Acid Sphingomyelinase Regulates Platelet Cell Membrane Scrambling, Secretion, and Thrombus Formation

Patrick Münzer,* Oliver Borst,* Britta Walker, Evi Schmid, Marion A. H. Feijge, Judith M. E. M. Cosemans, Madhumita Chatterjee, Eva-Maria Schmidt, Sebastian Schmidt, Syeda T. Towhid, Christina Leibrock, Margitta Elvers, Martin Schaller, Peter Seizer, Klaus Ferlinz, Andreas E. May, Erich Gulbins, Johan W. M. Heemskerk, Meinrad Gawaz, Florian Lang

Objective—Platelet activation is essential for primary hemostasis and acute thrombotic vascular occlusions. On activation, platelets release their prothrombotic granules and expose phosphatidylserine, thus fostering thrombin generation and thrombus formation. In other cell types, both degranulation and phosphatidylserine exposure are modified by sphingomyelinase-dependent formation of ceramide. The present study thus explored whether acid sphingomyelinase participates in the regulation of platelet secretion, phosphatidylserine exposure, and thrombus formation.

Approach and Results—Collagen-related peptide–induced or thrombin-induced ATP release and P-selectin exposure were significantly blunted in platelets from Asm-deficient mice (Smpd1−/−) when compared with platelets from wild-type mice (Smpd1+/+). Moreover, phosphatidylserine exposure and thrombin generation were significantly less pronounced in Smpd1−/− platelets than in Smpd1+/+ platelets. In contrast, platelet integrin αIIbβ3 activation and aggregation, as well as activation-dependent Ca2+ flux, were not significantly different between Smpd1−/− and Smpd1+/+ platelets. In vitro thrombus formation at shear rates of 1700 s−1 and in vivo thrombus formation after FeCl3 injury were significantly blunted in Smpd1−/− mice while bleeding time was unaffected. Asm-deficient platelets showed significantly reduced activation-dependent ceramide formation, whereas exogenous ceramide rescued diminished platelet secretion and thrombus formation caused by Asm deficiency. Treatment of Smpd1−/− platelets with bacterial sphingomyelinase (0.01 U/mL) increased, whereas treatment with functional acid sphingomyelinase-inhibitors, amitriptyline or fluoxetine (5 μmol/L), blunted activation-dependent platelet degranulation, phosphatidylserine exposure, and thrombus formation. Impaired degranulation and thrombus formation of Smpd1−/− platelets were again overcome by exogenous bacterial sphingomyelinase.

Conclusions—Acid sphingomyelinase is a completely novel element in the regulation of platelet plasma membrane properties, secretion, and thrombus formation. (Arterioscler Thromb Vasc Biol. 2014;34:61-71.)

Key Words: acid sphingomyelinase ■ amitriptyline ■ granule secretion ■ phosphatidylserine exposure ■ platelets ■ thrombus formation ■ thrombin generation

Platelets are critically important for primary hemostasis after vascular injury and are pivotal elements in the development of acute thrombotic occlusion and subsequent myocardial infarction and ischemic stroke.1,2 Moreover, platelets may contribute to the pathophysiology of disorders not directly related to hemostasis or thrombosis, such as cancer,3 inflammation,4,5 host–pathogen interaction,6 and lymphatic development.7 Platelets are activated by a wide variety of stimulators, such as subendothelial collagen, ADP released from activated platelets, or activated thrombin and collagen-related peptide (CRP).4,9,9 After activation, platelets secrete their granules, undergo cell membrane scrambling with phosphatidylserine exposure, aggregate, and form thrombi resulting in vascular occlusion.10 Thereby phosphatidylserine exposure at the outer membrane surface provides binding sites for coagulation factors and promotes formation of factor Xa and thrombin by facilitating assembly of tenase and prothrombinase complexes.12,13

In other cell types, cell membrane scrambling14,15 and degranulation16 are modified by ceramide producing acid sphingomyelinase (ASM). Sphingomyelinases break down membrane sphingomyelin with ceramide production and play

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an important role in lipid signaling of different cardiovascular diseases resulting in vascular inflammation.\(^1\)\(^7\) Ceramides form ceramide-rich platforms thus increasing membrane curvature and eventual fusion and fission of vesicles.\(^1\)\(^8\) Ceramides participate in vascular inflammation and thrombosis by triggering exocytosis of Weibel–Palade bodies from endothelial cells, an effect inhibited by exogenous NO.\(^1\)\(^9\) ASM is a key regulator of T-lymphocyte granule secretion, degranulation, vesicle shedding, and especially phosphatidylserine-exposing microparticle release from glial cells.\(^1\)\(^6\)\(^,\)\(^2\) By producing ceramide, ASM may trigger cell membrane scrambling with phosphatidylserine exposure and thus suicidal death of other blood cells, such as erythrocytes.\(^2\)\(^1\)\(^,\)\(^2\)\(^2\)

