Supplemental Data

Sphingomyelin encrypts tissue factor: ATP-Induced activation of A-SMase leads to tissue factor decryption and microvesicle shedding

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Materials and Methods

Reagents

Recombinant human FVIIa was provided by the late Walter Kisiel, the University of New Mexico Health Science Center, Albuquerque, NM, USA. Purified human clotting factors X, Xa, and prothrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Purified human alpha-thrombin and factor Va were obtained from Hematologic Technologies, Inc. (Essex Junction, VT). 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (Bz-ATP), PACMA 31, desipramine and imipramine were purchased from Sigma-Aldrich (St.Louis, MO). Rabbit polyclonal antibodies against human acid sphingomyelinase (A-SMase) were from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor (AF) 488, 546, 567 or 647 conjugated secondary antibodies were obtained from Thermo Fisher Scientific (Waltham, MA). siRNAs for A-SMase and PDI and control scrambled siRNA (scRNA) were obtained from MWG Operon (Louisville, KY). Macrophage colony stimulating factor (MCSF) was from Peprotech (Rocky Hill, NJ). Cell culture medium DMEM, RPMI 1640, and fetal bovine serum (FBS) were from GIBCO (Life Technologies, Grand Island, NY). [methyl-^{14}C]-Choline Chloride was obtained from PerkinElmer (Waltham, USA).

Preparation of TF proteoliposomes

Full-length recombinant human TF expressed in human umbilical vein endothelial cells was purified by affinity chromatography using anti-human TF antibody column. Purified TF protein was reconstituted into liposomes using phosphatidylcholine (PC) alone, PC with varying molar ratios of SM or other defined phospholipid compositions as described earlier using octyl glucopyranoside as the detergent.[1] The molar ratio of TF: Phospholipid in the reconstituted liposomes was 1:10,000.

Human monocyte-derived macrophages (MDMs)

Human peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Pittsburg, PA; density 1.0776 ± 0.001 g/ml). PBMCs were washed twice by suspending them in HEPES buffer based Tyrode solution (10 mM HEPES, 135 mM NaCl, 10 mM glucose, 5.4 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) and sedimenting the cells by a brief centrifugation (400 x g for 10 min). Washed PBMCs were plated into 24-well plates and allowed to adhere for 2 h in RPMI 1640 medium containing 10% FBS. For some experiments, cells were plated on a glass coverslip placed in a culture dish. After 2 h, non-adherent cells were removed by aspiration and the adherent monocytes were allowed to mature into MDMs by culturing for six days in RPMI medium containing serum and supplemented with MCSF (5 ng/ml). All cells were cultured at 37°C and 5% CO_2 in a humidified incubator.

Inhibition of A-SMase and PDI by siRNA

MDMs cultured in a 24-well plate were transfected with a control transfection reagent (mock transfection), scrambled oligonucleotide (scRNA) as a control, or siRNA specific for A-SMase or PDI using Lipofectamine RNAiMax transfection reagent (ThermoFisher, Waltham, MA). Briefly, 1.5 µl RNAiMax was added to 50 µl of serum-free RPMI medium containing control scRNA or specific siRNA (50 pmol). After 5 min, the mixture was added to cells in 450 µl of RPMI growth medium. After 48 h, the transfected cells were analyzed for the expression of A-SMase by Western blot analysis and used in experiments. The sequences of siRNAs are as follows: A-SMase (SMPD1), 5'GAGCUGGAAUUAUUACCGAAUUGUA3'; PDI, 5' GACCUCCCCUUCAAAGUUGTT 3'.

Measurement of tissue factor and prothrombinase activities

Cell surface TF activity was measured as the ability of monolayers to support activation of FX with the addition of FVIIa and FX as described earlier.[2] Briefly, macrophages cultured in a 24-well plate were treated with 200 µM Bz-ATP in serum free (SF) RPMI at 37°C for 15 min. The supernatant was removed (and processed for isolation of microvesicles), and the cells were washed twice with buffer A (10 mM HEPES, 0.15 M NaCl, 4 mM KCl, 11 mM glucose, pH 7.5). The cells were incubated with FVIIa (10 nM) in buffer B (buffer A containing 5 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mg/ml BSA) for 5 min at 37°C, followed by the addition of FX (175 nM) to initiate TF-FVIIa mediated activation of factor X. After 20 min, the reaction was terminated by the addition of EDTA (10 mM), and the amount of FXa generated was measured in a chromogenic assay using chromogenic substrate Biophen CS-11(65). Cell surface prothrombinase activity was determined as described earlier.[2;3] Briefly, cells were incubated with FXa (1 nM), FVa (10 nM) in buffer

B for 5 min at 37°C, followed by prothrombin (1.4 μM). The reaction was terminated at the end of 5-min activation period by the addition of EDTA (10 mM), and the amount of thrombin generated was measured in a chromogenic assay using Chromozym TH. Similar assay procedures were used for measuring TF and prothrombinase activities in liposomes and microvesicles. To isolate MPs from cell supernatants, supernatant were centrifuged first at 400 x g for 5 min to remove cell debris and then 21,000 x g for 60 min to pellet MPs. MPs were resuspended in buffer B to the original volume.

