Sphingolipids as Bioactive Regulators of Thrombin Generation*

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Sphingolipids contribute to modulation of two opposing cell processes, cell growth and apoptotic cell death; ceramide and sphingosine promote the latter and sphingosine-1-phosphate triggers the former. Thrombin, a pro-inflammatory protease that is regulated by the blood coagulation cascade, exerts similar effects depending on cell type. Here we report a new mechanism for cross-talk between sphingolipid metabolism and thrombin generation. Sphingosine and sphinganine, but not ceramide or sphingosine-1-phosphate, down-regulated thrombin generation on platelet surfaces (IC50 = 2.4 and 1.4 μM for sphingosine and sphinganine, respectively) as well as in whole plasma clotting assays. Thrombin generation was also inhibited by glucosylsphingosine, lysosphingomyelin, phytosphingosine, and primary alkylamines with >10 carbons. Acylation of the amino group ablated anticoagulant activities. Factor Va was required for the anticoagulant property of sphingosine because prothrombin activation was inhibited by sphingosine, sphinganine, and stearylamine in the presence but not in the absence of factor Va. Sphingosine did not inhibit thrombin generation when Gla-domainless factor Xa was used in prothrombinase assays, whereas sphingosine inhibited activation of Gla-domainless prothrombin by factor Xa/factor Va in the absence of phospholipids (IC50 = 0.49 μM). Fluorescence spectroscopy studies showed that sphingosine binds to fluorescein-labeled factor Xa and that this interaction required the Gla domain. These results imply that sphingosine disrupts interactions between factor Va and the Gla domain of factor Xa in the prothrombinase complex. Thus, certain sphingolipids may be bioactive lipid mediators of thrombin generation such that certain sphingolipid metabolites may modulate proteases that affect cell growth and death, blood coagulation, and inflammation.

During inflammation, cytokines induce tissue factor on some cell surfaces to activate the coagulation system to generate the multifunctional serine protease, thrombin. Thrombin is one of the key factors in inflammation-inducing cell proliferation as well as blood clotting by forming fibrin (1, 2). Thrombin is generated by the conversion of prothrombin to thrombin on negatively charged phospholipids of cells such as endothelial cell and platelet membranes by the proteolytic activity of the prothrombinase complex composed of factor Xa, factor Va, phospholipids, and Ca2+ (3). This thrombin formation is regulated by the protein C anticoagulant system by inactivation of factor Va and factor VIIIa (4). Dysfunction or inefficiency of this anticoagulant protein C system is closely related to thrombotic events (5–9), suggesting the importance of down-regulating the coagulation system, e.g. down-regulating prothrombinase activity in vivo.

Sphingolipids, which are defined by the presence of a long-chain sphingoid backbone, contribute to modulation of two opposing cell processes, cell growth and apoptotic cell death (10–14). For instance, one sphingomyelin metabolite, ceramide (N-acetyl sphingosine) induces apoptosis, whereas another metabolic product, sphingosine-1-phosphate (SIP), induces cell proliferation (11, 13). Sphingolipid metabolism can be regulated by inflammatory cytokines (12, 14), and some sphingolipids are released to circulating blood to act as extracellular mediators (15). Certain sphingolipids are reported to down-regulate thrombin generation by inhibiting the extrinsic pathway of coagulation (16) or by enhancing the protein C anticoagulant pathway (17, 18). These findings caused us to explore the effects of the major sphingolipids on thrombin generation and blood coagulation. Here we tested the effect of major sphingolipids on the blood coagulation system and discovered that thrombin generation can be strongly influenced by a variety of sphingolipids and related lipids that possess a free amino group.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fatty acid-free bovine serum albumin (BSA) came from Calbiochem. Human factor Va, factor Xa, and Gla-domainless (DG)-factor Xa were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Factor VIIa was from Enzyme Research Laboratories (South Bend, IN). DG-prothrombin was made from purified prothrombin as described previously with some modification (19). Sphingosine (Sph), glucosylsphingosine (Glc-Sph), trimethyl-Sph, dimethyl-Sph, phytosphingosine, sphingosine-1-phosphate (SIP), and ceramide were purchased from Avanti Polar Lipids (Alabaster, AL). Stearylamine, hexadecylamine, tetradecylamine, dodecylamine, decylamine, octylamine, butylamine, octadecylamine, diamines (spermidine, putrescine, cadaverine), and lysine, thiamine, and tyramine were from Sigma. Isomers (N-erythro, N-threo, N-threo, and L-threo) of Sph and sphinganine were purchased from Matreya Inc. (State College, PA). RGDS peptide was obtained from Innovin from DADE, Marburg, Germany. N-fluorescein-p benzyol phenylalanine-lysyl (N'-bromoacetyl) amide (Fl) was prepared as described before (20). Diphenyl hexatriene (DPH) was purchased from Fisher Scientific (Pittsburgh, PA).

