from C. pseudotuberculosis; Sc-PLD, PLD from S. chromofuscus; DMEM, Dulbecco’s modified Eagle’s medium; HBS, HEPES-buffered saline; HA, hemagglutinin; PS, phosphatidylserine.

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10833
Toxic SMases D Target LPA Receptors

No significant contribution to SMaseD-mediated pathogenicity. Our results suggest that degradation of circulating LPC to LPA, with consequent activation of LPA receptors in cells of the circulatory and vascular systems, may significantly contribute to SMaseD-mediated pathogenicity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**—Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum. Cells were exposed to serum-free DMEM for 24 h prior to experimentation, unless indicated otherwise. All phospholipids were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) at the highest purity grade available. Fatty acid-free bovine serum albumin and monoclonal anti-phospho-MAP kinase antibody were from Sigma. Secondary antibodies (rabbit anti-mouse and swine anti-rabbit) conjugated to horseradish peroxidase were from Dako (Glostrup, Denmark). Highly purified PLD (57 kDa) from *S. chromofuscus* (24) was kindly provided by Dr. Mary F. Roberts (Boston College, Chestnut Hill, MA).

**Recombinant SMaseD from Loxosceles laeta**—Recombinant SMaseD (SMase D) from *L. laeta* was produced as described previously (7). In brief, mature *L. laeta* enzyme was expressed in *E. coli* as a fusion protein, including a His6-tag at the N terminus and a 33-amino acid linker. The cells were collected by centrifugation and the bacterial pellet was resuspended in extraction buffer (300 mM NaCl, 100 mM Tris-HCl, pH 8.0) and disrupted by French pressure. The supernatant was loaded onto an Ni2+–chelating Sepharose Fast Flow column (Amersham Biosciences, Sweden, 1.0 × 6.4 cm), and the recombinant protein was eluted with buffer (300 mM NaCl, 100 mM Tris-HCl, pH 8.0, 0.8 M imidazole). Fractions of 1 ml were collected and analyzed by SDS-PAGE. Stock solutions were prepared in PBS at 1.0 mg of protein/ml.

**Choline Release Assay**—SMaseD/PLD enzymatic activity was estimated by determining choline liberated from exogenously added phospholipid substrates, using a fluorimetric assay modified from Tokumura *et al.* (25). In the standard assay, the substrate was diluted in 100 μl of DMEM or HEPES-buffered saline (HBS; 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, pH 7.4). SM and PC substrates were applied as liposomes, whereas the lysophospholipids were complexed to fatty acid-free bovine serum albumin (5 mg/ml). After SMaseD/PLD addition, the reaction was left to proceed for 20 min at 37°. By adding 10 μl of a second assay mixture, the liberated free choline was oxidized (in 10 min) to betaine, and the H2O2 concomitantly generated was determined by fluorometry. The second reaction mixture consisted of 1 unit/ml choline oxidase (Sigma), 0.06 unit/ml horseradish peroxidase, and 50 μM 3,4-dihydroxy-phenylglycpropanic acid in HBS. The second reaction was left to proceed for 10 min. Fluorescence of the oxidized substrate was measured at an excitation of 320 nm and emission of 405 nm using a 96-well plate reader. SMaseD-induced choline release from LPC proceeded at a constant rate for at least 1 h at 37°, with the rate being proportional to the enzyme concentration.

**LPA, cDNA and Retroviral Transduction**—Human LPA cDNA (GenBank™ accession number U78192) was amplified by PCR using primers carrying 5’Xhol + HA3’NotI sites. N-terminally HA-tagged and C-terminally green fluorescent protein (GFP)-fused LPA1 receptor cDNAs were cloned into retroviral LZRS-IRES-Neo. Recombinant retroviruses produced in Phoenix packaging cells was used to infect rat B103 neuroblastoma cells or human HEK293 cells, essentially as described previously (26). After 48 h, transduced cells were selected in medium containing 1 mg/ml G418. Correct expression of LPA1 was confirmed by Western blotting and immunofluorescence.

**LPA Receptor Internalization**—HEK293 cells stably transfected with GFP-LPA1 receptor cDNA were fixed in 3.7% formaldehyde in PBS.

After treatment with agonist, cells were washed and coverslips were mounted with vectashield (Vector Laboratories Inc., Burlingame, CA). GFP-LPA1 receptors were visualized by confocal microscopy.

**RESULTS AND DISCUSSION**

The secreted SMases D from *L. laeta* (Lox-SMaseD) (7) and *C. pseudotuberculosis* (Cp-SMaseD) (10) have the same molecular mass (31–32 kDa) and share 32% sequence similarity and 20% identity, including a conserved N-terminal histidine residue required for the enzymatic activity of Cp-SMaseD (see Supplemental Fig. 1, for sequence alignment, and Ref. 27). Despite being de facto PLDs, both SMases D are unique in that they show no homology to other phospholipid-metabolizing enzymes, and along with the broad specificity PLD from *S. chromofuscus* (Sc-PLD, 10 nm) was used as a positive control. Qualitatively similar results were obtained with enzyme concentrations up to 100 nm. Control denotes no enzyme addition. α, hydrolysis of PC and SM, added at 50 μM as multilamellar vesicles (liposomes). β, hydrolysis of LPC (1-oleoyl) and SPC, added at 50 μM and complexed to fatty acid-free albumin (5 mg/ml). κ, dependence of LPC hydrolysis on the presence of EDTA or EGTA (4 mm). Error bars represent S.E. of the mean (n = 5).