Measurement of SM hydrolysis by [methyl-14C]-choline chloride hydrolysis

To evaluate the hydrolysis of SM in the outer leaflet, the cells were metabolically labeled with [methyl-^{14}C]-choline chloride, and the release of the ^{14}C-phosphorylcholine group into the supernatant medium was monitored. Briefly, MDMs cultured in 24-well plates were metabolically labeled with [methyl-^{14}C]-choline chloride (0.2 μCi/ml) for 48 h. After removing the unincorporated label and washing cells thrice with serum-free RPMI medium, the cells were subjected to experimental treatments – control vehicle, bacterial SMase or Bz-ATP in in serum-free RPMI medium at 37°C. The supernatants were collected and supernatants containing nonhydrolyzed SM associated with membrane fragments. The supernatant was counted for the radioactivity ([^{14}C]-phosphorylcholine) released into the medium using LS 6500IC scintillation counter (Beckman).

FACS analysis

MDMs were detached from the culture dish using EDTA (10 mM) solution. After blocking with 10% FBS, cells were labeled with control IgG or rabbit anti-human TF IgG (10 μg/ml) followed by AF488-conjugated secondary antibody and analyzed using FACSCalibur (Becton and Dickinson, San Jose, CA).

Lysenin binding and immunostaining

PBMC-derived macrophages cultured on glass coverslips were treated with a control vehicle, b-SMase or Bz-ATP (200 μM) as described in Results or figure legends. The cells were fixed by incubating them with 4% paraformaldehyde (in serum-free RPMI medium) for 1 h at room temperature. At the end of 1 h, the fixative was removed, and excessive formaldehyde was quenched with 0.05% glycine, pH 8.0, for 5 min. The fixed cells were left either intact (nonpermeabilized) or permeabilized with 0.05% Triton X-100 in PBS for 5 min and blocked with 10% fetal bovine serum (FBS) in PBS for 1 h at RT. To label SM with lysenin, nonpermeabilized cells were incubated with 0.5 μg/ml lysenin (Peptide Institute Inc., Osaka, Japan) in 2% BSA/PBS for 60 min. After removing the unbound lysenin and washing cells, the cells were incubated with rabbit polyclonal anti-lysenin antiserum (Peptide Institute Inc., Osaka, Japan; 200 x dilution) for 60 min. For staining A-SMase, permeabilized MDMs were incubated with rabbit anti-human A-SMase IgG (0.5

μg/mL) for overnight. After removing the unbound primary antibodies and washing the cells twice with PBS, the cells were incubated with DAPI (5 μg/mL), AF546 donkey anti-rabbit IgG (2 μg/mL) for 90 min before washing and mounting them on a glass slide using aqueous gel mounting medium (Biomedia) containing antifading agent. TF was labeled similarly using goat anti-human TF IgG (10 μg/ml). In some studies to view the plasma membrane, the live cells were labeled with general membrane marker using PKH67 Green Fluorescent Cell Linker Mini Kit (Sigma) for 5 min. To label PS, the fixed cells were incubated with AF488-annexin V for 60 min.

Immunofluorescence microscopy, image acquisition, and quantification of TF and A-SMase

The confocal images were obtained using LSM 510 Meta confocal system (Carl Zeiss) equipped with an inverted microscope (Axio Observer Z1; Carl Zeiss). Immunostained cells were viewed using Plan-APOCHROMAT 63×/1.4 NA oil objective lens. Argon ion 488 nm laser line was used for the excitation of AF488, HeNe 543 nm laser line for the excitation of AF546 and HeNe 633 nm laser line for the excitation of AF647. Diode 405-30 laser line was used to scan images for DAPI. Zen 2009 software (Carl Zeiss) was used for the image acquisition and determining of colocalization. The scanned images were exported and processed using FIJI software (Image J2, Wayne Rasband, National Institute of Mental Health). To quantify fluorescence signals in the plasma membrane, a region of interest (ROI) in the plasma membrane was selected with a defined area and a mean pixel value was obtained. A mean pixel value of the same ROI size outside of the staining area was used to correct for the background fluorescence. At least 3 randomly selected areas were chosen for each cell, and 30 to 60 cells were analyzed for each experimental variable. Details of image acquisition, processing, and quantification of immunostaining signals were described in our earlier publications.[4-6]

Data analysis

All experiments were performed at least three times in duplicates. The values were shown as mean ± SEM. Statistical significance between the groups was determined by Mann-Whitney test.

References

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Figure S1. Structures of ceramide, SM, and PC. Encircled in green is the phosphorylcholine headgroup common between PC and SM but absent in ceramide. The red box shows the presence of double bonds (which could be 1-6) in the hydrocarbon chain attached to C-2 in PC and their absence in SM. Arrowheads point to the positions of the carbon to which the hydrocarbon chains, a hydroxyl group or the phosphorylcholine headgroup is attached.