**Preparation of Sphingosine and Its Analog Solutions**—Sph and related lipids were dissolved in ethanol (stock solution: final 50 mM), and the stock solutions were diluted using Tris-buffled saline (TBS) containing 3 The abbreviations and trivial terms used are: SIP, sphingosine-1-phosphate; Sph, sphingosine; Glc-Sph, glucosylsphingosine; DG, Gla-domainless; Fl, N'-fluorescein-p benzyol phenylalanine-lysyl (N'-bromoacetyl) amide (Fl) was prepared as described before (20). Diphenyl hexatriene (DPH) was purchased from Fisher Scientific (Pittsburgh, PA).

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ing BSA. The diluted lipid solutions contained equimolar amounts of lipid and BSA as carrier. In some experiments, Sph was dissolved directly in TBS in the absence of BSA. Although BSA helped to solubilize Sph in TBS, it had little effect on the assays and Sph behaved similarly in the absence and presence of BSA as carrier protein. The aliphatic side chain of Sph and its derivatives contains 18 carbons unless otherwise noted.

Proteins Preparation of Washed Platelets—Washed human platelets were obtained from healthy consenting donors by vein puncture. Briefly, six volumes of blood were collected into one volume containing ACDB (0.022 mol/liter citrate, 0.014 mol/liter dextrose, final) and prostaglandin E1 (5 μmol/liter, final). The blood was centrifuged for 15 min at 250 × g at room temperature. After collecting platelet-rich plasma (PRP), an additional one-third of centrifugation was performed to remove any remaining erythrocytes and leukocytes. The PRP was subsequently centrifuged for 15 min at 500 × g, and the platelet pellet was resuspended into a buffer consisting of 10 mM HEPES (pH 6.6), 136 mM NaCl, 2.68 mM KCl, 2 mM MgCl2, 25 mM dextrose. Platelet concentration was determined by cell counting (ABX Diagnostics Inc., Irvine, CA).

Clotting Assays—The procoagulant and anticoagulant properties of sphingolipids were determined using Factor Xa-initiated and thrombin-initiated clotting assays using normal pooled plasma. Briefly, 7.5 μl of plasma was mixed with Sph or analogous compounds at varying doses and fibrinogen (0.6 mg/ml final) and incubated for 3 min at 37 °C (87.5 μl total). After addition of 50 μl of factor Xa (0.9 μl/ml, final) or thrombin (0.43 units/ml, final) containing 30 mM CaCl2, clotting times were determined by a coagulometer (Amelung KG4 micro coagulometer, Sigma). The rate of thrombin formation was monitored using an Optimax microplate reader (Molecular Devices, Sunnyvale, CA). The CMC of sphingolipids was determined using either factor Xa alone or factor Xa plus factor Va. In the assay with factor Xa plus factor Va, prothrombin was mixed with various concentrations of sphingosine or its analogs at room temperature for 5 min before addition of factor Xa with or without factor Va. In separate experiments, either DG-prothrombin or DG-factor Xa was used instead of thrombin or factor Xa following the same procedure. After detectable thrombin was generated, the prothrombinase reaction was quenched by EDTA, and the rate of thrombin formation was determined by adding the clotting times.

Factor X Activation by Factor Xa in the Absence of Phospholipids—Prothrombin activation in the absence of phospholipids was determined using either factor Xa alone or factor Xa plus factor Va. In the assay with factor Xa plus factor Va, prothrombin was mixed with various concentrations of sphingosine or its analogs at room temperature for 5 min before addition of factor Xa with or without factor Va. In separate experiments, either DG-prothrombin or DG-factor Xa was used instead of thrombin or factor Xa following the same procedure. After detectable thrombin was generated, the prothrombinase reaction was quenched by EDTA, and the rate of thrombin formation was determined by adding the clotting times.