FIG. 1. Choline release from phospholipids induced by recombinant SMase D from *L. laeta* and *C. pseudotuberculosis*. Choline release was determined fluorimetrically in HEPES-buffered DMEM, pH 7.4, at 37°C (see “Experimental Procedures”). Lox-SMaseD and Cp-SMaseD were used at 10 nm. The nonspecific PLD from *S. chromofuscus* (Sc-PLD; 10 nm) was used as a positive control. Qualitatively similar results were obtained with enzyme concentrations up to 100 nm. Control denotes no enzyme addition. a, hydrolysis of PC and SM, added at 50 μM as multilamellar vesicles (liposomes). b, hydrolysis of LPC (1-oleoyl) and SPC, added at 50 μM and complexed to fatty acid-free albumin (5 mg/ml). c, dependence of LPC hydrolysis on the presence of EDTA or EGTA (4 mm). Error bars represent S.E. of the mean (n = 5).

We confirmed that the recombinant Lox- and Cp-SMases D catalyze the release of choline from SM but not PC, whereas the unrelated PLD from *S. chromofuscus* (Sc-PLD) cleaves both SM and PC (Fig. 1A). The intrinsic lyso-PLD activity of the recombinant SMases D was then assessed using 1-oleoyl-LPC and sphingosylphosphorylcholine (SPC or lyso-SM) as substrates; SPC is a normal plasma constituent (30) that would yield the

cell.
potent lipid mediator sphingosine 1-phosphate following choline release. As shown in Fig. 1 (B and C), the spider and bacterial SMases D were both capable of liberating choline from albumin-bound LPC in physiological medium, thereby producing LPA (as confirmed by thin layer chromatography analysis). Surprisingly, SPC did not serve as a substrate for either SMaseD, even when SPC was applied at supra-physiological concentrations and acted only at still higher concentrations (50 μM) was not hydrolyzed by either SMaseD nor did it act as an inhibitor of the enzymes (20 nM) or 1-oleoyl-LPA (1 μM), in the presence or absence of 1-oleoyl-LPC (10 μM; complexed to 5 mg/ml fatty acid-free albumin).

logical substrate. For Lox-SMaseD, the apparent V_max for LPC was 212 ± 6 nmol/min/mg of protein; for Cp-SMaseD, the V_max value was 68 ± 2 nmol/min/mg (Fig. 2). Taken together, these results indicate that the spider and bacterial SMases D possess Mg²⁺-dependent lyso-PLD activity toward acyl- and alkyl-LPC, but not toward SPC, with both enzymes showing similar catalytic efficiency (V_max/K_m, if V_max is considered an approximation of K_m). For comparison, the estimated efficiency of SMaseD for LPC hydrolysis is approximately 1 order of magnitude lower than that of mammalian lyso-PLD or “autotaxin” (K_m = 250 μM and V_max = 9 nmol/min/mg (34)).

We next examined the responsiveness of mammalian cells to SMaseD. While the nonspecific Sc-PLD can trigger diverse signaling events (35, 36), the SMases D failed to induce cellular responses under the same conditions. When preincubated with albumin-LPC, however, the SMases D exhibited biological activity as evidenced by the induction of MAP kinase activity and cellular shape changes, consistent with SMaseD hydrolyzing extracellular LPC to receptor-active LPA. To prove this directly, we used rat B103 neuroblastoma cells that lack endogenous LPA receptors. Fig. 3 shows that SMaseD failed to activate MAP kinase (ERK1/2) in native B103 cells, either in the presence or absence of albumin-LPC. After forced expression of the prototypic LPA1 receptor in these cells, both SMases D mimicked LPA in activating MAP kinase, but only when preincubated with albumin-LPC (Fig. 3). Likewise, SMaseD mimicked LPA in inducing rapid cytoskeletal contraction in N1E-115 neuroblastoma cells, a typical RhoA-mediated response that also underlies LPA-induced endothelial barrier dysfunction (22, 37); but again, SMaseD acted only in the presence of albumin-LPC (see Supplemental Fig. 2).

Fig. 2. Saturation kinetics of SMaseD from L. laeta (a) and C. pseudotuberculosis (b). Rates of choline release from 1-oleoyl-LPC are plotted against increasing concentration of 1-oleoyl-LPC. Assays were carried out in HEPES-buffered DMEM (pH 7.4) at 37 °C (see "Experimental Procedures"). Data were fitted to the Michaelis-Menten equation, yielding the indicated apparent K_m values. The V_max values were 212 ± 6 nmol/min/mg for Lox-SMaseD and 56 ± 2 nmol/min/mg for Cp-SMaseD. Data points are the mean of three independent experiments each performed in triplicate. Error bars represent S.E. values.
blood are normally very low, but the lipid accumulates during blood clotting to promote wound healing processes (39, 40). However, aberrant accumulation of LPA in blood may have deleterious effects, including endothelial barrier dysfunction (37), intravascular coagulation, and infiltration of inflammatory cells such as neutrophils (31, 38, 41, 42), responses that are strongly reminiscent of those to SMaseD (37), intravascular coagulation, and infiltration of inflammatory cells such as neutrophils (31, 38, 41, 42), responses that are strongly reminiscent of those to SMaseD.

Unlike envenomation by a Loxosceles spider bite, infections such as lymphadenitis caused by C. pseudotuberculosis result not only from the toxic effects of SMaseD per se but also from bacterial dissemination to host tissues such as the lymph nodes. A SMaseD-deficient mutant of C. pseudotuberculosis is unable to disseminate from the site of inoculation (13), and a popular theory is that vascular hyperpermeability caused by SMaseD, shown here to be likely a result of LPA production, aids in the escape of bacteria into the lymphatic system and subsequent spread to regional lymph nodes (44).

In conclusion, by building on early but largely overlooked evidence (21), we have demonstrated here that spider and bacterial SMases D have intrinsic lyso-PLD activity in the hematolymphatic action of SMaseD obviously warrants further study.

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