ASM activity and ceramide formation thus contribute to the pathophysiology of a wide variety of disorders, including ath- erosclerosis,\(^2\)\(^3\)\(^–\)\(^2\)\(^5\) inflammation, fibrosis and infection,\(^2\)\(^6\) cystic fibrosis,\(^2\)\(^6\)\(^–\)\(^2\)\(^8\) Wilson disease,\(^2\)\(^2\) diabetes mellitus,\(^2\)\(^9\) cardiovascular disease,\(^3\)\(^0\)\(^,\)\(^3\)\(^1\) cerebral disease,\(^3\)\(^2\) multiple sclerosis\(^3\)\(^3\) major depression,\(^1\)\(^4\) Parkinson disease,\(^1\)\(^4\) and Alzheimer disease.\(^1\)\(^4\) Tricyclic antidepressant medications, such as amitriptyline or fluoxetine, are widely used as experimental functional ASM-inhibitors.\(^3\)\(^5\)

Although it is known that platelets secrete ASM on thrombin stimulation,\(^3\)\(^6\)\(^,\)\(^3\)\(^7\) nothing is known about the role of ASM in platelet physiology. The present study thus explored the role of ASM in platelet function. To this end, platelets have been isolated from gene-targeted mice lacking functional ASM (Smpd1\(^{−/−}\)) and from their wild-type littermates (Smpd1\(^{+/+}\)).

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

To study the functional role of ASM in the regulation of platelet function, platelets were isolated from gene-targeted mice lacking functional Asm (Smpd1\(^{−/−}\)) and their respective wild-type littermates (Smpd1\(^{+/+}\)).

Smpd1\(^{−/−}\) mice did not exhibit spontaneous bleeding. Blood platelet counts and mean platelet volume were similar in Smpd1\(^{+/+}\) and Smpd1\(^{−/−}\) mice (Table), indicating that Asm is not essential for platelet generation. No differences were found in other hematologic parameters (Table). Furthermore, transmission electron microscopy of Asm-deficient platelets revealed no significant morphological differences and especially no difference in number and morphology of α-granules and dense granules when compared with platelets from wild-type mice (Figure 1 in the online-only Data Supplement).

To elucidate the effect of Asm on platelet secretion, the activation-dependent release of platelet α (P-selectin exposure) and dense granules (ATP release) was quantified before and after platelet stimulation with CRP or thrombin. As illustrated in Figure 1A, P-selectin abundance at the platelet surface after stimulation with low concentrations of CRP (0.1 and 1.0 μg/mL) and thrombin (0.005 U/mL) was significantly lower in Smpd1\(^{−/−}\) platelets than in Smpd1\(^{+/+}\) platelets. Degranulation after stimulation with high concentrations of CRP (5 μg/mL; data not shown) or thrombin (0.02 U/mL) was not significantly different. To explore the effect of Asm on degranulation of platelet dense granules further, activation-dependent ATP release was determined in Smpd1\(^{+/+}\) and Smpd1\(^{−/−}\) platelets. The increase of ATP concentration in the supernatant after stimulation with lower concentrations of CRP (0.5 and 1 μg/mL) or thrombin (0.005 and 0.02 U/mL) was significantly diminished in Smpd1\(^{−/−}\) platelets as compared to Smpd1\(^{+/+}\) platelets (Figure 1C and 1D). At high CRP (≥5 μg/mL) or thrombin (0.1 U/mL) concentrations, no significant difference was found.

Although the activation-dependent platelet secretion was significantly diminished in Asm-deficient platelets, integrin α\(^{\pi}\)β\(^{3}\) activation (Figure 1B) and aggregation (Figure 1E and 1F), after low dose and high dose of thrombin or CRP stimulation were not significantly different in Smpd1\(^{+/+}\) and Smpd1\(^{−/−}\) platelets.

To examine the role of Asm in activation-dependent platelet phosphatidylserine exposure after cell membrane scrambling and induction of the coagulation cascade by activation of prothrombinase complexes, phosphatidylserine exposure (annexin-V binding) and thrombin generation were quantified before and after platelet stimulation (Figure 2). The effect of Asm on activation-dependent platelet cell membrane scrambling with procoagulant phosphatidylserine exposure was determined by flow cytometric analysis of platelet annexin-V binding after stimulation with thrombin (1 U/mL) or a combination of thrombin (0.01 U/mL) and CRP (5 μg/mL). As shown in Figure 2A, activation-dependent phosphatidylserine exposure was significantly blunted in Asm-deficient platelets than in wild-type platelets. In a further series of experiments, we found that thrombin generation is significantly affected in Smpd1\(^{−/−}\) platelets. As illustrated in Figure 2B through 2D, platelet-rich plasma from Asm-deficient mice displayed significantly reduced peak levels of thrombin generation, whereas thrombin levels in platelet-poor plasma were similar in Smpd1\(^{+/+}\) and Smpd1\(^{−/−}\) mice.