Effect of Sph and Related Compounds on Prothrombinase Activity on Platelets—Platelet surfaces provide physiological surfaces where thrombin generation and subsequent blood coagulation can occur. At physiologic concentrations, platelets (1.5 × 107/ml) were activated by thrombin and tested for the effects of Sph and related compounds on thrombin generation by factor Xa. Sph, sphinganine, and stearylamine inhibited the prothrombinase activity in a dose-dependent fashion, whereas ceramide and S1P did not (Fig. 1). The analogs of Sph showed anticoagulant activity similar to Sph in the clotting assays (Fig. 2A).

RESULTS

Effect of Sph and Related Compounds on Prothrombinase Activity on Platelets—Platelet surfaces provide physiological surfaces where thrombin generation and subsequent blood coagulation can occur. At physiologic concentrations, platelets (1.5 × 107/ml) were activated by thrombin and tested for the effects of Sph and related compounds on thrombin generation by factor Xa. Sph, sphinganine, and stearylamine inhibited the prothrombinase activity in a dose-dependent fashion, whereas ceramide and S1P did not (Fig. 1). The analogs of Sph showed anticoagulant activity similar to the sphingosine analogs. The analogs of Sph showed anticoagulant activity similar to Sph in the clotting assays (Fig. 2A). Sphinganine and stearylamine prolonged clotting times more potently than Sph (Fig. 2B). Sph and...
primary alkylamines was reduced in parallel with the decrease of carbon number, and octylamine and butylamine, which contain 8 and 4 carbons, respectively, did not prolong the clotting time (Fig. 2B). The octadecane, which has 18 carbons but no amino group, did not prolong the clotting time (Fig. 2B). Thus, the primary amino moiety and an aliphatic chain length of greater than 8 carbons were required for anticoagulant activity.

Effect of Sph and Related Compounds on Prothrombin Activation by Factor Xa in the Absence of Phospholipid—In purified reaction mixtures lacking phospholipids, thrombin generation by factor Xa/factor Va was inhibited by Sph in a dose-dependent fashion (Fig. 3A). Sphinganine and stearylamine also inhibited thrombin generation with potency similar to Sph, whereas ceramide did not inhibit prothrombin activation. In the absence of factor Va, prothrombin activation by factor Xa was not inhibited by Sph, sphinganine, or stearylamine (data not shown), indicating that factor Va was required for the anticoagulant property of Sph.

DG-factor Xa, lacking the N-terminal Gla domain of the enzyme, showed catalytic activity similar to normal factor Xa both on the chromogenic substrate S2222 and in assays of prothrombin activation in the absence of factor Va (data not shown). However, in the presence of factor Va, thrombin generation by DG-factor Xa was about one-thirtieth of that observed for normal factor Xa, reflecting the fact that the Gla domain of factor Xa is required for exerting optimal prothrombinase activity in the absence of phospholipid (Fig. 3B) (23). Notably, Sph did not inhibit thrombin generation in the presence of factor Va when DG-factor Xa was used in prothrombinase assays (Fig. 3B). Thus, both the factor Va Gla domain and factor Va were required for Sph to exert its anticoagulant activity, implying that Sph disrupts interactions between factor Va and the Gla domain of factor Xa.

When DG-prothrombin and normal prothrombin were used as substrates for factor Xa in the absence of factor Va, the observed rates of thrombin activation were the same (data not shown). However, when DG-prothrombin was activated by factor Xa plus factor Va, the rate of thrombin generation was approximately a third of the rate observed when normal prothrombin was the substrate (Fig. 3C), suggesting the importance of prothrombin Gla domain interactions with factor Va as described previously (23). Interestingly, Sph inhibited thrombin generation when DG-prothrombin was the substrate for factor Xa in the presence of factor Va in the absence of phospholipids (IC\(_{50}\) = 0.49 \(\mu\)M) (Fig. 3C), consistent with the hypothesis that Sph does not interfere with interactions between factor Va and the Gla domain of prothrombin.

Factor X Activation by Factor VIIa/Tissue Factor—The ability of Sph to inhibit Factor Xa generation was also studied. Sph inhibited in a dose-dependent manner factor X activation by factor VIIa/tissue factor/phospholipid vesicles with ~30% inhibition observed at 15 \(\mu\)M lipid (Fig. 3D). Sphinganine and stearylamine inhibited factor X activation by factor VIIa/tissue factor more efficiently than did Sph, giving 60 and 80% inhibition at 15 \(\mu\)M lipid (Fig. 3D). It is noteworthy that the potency of Sph to inhibit factor Xa generation was at least an order of magnitude weaker than its potency to inhibit thrombin generation.

CMC of Sph—DHP is an aggregation-sensitive fluorescence probe and exhibits minimal fluorescence in aqueous solution but displays significant fluorescence when it binds to a water membrane interface. This property was used to determine the CMC of Sph in the absence or presence of 200 \(\mu\)M factor Xa. When Sph was added at room temperature to a buffer solution containing DHP in 50 mM HEPES (pH 7.4), 150 mM NaCl, and

**Fig. 2. Inhibition of blood clotting assays by sphingosine and related lipid compounds.** Normal pooled human plasma (17.5 \(\mu\)l) was mixed with varying doses of Sph or related lipids and fibrinogen (0.6 mg/ml final) and incubated 3 min at 37 °C. Clotting times were measured after the addition of factor Xa with 30 mM CaCl\(_2\) to initiate clotting. A, ●, Sph; ○, Glc-Sph; ▲, trimethyl-Sph; △, dimethyl-Sph; ▼, phyto-ceramide; ▲, phyto-Sph; □, S1P; ■, lysosphingomyelin; ○, ceramide; ●, stearylamine; □, hexadecylamine; △, tetradecylamine; ▲, dodecylamine; ▼, decylamine; □, octylamine; □, butylamine; ■, sphinganine; ●, spermidine.

lyso-sphingomyelin showed anticoagulant activity, but their potencies were not as great as Sph. Ceramide, phyto-ceramide, and S1P had no significant effects on clotting times (Fig. 2, A and B). Thrombin-induced clotting of plasma (i.e., the thrombin time assay) was not affected by any sphingolipids at the concentrations employed in factor Xa-induced clotting assays (data not shown), indicating Sph and its analogs inhibit activation of prothrombin but not the clotting activity of thrombin on fibrinogen.

Sph and sphinganine have two asymmetric carbon atoms (at positions 2 and 3) (Table I) and exist in four stereoisomeric configurations ([\(\alpha\)-erythro, \(\alpha\)-threo, \(\beta\)-erythro, and \(\beta\)-threo). When different isomers of Sph and sphinganine were studied, there were no obvious differences in dose responses among the four stereoisomeric configurations of Sph and sphinganine in anticoagulant activity as measured in clotting assays (data not shown).

To characterize the nonspecific charge effect of amino groups, we tested the anticoagulant activity of other amino compounds such as diamines (spermidine, putrescine, cadaverine) or positively charged amines such as lysine, thiamine, tyramine. None of these amino compounds showed anticoagulant properties in factor Xa-1-stage clotting assays when tested at up to 500 \(\mu\)M (data not shown).

**Effect of Side Chain Length of Primary Alkylamine**—To define the effect of the aliphatic side chain length on the anticoagulant activity of primary alkylamines, primary amines with varying saturated chain lengths were assayed in the factor Xa-induced clotting time assay. As shown in Fig. 2B, stearylamine and hexadecylamine, which contain linear chains of 18 and 16 carbons, respectively, prolonged clotting times more significantly than did Sph. The anticoagulant activity of...
5 mM CaCl₂, a modest concentration-dependent increase in DPH fluorescence was observed below the CMC with a steeper increase above the CMC (Fig. 4). Based on the inflection point, the CMC of Sph under this condition was 112 μM (Fig. 4), showing Sph exists as a monomer in the solution conditions of our experiments. In the presence of 200 nM factor Xa, the CMC of Sph was similar to that in the absence of factor Xa (Fig. 4). Therefore, factor Xa did not nucleate the formation of Sph micelles at our experimental conditions. Further, we concluded that because the CMC of Sph is ~100-fold higher than the Sph IC₅₀ in functional assays (~1 μM) (Fig. 3A), Sph micelles presumably do not account for the anticoagulant function in the prothrombinase assays.