Platelet activation, degranulation, and subsequent thrombus formation are triggered by an increase of intracellular

### Table. Blood Count of Smpd1\(^{+/+}\) and Smpd1\(^{−/−}\) mice

<table>
<thead>
<tr>
<th></th>
<th>Smpd1(^{+/+})</th>
<th>Smpd1(^{−/−})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets, ×10(^{11})/μL</td>
<td>1097±39</td>
<td>1105±108</td>
</tr>
<tr>
<td>MPV, fl</td>
<td>6.6±0.1</td>
<td>6.4±0.1</td>
</tr>
<tr>
<td>Erythrocytes, ×10(^{12})/L</td>
<td>7.7±0.7</td>
<td>7.9±0.5</td>
</tr>
<tr>
<td>HGB, g/dL</td>
<td>11.2±1.1</td>
<td>11.8±0.9</td>
</tr>
<tr>
<td>HCT, %</td>
<td>37.2±4.6</td>
<td>38.2±4.0</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>48.4±1.8</td>
<td>49.2±1.5</td>
</tr>
</tbody>
</table>

Arithmetic mean±SEM (n=8) of platelet count, mean platelet volume (MPV), red blood cell (RBC) count (erythrocytes), hemoglobin concentration (HGB), hematocrit (HCT), and mean RBC volume (MCV).
Ca\(^{2+}\) concentration ([Ca\(^{2+}\)_i]), which is accomplished by Ca\(^{2+}\) store depletion and store operated Ca\(^{2+}\) entry. In theory, ASM could have an effect on degranulation, phosphatidylserine exposure, and thrombus formation by modifying [Ca\(^{2+}\)_i]. However, spectrofluorimetric measurements revealed that activation-dependent [Ca\(^{2+}\)_i] increases were similar in Smpd1\(^{-/-}\) platelets and in Smpd1\(^{+/+}\) platelets after stimulation with CRP or thrombin (Figure IIB in the online-only Data Figures).
Furthermore, as shown in Figure IIA in the online-only Data Supplement, Asm deficiency did not appreciably influence intracellular Ca\(^2+\) release or store operated Ca\(^2+\) entry induced in Smpd1\(^{+/+}\) and Smpd1\(^{-/-}\) platelets with sarco/endoplasmic reticulum (SR/ER) Ca\(^{2+}\) ATPase pump inhibitor thapsigargin.

To elucidate the relevance of Asm in pathological thrombus formation, we examined in vitro platelet adhesion and thrombus formation to collagen-coated surfaces under flow at low (500 s\(^{-1}\)) and high (1700 s\(^{-1}\)) wall shear rates. As illustrated in Figure 3A, Smpd1\(^{+/+}\) platelets formed massive and dense thrombi after 5 minutes of perfusion. At shear rates of 500 s\(^{-1}\), no significant reduction of thrombus formation was found, but at high arterial shear rates Smpd1\(^{-/-}\) platelets formed only smaller single thrombi with a significantly reduced thrombus surface coverage (Figure 3A and 3B). Real-time imaging of thrombus formation under flow by capturing images of fluorescent-labeled platelets indicated a consistent and gradual increase in fluorescence intensity from deposited Smpd1\(^{+/+}\) platelets (Figure 3E). With Smpd1\(^{-/-}\) blood, the slope of fluorescence increase was diminished, and time traces pointed to a slow, but gradual thrombus buildup. Notably, time traces from none of the experiments or inspection of consecutive images showed evidence for increased thrombus instability or embolization events with Smpd1\(^{-/-}\) blood.

To assess the significance of Asm for arterial thrombus formation in vivo, time to occlusion of mesenteric arterioles caused by thrombus formation after FeCl\(_3\)-induced injury have been analyzed. As shown in Figure 3C and 3D, vascular occlusion caused by arterial thrombus formation was significantly impaired in Smpd1\(^{-/-}\) mice. Nevertheless, the tail bleeding time was nearly unaffected in Asm-deficient mice (Figure 3F).

Because Asm has been identified as critical enzyme regulating ceramide production, we examined the activation-dependent ceramide formation in Smpd1\(^{-/-}\) platelets. As a result, we found significantly impaired ceramide formation in Asm-deficient platelets when compared with wild-type platelets on stimulation with thrombin or the combination of thrombin and CRP (Figure 4A). In a further series of experiments, we tried to restore the ceramide defect in Smpd1\(^{-/-}\) platelets by the addition of exogenous ceramide C16. As illustrated in Figure 4, addition of exogenous ceramide rescued the defective granule secretion and as the blunted thrombus formation of Smpd1\(^{-/-}\) platelets (Figure 4D and 4E) up to a level comparable with that of wild-type platelets.