Binding of Sph to Factor Xa—To assess whether Sph can directly bind to factor Xa, we performed binding assays using Fl-factor Xa. When Fl-factor Xa was titrated with Sph, the fluorescence emission intensity of fluorescein decreased and reached a plateau value at F/F₀ of 0.67 at ~48 μM Sph with an EC₅₀ of ~16 μM (Fig. 5). The signal change was not affected by the addition of EDTA, suggesting the binding of Sph to factor Xa is not Ca²⁺-dependent (data not shown). Octylamine, which is functionally inactive as an anticoagulant, did not cause a significant decrease of fluorescence emission intensity (data not shown). Because Sph did not inhibit the activity of DG-factor Xa to activate prothrombin (Fig. 3B), Fl-DG-factor Xa was also titrated with Sph. No change in fluorescein fluorescence was observed upon titration of Fl-DG-factor Xa with Sph (Fig. 5). Thus, the spectroscopic data for the effects of Sph on fluorescein-labeled factor Xa were in agreement with the functional data in suggesting that Sph binds specifically to factor Xa via the Gla domain to exert its inhibitory effect on the prothrombinase complex.

DISCUSSION
Here we report that thrombin generation can be down-regulated by certain sphingolipids. Sph, the intermediate sphingolipid metabolite between ceramide and S1P, potently blocked thrombin generation in a physiologic setting, i.e. on platelet surfaces. Sph was reported to down-regulate thrombin generation on bovine pulmonary endothelial cells, although the mechanism for inhibition of thrombin generation was unclear (24). Based on our data, the ability of Sph to inhibit potently and directly thrombin generation extends to reaction mixtures containing purified clotting factors (see below). The strong inhibi-
Sphingosine Down-regulates Thrombin Generation

**Fig. 3. Inhibition of prothrombinase activity and of factor X activation by sphingolipids.** Prothrombinase components (panels A–C) were allowed to react for 5 min at room temperature to generate thrombin in the absence of added phospholipids. Then the reaction was stopped by EDTA addition and thrombin generation was quantitated. A, factor Xa (0.7 nM), factor Va (15.5 nM), and prothrombin (0.76 μM) were incubated with Sph or other lipids (●, Sph; ○, sphinganine; ▲, ceramide; △, stearylamine), and thrombin generation was measured. B, factor Xa (○) or DG-factor Xa (●) (0.7 nM), FVa (15.5 nM), and prothrombin (0.76 μM) were incubated with Sph for 5 min at room temperature, and thrombin generation was measured. Note different scales for factor Xa and DG-factor Xa. C, factor Xa (0.7 nM), factor Va (15.5 nM), DG-prothrombin (○), or prothrombin (●) (0.76 μM) were incubated with Sph for 5 min at room temperature, and thrombin generation was measured. D, lipids (as indicated), factor VIIa (8.4 nM), factor X (630 nM), and tissue factor (1:1000 dilution of Innovin) containing 5 mM CaCl$_2$ were incubated for 5 min to generate factor Xa. The reaction was stopped by adding EDTA, and factor Xa generation was quantitated as described. Lipids included ●, Sph; ○, sphinganine; ▲, ceramide; △, stearylamine.

**Fig. 4. Sphingosine CMC determination.** The relative fluorescence intensity of DPH was measured as function of increasing concentration of Sph in the absence (○) or presence (▲) of 200 nM factor Xa.

Sphingosine Down-regulates Thrombin Generation

Sph raises the question of whether Sph may exert anticoagulant effects in vivo as well as in vitro. Because physiological concentrations of Sph range from 100 nM in plasma to 260 nM in serum (25, 26) and because Sph that is locally generated could achieve much higher levels, we speculate that Sph anticoagulant activity could potentially be physiologic.

The structural properties of the sphingoid base (e.g. chain length, presence of double bonds, steric configuration of functional groups, and the role of hydroxyl or amino groups) were studied for their functional implications for anticoagulant activity. Sphinganine (dihydrosphingosine) and stearylamine, a simple aliphatic primary amine, also showed potent anticoagulant ability in plasma clotting assays and also inhibited thrombin generation in assays using purified reagents or on platelets. These results suggest that the free amino group on the aliphatic side chain, but not the hydroxyl group, appears to be a key component for the observed anticoagulant activity. Modification of the amino group with two or three methyl groups, which may alter the ionization constant but still allow a positive charge, showed similar anticoagulant activity compared with Sph. However, acylation of the amino group of Sph that produces ceramide (N-acyl Sph) ablated this anticoagulant activity. Interestingly, synthetic stearylamine, which at neutral pH has a positively charged amino group, showed potent anticoagulant activity.