In the next step, we explored the effect of pharmacological ASM inhibition with amitriptyline (5 \(\mu\)mol/L) in wild-type mouse platelets (Figure 5). The increase of \(\alpha\)-granule...
secretion (P-selectin exposure; Figure 5A) and dense granule secretion (ATP release; Figure 5C and 5D) after stimulation with lower concentrations of CRP (0.1 and 1 μg/mL) or thrombin (0.005–0.02 U/mL) was significantly decreased in the presence of the functional ASM inhibitor amitriptyline (5 μmol/L). At high CRP (≥ 5 μg/mL) or thrombin (0.1 U/mL) concentrations, degranulation tended to be lower in the presence than in the absence of amitriptyline; however, the difference did not reach statistical significance (data not shown). Phosphatidylserine exposure after platelet stimulation with thrombin (1.0 U/mL) or the combination of thrombin (0.01 U/mL) and CRP (5 μg/mL) was significantly diminished after preincubation with amitriptyline (5 μmol/L) when compared with solvent control (Figure 5B). Similar observations were made in arterial thrombus formation at shear rates of 1700 s⁻¹ after inhibition of ASM with 5 μmol/L amitriptyline. As illustrated in Figure 5E, thrombi formed by Smpd1⁺/⁻ platelets after 5-minute perfusion were significantly blunted in the presence of amitriptyline (5 μmol/L), whereas amitriptyline showed no further effect on reduced thrombus formation of Smpd1⁻/⁻ platelets.

Figure 3. Formation and stability of thrombi formed by Smpd1⁻/⁻ and Smpd1⁺/⁺ platelets, arterial thrombotic occlusion in vivo, and tail bleeding time in Smpd1⁻/⁻ and Smpd1⁺/⁺ mice. A, Original phase-contrast images of surface coverage by adherent platelets after perfusion of whole blood from Smpd1⁻/⁻ (top) and Smpd1⁺/⁺ (bottom) mice over a collagen-coated surface for 5 minutes at low (500 s⁻¹, left) and high (1700 s⁻¹, right) arterial shear rates. Scale bar, 50 μm. B, Arithmetic means±SEM (n=5) of surface coverage by adherent platelets after perfusion of whole blood from Smpd1⁺/⁺ (black bars) and Smpd1⁻/⁻ (gray bars) mice over a collagen-coated surface for 5 minutes at low (500 s⁻¹, left) and high (1700 s⁻¹, right) arterial shear rates. *P<0.05 indicates statistically significant difference. C, Representative images of occlusive in vivo thrombus formation 0, 5, 10, and 20 minutes after injury of mesenteric arterioles with FeCl₃ in Smpd1⁺/⁺ (left) and Smpd1⁻/⁻ (right) mice. Scale bar, 50 μm. D, Arithmetic means±SEM (n=10) of time to arterial occlusion after FeCl₃-induced injury of mesenteric arterioles in Smpd1⁺/⁺ (black bar) and Smpd1⁻/⁻ (gray bar) mice. **P<0.01 indicates statistically significant difference. E, Arithmetic means±SEM (n=4) of integrated fluorescence (FL) intensity of adherent platelets after perfusion of whole blood from Smpd1⁺/⁺ (black diamonds) and Smpd1⁻/⁻ (gray diamonds) mice over a collagen-coated surface representing thrombus growth and stability in vitro >5 minutes. F, Tail bleeding time measured after amputating the tail tip of Smpd1⁺/⁺ (black dots) and Smpd1⁻/⁻ (gray dots) mice. Each dot represents 1 individual; black bar represents the mean value (n=12; P=0.21). n.s. indicates nonsignificant.
and 1 μg/mL) or thrombin (0.005 U/mL) were significantly increased in the presence of bacterial sphingomyelinase (bSM; 0.01 U/mL). At high CRP (≥ 5 μg/mL) or thrombin (0.1 U/mL) concentrations, degranulation again tended to be higher in the presence than in the absence of bacterial ASM; however, the difference did not reach statistical significance (data not shown). A significantly increased phosphatidylserine exposure was observed in thrombin-stimulated platelets (1.0 U/mL) or the combination of thrombin (0.01 U/mL) and CRP (5 μg/mL)-stimulated platelets after treatment with bSM when compared with solvent control treatment (Figure 7B).

Exposure of the platelets to bSM (0.01 U/mL) significantly augmented platelet secretion, phosphatidylserine exposure, and formation of thrombin in Smpd1−/− platelets up to the values observed in wild-type platelets (Figure 8). In detail, bSM rescued defective activation-dependent ATP release on stimulation with CRP (0.5 μg/mL) or thrombin (0.005 U/mL) (Figure 8A and 8B), as well as CRP-/thrombin-triggered phosphatidylserine exposure (Figure 8C) and thrombus formation at 1700 s⁻¹ (Figure 8D and 8E). Moreover, bSM significantly increased platelet secretion on low agonist stimulation, cell scrambling, and thrombus formation at high arterial shear rates.