A positive charge in the sphingolipid molecule appears to affect its anticoagulant activity. Substitution of the hydroxyl group in position 1 by a negatively charged phosphate group, as in SIP, ablated anticoagulant activity. Lysosphingomyelin, where the OH group in position 1 is replaced by phosphorylcholine, caused dose-dependent prolongation of Xa-1-stage clotting assay, but not as potently as Sph. Glc-Sph, where the OH group in position 1 is replaced by neutral glucose, caused dose-dependent prolongation of the clotting assay similar to Sph. Therefore, replacing the OH group in position 1 on a sphingolipid either with a neutral sugar or with a moiety carrying both a negative −1 charge and a positive +1 charge maintained anticoagulant activity, whereas a phosphate moiety carrying a negative charge of −1 or −2 abrogated activity. However, positively charged amino compounds such as spermidine or lysine, which are less hydrophobic, did not exhibit anticoagulant activity, suggesting that anticoagulant activity is not caused by only a simple charge effect.

Studies of the four stereoisomeric configurations of Sph and sphinganine in clotting assays indicated that the anticoagulant activity of Sph and sphinganine does not depend on the stereoisomeric configurations. In contrast, the anticoagulant activity clearly depended on the length of aliphatic carbon chains. When primary amines of varying lengths were assayed, at least 10 carbons were required to exert substantial anticoagulant activity; 16 or 18 carbons, the physiological carbon number of many sphingolipids, were required to exhibit maximum anticoagulant activity. Taken together, the anticoagulant property of Sph and related compounds requires a hydrophobic side chain of more than 10 carbons as well as a positive charge.

How do the biologically active sphingolipids and primary alkylamines act as anticoagulants and down-regulate thrombin generation? To study mechanisms, purified clotting factor Xa and factor Va were used to convert prothrombin to thrombin in the absence of phospholipids. The anticoagulant activity of Sph, i.e. inhibition of thrombin generation, was readily observed in the presence of factor Va. In contrast, Sph, sphinganine, and stearylamine did not inhibit thrombin generation in the absence of factor Va in the purified system assay, indicating that factor Va is required for expression of the anticoagulant activity of those lipids. Overall, Sph, sphinganine, and stearylamine manifest quite similar potency in their inhibitory
effects on thrombin generation, whereas ceramide does not inhibit thrombin generation. These results with purified clotting factors agree well with the results of the whole plasma clotting assays.

When prothrombin activation assays in the absence of phospholipids were performed using DG-factor Xa that lacked the N-terminal Gla domain, Sph exerted no anticoagulant activity, indicating that the Gla domain of factor Xa is a key component for Sph to exert its anticoagulant activity. In assays in the absence of added phospholipid, human factor Va showed only 5% of cofactor activity with DG-factor Xa, suggesting that interaction of the Gla domain of factor Xa and factor Va is very important for human prothrombinase activity (23). In contrast to DG-factor Xa, when DG-prothrombin was activated by factors Xa and Va, Sph potently inhibited thrombin generation (IC50 = 0.49 μM). Thus, the Gla domain of factor Xa, but not the Gla domain of prothrombin, is notably important for Sph to exert its anticoagulant activity. Overall, our data support the hypothesis that Sph interferes with protein-protein interactions essential for normal prothrombinase activity involving factor Va and the Gla domain of factor Xa (23).

Some reports suggested possible interactions of Sph with negatively charged procoagulant phospholipids in membranes that could thereby affect prothrombin activation on membranes (24–27). However, our data showed that Sph could directly bind to factor Xa below its CMC in the absence of any other lipids. Removal of the Gla domain from factor Xa ablated both the anticoagulant activity of Sph and binding of Sph to fluorescein-labeled factor Xa. These results therefore suggest the existence of functionally significant Sph binding site(s) on the factor Xa Gla domain.

Our functional and spectroscopic data suggest that the Gla domain of factor Xa is involved in Sph binding in a Ca2+-independent manner. Substantial structural changes are associated with divalent metal ion binding to the Gla domains of vitamin K-dependent clotting factors (28, 29). However, this does not preclude the existence of a Ca2+-independent Sph binding site on the Gla domain of factor Xa that contains a remarkable cluster of negatively charged amino acids that are not implicated in coordinating the array of Ca2+ ions found in Gla domain structures (30, 31). These residues, Gla-32, Asp-33, and Asp-35, are potential candidates for binding the positive charge of Sph or its analogs. The presence of Asp-33 and Asp-35 in factor Xa compared with the presence of Ser-33 and Val-35 in prothrombin (32–34) could explain why the factor Xa Gla domain, but not the prothrombin Gla domain, is necessary for Sph anticoagulant effects. Alternatively, the requirement for the Factor Xa Gla domain could be explained if Sph binds to a portion of the EGFP1 domain of factor Xa that is stabilized by the Gla domain (either plus or minus Ca2+ ions) (35). Additionally, it cannot be excluded that Sph also binds to factor Va in a manner that interferes with interactions between the factor Xa Gla domain and factor Va, presumably its C2 domain. More studies are needed to define Sph binding site(s) on factor Xa or factor Va that mediate inhibition of thrombin generation.