**Discussion**

The present study discloses an important role of ASM in the regulation of platelet secretion and initiation of platelet procoagulant activity. According to the present observations, Asm does not appreciably alter agonist-induced increases of platelet cytosolic Ca²⁺ and integrin αIIbβ₃ activation or aggregation, but critically enhances activation-dependent platelet degranulation and platelet-dependent thrombin generation after activation-dependent exposure of platelet phosphatidylserine, resulting in enhanced arterial thrombus formation. Similar to genetic knockout of ASM, pharmacological inhibition of the enzyme with the functional inhibitors amitriptyline or fluoxetine blunted ATP release and thrombus formation. Conversely, ATP release and thrombus formation were augmented in the presence of bSM.
The decreased activation-dependent granule secretion and phosphatidylserine exposure in Smpd1\(^{-/-}\) platelets paralleled their impaired ability to generate adhesion to collagen-coated surfaces under high arterial shear rates in vitro and defective arterial thrombus formation in vivo without affecting stability of formed thrombi. Interestingly, the effect of CRP and thrombin on P-selectin exposure and ATP release was apparent only at low concentrations of the agonists. Increasing the agonist concentration dissipated the differences between Smpd1\(^{-/-}\) and Smpd1\(^{+/+}\) platelets, indicating that Asm deficiency decreases the sensitivity of platelets to activating agonists but does not modify platelet function after maximal activation.

Although Asm-deficient platelets showed a significantly diminished activation-dependent secretion, number and morphology of \(\alpha\)-granules and dense granules of Smpd1\(^{-/-}\) platelets were normal and comparable with platelets from wild-type mice, indicating that the observed effects are not because of a regulatory role of Asm in platelet granules biogenesis.

Interestingly, the defects in secretion and phosphatidylserine exposure of Smpd1\(^{-/-}\) platelets were obviously not secondary to altered increases of cytosolic Ca\(^{2+}\) because activation-dependent increase of [Ca\(^{2+}\)] was lost, and store operated Ca\(^{2+}\) entry were found to be similar in Smpd1\(^{-/-}\) and Smpd1\(^{+/+}\) platelets. This is a remarkable finding because platelet secretion and phosphatidylserine exposure are known to be Ca\(^{2+}\)-dependent and, accordingly, suggest that a potential signaling defect is downstream of phospholipase C activation. Furthermore, Ca\(^{2+}\)-sensitive integrin \(\alpha_i\beta_{\text{IIb}}\) activation and platelet aggregation were insensitive to sphingomyelinase deficiency.

As shown previously,\(^{38}\) Asm modifies the organization of the plasma membrane and thus participates in the release of cytotoxic granules in T-lymphocytes. Indeed, the cellular process of degranulation and vesiculation is connected with membrane fusion and fission. Asm may have an effect on platelet function by modifying the membrane turnover secondary to altered increases of cytosolic Ca\(^{2+}\) because activation-dependent increase of [Ca\(^{2+}\)] was lost, and store operated Ca\(^{2+}\) entry were found to be similar in Smpd1\(^{-/-}\) and Smpd1\(^{+/+}\) platelets. This is a remarkable finding because platelet secretion and phosphatidylserine exposure are known to be Ca\(^{2+}\)-dependent and, accordingly, suggest that a potential signaling defect is downstream of phospholipase C activation. Furthermore, Ca\(^{2+}\)-sensitive integrin \(\alpha_i\beta_{\text{IIb}}\) activation and platelet aggregation were insensitive to sphingomyelinase deficiency.

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As ceramide increases the curvature and the bending rigidity of the membrane,\textsuperscript{15,41} it can change the membrane properties and, therefore, influence the degranulation of cells. Because platelets cannot accomplish de novo synthesis of ceramide,\textsuperscript{42} platelets do depend on their sphingomyelinase activity for ceramide production. Sphingomyelinase mediated ceramide generation occurs within seconds,\textsuperscript{43} thus influencing rapid activation-dependent cellular functions. As shown in the present study, the ASM seems to play a major role in platelet ceramide metabolism because Smpd1\textsuperscript{-/-} platelets showed a significantly decreased ceramide production. Furthermore, we could show that exogenous ceramide was able to rescue the phenotype of defective platelet secretion and thrombus formation.

ASM-dependent regulation of platelet secretion and phosphatidylserine exposure could result from direct regulation of platelet membrane property. As a matter of fact, ASM-deficient platelets showed a significant reduction in activation-dependent thrombin generation because of reduced exposure of phosphatidylserine, the assembly sites for tenase and prothrombinase complexes. Notably, reduced thrombin generation was only found in Asm-deficient platelet-rich plasma, whereas platelet-poor plasma of Smpd1\textsuperscript{-/-} mice showed no differences in thrombin levels when compared with that of wild-type samples.