The Gla domain of factor X is also important for factor X activation by factor VIIa/tissue factor. We found that factor X activation by tissue factor/factor VIIa with phospholipid vesicles is also inhibited by Sph in agreement with a previous report (16). In studies using purified clotting factors, Sph inhibited factor X activation by factor VIIa and tissue factor in a dose-dependent manner. Ceramide did not inhibit factor X activation. Factor X activation was inhibited by only 30% at 15 μM Sph, and inhibition of factor X activation by sphinganine and stearylamine was much more potent than by Sph. Although this inhibitory effect of sphingolipids on factor X activation by tissue factor and factor VIIa was notably less potent than the inhibitory effects of these lipids on prothrombin activation by factor Xa/factor Va under the experimental conditions employed, it remains possible that Sph or one of its analogs might significantly inhibit the extrinsic pathway.

Interconversions of the various sphingolipids modulate sphingolipid bioactivities from one function to another, e.g., switching activities among effects on inflammation, thrombin generation, apoptosis, or cell proliferation. Because some sphingolipids can act as extracellular mediators, they could also interact with coagulation factors in circulating blood on lipoproteins, on cells, or on circulating cellular microparticles. Glucosylceramide, one of the sphingolipids and a major metabolic product of ceramide, exerts anticoagulant cofactor activity in the protein C anticoagulant system, whereas ceramide does not (17, 18). In this report, we demonstrate the anticoagulant activity of other metabolites related to ceramide, namely Sph and sphingolipid metabolites containing an aliphatic side chain with a free amino group. Therefore, structural changes of sphingolipids during their metabolism may cause significant changes in their ability to down-regulate thrombin generation and clotting. Notably, Sph appears to interfere with protein-protein interactions even in the absence of clotting factors being bound to phospholipid vesicles.

The direct anticoagulant mechanism involving sphingolipids shown in this report could represent a significant physiologic system for modulating thrombin generation. This sphingolipid regulatory system that down-regulates thrombin formation could be involved in an inflammation network associated with apoptosis and cell proliferation. When Sph increases because of hydrolysis of S1P during apoptosis (36), the increased Sph could help suppress thrombin generation, thus decreasing the inflammatory reaction during apoptosis. Anticoagulant Sph would counterbalance procoagulant phosphatidylerine. This suggested mechanism agrees with the concept of apoptosis that generally allows for the elimination of cells without tissue damage, thrombosis, or an inflammatory response. In contrast, inflammation accompanied by large cytokine release promotes the metabolism of ceramide toward S1P via Sph. The initial increase of Sph may down-regulate inflammatory reactions by inhibiting thrombin generation. However, greater inflammation would cause S1P to increase, accompanied by a decrease in Sph, which reduces anticoagulant activity due to Sph, possibly permitting thrombin generation. The newly generated thrombin further activates coagulation, stimulating cells to activate and release S1P from platelets (37). The released S1P could stimulate cells together with thrombin to produce tissue factor to generate more thrombin (38). Thus, thrombin and S1P might work synergistically to stimulate cells and induce a proinflammatory environment.

In summary, the results presented here suggest a new mechanism for cross-talk between inflammation and coagulation via the highly dynamic sphingolipid metabolic network (12, 13). The shift from certain sphingolipid metabolites to others may regulate inflammatory events such as cell proliferation and also down-regulate blood coagulation and thrombin generation. A variety of enzymes that regulate sphingolipid metabolism are capable of shifting the balance between Sph and ceramide, Sph and S1P, glucosylceramide and ceramide, etc. (10–14). The sphingolipid rheostat (10) that influences the balance between cell survival and cell death may also provide a rheostat that influences the generation and the biological activities of key proteases, e.g., thrombin and activated protein C, which themselves can influence inflammation, cell survival, and apoptosis (39, 40).
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