Tricyclic antidepressant medications, including amitriptyline or fluoxetine, are widely used functional experimental inhibitors of ASM because it has been shown that these cationic amphiphiles trigger proteolysis of ASM, resulting in a significantly reduced ASM activity.\textsuperscript{39} Treatment with amitriptyline or fluoxetine at concentrations of 5 \( \mu \text{mol/L} \) leads to an effective inhibition of ASM activity\textsuperscript{44} because granule secretion and phosphatidylserine exposure after stimulation of platelets with CRP or thrombin were significantly impaired in the presence of amitriptyline or fluoxetine, a finding similar to that in Asm-deficient platelets. Treatment of platelets with amitriptyline showed no further inhibitory effect on impaired thrombus formation of Smpd1\textsuperscript{-/-} platelets, confirming that there are no significant further targets of amitriptyline affecting platelet function unrelated to ASM inhibition, even though we cannot rule out that amitriptyline and fluoxetine exert additional small effects in platelets.

The addition of bSM could mimic the effect of ASM on platelet degranulation and phosphatidylserine exposure. The stimulation-dependent secretion of platelet \( \alpha \)-granules and dense granules shown by P-selectin expression, ATP release, and phosphatidylserine translocation to the outer leaflet of the plasma membrane was significantly increased in the presence of bSM. According to the present observations, ASM activity emerged as a critical determinant of platelet function and thrombus formation. Activation of ASM is thus expected to enhance the activation-dependent platelet degranulation and phosphatidylserine translocation by modifying membrane properties. ASM is upregulated or activated by a wide variety of mediators, including thrombin,\textsuperscript{37} platelet-activating factor,\textsuperscript{30} amyloid,\textsuperscript{45,46} and plasminogen activator inhibitor 1.\textsuperscript{47} Pathophysiologically, ASM plays a major role in vascular inflammation and the development of atherosclerosis\textsuperscript{31} by regulating the release of Weibel–Palade bodies from stimulated endothelial cells.\textsuperscript{19} According to the present study, ASM might,
tenase and prothrombinase complex. This conversion of activation process by facilitating the assembly and activation of membrane blebs and microvesicles propagating the coagulation machinery of platelets to support thrombin generation, is associated with specific biochemical and morphological changes, some of which are similar to those occurring in apoptotic cells. Platelets with impaired Asm are less prone to undergo collagen-induced thrombus formation under high shear stress. In line with these findings, even though thrombus buildup is significantly impaired, thrombus stability is unaffected in Asm-deficient mice. The possibility remains that Asm and Asm-dependent ceramide production may affect platelet membrane structure directly and are involved in the signaling cascade triggering platelet secretion and phosphatidylserine exposure but are not required for platelet integrin activation and aggregation. Further studies will be required to define whether the respective signaling pathways dissociate.

thrombin generation plays a significant role for arterial thrombus formation in vivo. Asm-deficient mice were found to be protected against arterial thrombotic occlusion in a model of arterial thrombus formation in mesenteric arterioles after Fe3Cl-induced injury where thrombus formation is highly thrombin dependent. Accordingly, enhanced activity of ASM could increase platelet responsiveness and platelet-dependent thrombin generation, thus predisposing to thrombotic complications, such as ischemic stroke. Conversely, genetic or pharmacological knockout of ASM may be a therapeutic option to decrease the susceptibility to thrombotic complications. As a matter of fact, Asm-deficient mice displayed a significantly reduced infarct size and a better behavioral outcome after cerebral ischemia.

Despite the strong effect of Asm deficiency on degranulation, as well as thrombin generation and thrombus formation, we could, surprisingly, not observe a significant effect of Asm deficiency on platelet integrin activation and aggregation. In line with these findings, even though thrombus buildup is significantly impaired, thrombus stability is unaffected in Asm-deficient mice. The possibility remains that Asm and Asm-dependent ceramide production may affect platelet membrane structure directly and are involved in the signaling cascade triggering platelet secretion and phosphatidylserine exposure, in part, be effective by regulating platelet secretion that plays an active part in vascular inflammation and atherogenesis.

After rupture of atherosclerotic lesions with endothelial denudation, circulating platelets are exposed to thrombogenic subendothelial collagen resulting in platelet recruitment to the injured vessel wall. Platelets adhering to collagen, for example, via glycoprotein VI, expose phosphatidylserine at their outer surface and produce phosphatidylserine-exposing membrane blebs and microvesicles propagating the coagulation process by facilitating the assembly and activation of tenase and prothrombinase complex. This conversion of activated platelets to a procoagulant state, referring to the ability of platelets to support thrombin generation, is associated with specific biochemical and morphological changes, some of which are similar to those occurring in apoptotic cells.

Platelets with impaired Asm are less prone to undergo collagen-triggered thrombus formation under high shear stress, a property reversed by adding ceramide or (bacterial) sphingomyelinase. Along those lines, treatment with the functional Asm inhibitors, amitriptyline and fluoxetine, significantly reduced shear stress–induced thrombus formation on collagen. Platelet degranulation and phosphatidylserine exposure, as described in this article to be modified by Asm, are expected to be the central mechanism underlying thrombus formation under high arterial shear stress. In line with these findings, the present study unravels that Asm-dependent regulation of platelet membrane properties, secretion, and platelet-driven thrombin generation plays a significant role for arterial thrombus formation in vivo.
In conclusion, the present observations identify ASM as a novel regulator of platelet degranulation and thrombus formation. Thus, inhibition of ASM may prove useful in the treatment of thrombosis.

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Disclosures
None.

References


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Materials and Methods

Chemicals and antibodies

Platelets were activated using collagen-related peptide (Richard Farndale, University of Cambridge, United Kingdom) or thrombin (Roche, Germany). Fluorophore-labeled antibodies for P-selectin (Wug.E9-FITC) and activated integrin α\textsubscript{IIb}β\textsubscript{3} (JON/A-PE) from Emfret Analytics were used for flow cytometric analysis. Sphingomyelinase was functionally inhibited with amitriptyline (5 µM; Sigma-Aldrich) or fluoxetine (5 µM; Sigma-Aldrich). Where indicated, bacterial sphingomyelinase (0.01 U/ml; Enzo Life Science) or ceramide C16 (1µM; Enzo Life Science) were used.

Mice

Acid sphingomyelinase knockout mice (Smpd\textsubscript{1}\textsuperscript{-/-}) and wildtype littermates (Smpd\textsubscript{1}\textsuperscript{+/+}) were generated and bred as described \textsuperscript{1, 2}. Animals were genotyped by polymerase chain reaction (PCR). All animal experiments were conducted according to German law for the welfare of animals and were approved by local authorities. The investigation conforms to the Directive 2010/63/EU of the European Parliament.

Preparation of mouse platelets

Platelets were obtained from 10- to 12-week-old Smpd\textsubscript{1}\textsuperscript{-/-} and Smpd\textsubscript{1}\textsuperscript{+/+} mice of either sex. The mice were anesthetized and blood was drawn from the retroorbital plexus into heparinized tubes. Blood parameters were analyzed with pocH-100iv automatic hematology analyzer (Sysmex). Platelet rich plasma (PRP) was obtained by centrifugation at 260 g for 5 minutes. Afterwards PRP was centrifuged at 640 g for 5 minutes to pellet the platelets. Where necessary apyrase (0.02 U/ml; Sigma-Aldrich) and prostaglandin I\textsubscript{2} (0.5 µM; Calbiochem) were added to the PRP to prevent activation of platelets during isolation. After two washing steps the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl\textsubscript{2} where indicated).

ATP release

ATP release was determined to study secretion of dense granules as described previously \textsuperscript{3}. To this end, platelets were treated with different agonist concentrations. Where indicated platelets were pretreated with amitriptyline (5 µM), fluoxetine (5 µM), bacterial sphingomyelinase (0.01 U/ml) or ceramide (1 µM). For determination of ATP release, the isolated platelets were adjusted to a concentration of 250 x 10\textsuperscript{6} platelets per µl. After calibration of one sample with ATP standard (ChronoLog), the ATP concentration was determined utilizing the ChronoLume luciferin assay (ChronoLog, Havertown, USA) on a luminoaggregometer (Modell 700, ChronoLog) according to the manufacturer's protocol.

Flow cytometry

Two-colour analysis of mouse platelet activation was conducted using fluorophore-labeled antibodies for expression of P-selectin (Wug.E9-FITC) and activated integrin α\textsubscript{IIb}β\textsubscript{3} (JON/A-PE). Heparinized whole blood was diluted 1:20 in modified Tyrode buffer and washed twice. After adding 1 mM CaCl\textsubscript{2}, blood samples were mixed with antibodies and subsequently stimulated with agonists for 15 minutes at room temperature (RT). For analysis of phosphatidylserine exposure, washed platelets were diluted in Tyrode buffer containing 2 mM CaCl\textsubscript{2} and activated with thrombin or thrombin/CRP for 15 minutes and stained with annexin-V-FITC (Immunotools, Germany) at RT. The reaction was stopped by addition of PBS supplemented with calcium and samples were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences).
**Platelet aggregometry**

Aggregation of isolated platelets at a concentration of 250 x 10⁶ platelets per µl in Tyrode buffer pH 7.4 was estimated from light transmission determined with a luminoaggregometer Modell 700 (ChronoLog). After adjusting the measurement according to the manufacturer's protocol platelets were activated with thrombin or CRP at the indicated concentrations for 10 minutes, 37°C and a stirr speed of 1000 rpm. Analysis was performed with the aggrolink8 software (ChronoLog).

**Flow chamber**

Heparinized mouse whole blood was diluted 1:3 in modified Tyrode buffer (2 mM CaCl₂) and perfused through a transparent flow chamber (slit depth 50 µm) over a collagen-coated surface (200 µg/ml) with low (500 s⁻¹) or high (1700 s⁻¹) wall shear rates for 5 minutes as described previously⁴. After perfusion the chamber was rinsed for 5 minutes by perfusion with Tyrode buffer and pictures were taken from 5 to 6 different microscopic areas (using optical objectives 20x and 40x, Carl Zeiss). Analysis was done with AxioVision software (Carl Zeiss) and the mean percentage value of the covered area was determined.

To determine thrombus growth and stability, murine platelets in whole blood were stained in situ with non-blocking Dylight 488 anti-GPIb mAb (1:100) at 37°C for 5 minutes. Afterwards, blood was perfused over collagen (3.5 minutes at 1700 s⁻¹) as described above. Images were captured every 20 seconds during whole blood perfusion using an EVOS microscope (AMG, Bothell, USA) with an Olympus UPlanSApo 60x/1.35 NA oil immersion objective. A normalized quantification of integrated Dylight488 fluorescence intensity was calculated with ImageJ software.

**Calcium measurements**

Washed murine platelets were suspended in Tyrode buffer without calcium and loaded with 5 µM Fura-2 acetoxyxymethylester (Invitrogen) in the presence of 0.2 µg/ml Pluronic F-127 (Biotium) at 37°C for 30 minutes. Loaded platelets, washed once and resuspended in 500 µl Tyrode buffer containing 0.5 mM EGTA (Roth) or 1 mM Ca²⁺, were activated with agonists or treated with thapsigargin and subsequent calcium addition. Calcium responses were measured under stirring with a spectrofluorimeter (LS 55, PerkinElmer), at alternating excitation wavelengths of 340 and 380 nm. The 340/380 nm ratio values were converted into nanomolar concentrations of [Ca²⁺] by lysis with Triton X-100 (Sigma-Aldrich) and a surplus of EGTA. Analysis was done with the Flwinlab software (PerkinElmer).

**In vivo thrombus formation after FeCl₃ injury in mesenteric arterioles**

For examination of arterial thrombus formation in vivo, 5-6 weeks old Smpd1⁻/⁻ mice and wildtype littermates (Smpd1⁺/⁺) were anesthetized. After midline abdominal incision, the mesentery was exteriorized and arterioles free of fat tissue were injured by topical application of a filter paper saturated with 20% FeCl₃ for 20 seconds. Thrombus formation in arterioles was monitored for 40 minutes or until complete occlusion occurred (stop of blood flow for > 1 min). Digital images were recorded and analyzed off-line.

**Thrombin generation**

Thrombin generation in murine platelet poor plasma (PPP) or murine platelet rich plasma (PRP) was measured as described previously⁵. Platelet count of PRP was adjusted to a final concentration of 150x10⁶/µl whereas the PPP was supplemented with 4 mM phospholipids. Before adding PPP or PRP, wells of a 96 well plate were prefilled with 10 µl tissue factor (5 pM) in buffer pH 7.35 (20 mmol/L Hepes, 140 mmol/L NaCl, 5 mg/mL bovine serum albumin). Samples were run in duplicates and where indicated the samples were pretreated with 5 µg/ml collagen-related peptide (CRP). By adding 10 µl of the fluorescent
thrombin substrate (Z-Gly-Gly-Arg aminomethyl coumarin, 2.5 mmol/L) the coagulation reaction was started. The whole procedure was done under shaking and 37°C. Calibration was done using a thrombin calibrator. Finally the thrombin peak height (maximal rate of thrombin formation), and endogenous thrombin potential (area-under-the-curve, i.e. integrated thrombin activity) was measured by means of the Thrombinscope software (Stago, Germany).

**Ceramide production**

Activation-dependent ceramide production was measured using the DAG kinase assay. To this end isolated murine platelets were kept in a resting state or stimulated for 5 minutes with 1 U/ml or 0.1 U/ml thrombin and 5 µg/ml CRP. After pelleting the platelets the supernatant was trashed and the pellet was kept in liquid nitrogen. Afterwards membrane phospholipids were extracted and analysed as described previously 6.

**Bleeding time**

Mice were anesthetized and a 3-mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gentle absorption of the blood with filter paper at 20 seconds intervals without making contact with the wound site. When no blood was observed on the paper, bleeding was considered to have ceased. Experiments were stopped after 20 minutes.

**Transmission electron microscopy**

After washing with warmed PBS, freshly isolated platelets were fixed with warmed Karnovsky’s fixative for 1 h at room temperature and stored at 4°C. For electron microscopic analyses, the cell pellets were embedded in 3.5% agarose at 37°C, coagulated at room temperature, and fixed again in Karnovsky’s solution. Post-fixation was based on 1.0% osmium tetroxide containing 1.5% K-ferrocyanide in distilled water for 2 h. Following standard methods, blocks were embedded in glycide ether and cut using an ultra microtome (Ultracut, Reichert, Vienna, Austria). Ultra-thin sections (30 nm) were mounted on copper grids and analyzed using a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) operating at 120 kV.

**Statistical analysis**

Data are provided as means ± SEM, n represents the number of experiments. All data were tested for significance using paired or unpaired Student t-test or one-way ANOVA with Dunnett’s post-hoc test, as appropriate. Results with p<0.05 were considered statistically significant.
References


Suppl. Figure I

Granules per platelet

α granules  δ granules

n.s.
Suppl. Figure II