Inner mitochondrial membrane disruption links apoptotic and agonist-initiated phosphatidylserine externalization in platelets

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Abstract

Objectives—Phosphatidylserine (PSer) exposure mediates platelet procoagulant function and regulates platelet lifespan. Apoptotic, necrotic, and integrin-mediated mechanisms have been implicated as intracellular determinants of platelet PSer exposure. Here we investigate 1) the role of mitochondrial events in platelet PSer exposure initiated by these distinct stimuli and 2) the cellular interactions of the procoagulant platelet in vitro and in vivo.

Approach and results—Key mitochondrial events were examined, including cytochrome c release and inner mitochondrial membrane (IMM) disruption. In both ABT-737 (apoptotic) and agonist (necrotic)-treated platelets PSer externalization was temporally correlated with IMM disruption. Agonist stimulation resulted in rapid cyclophilin D-dependent IMM disruption that coincided with PSer exposure. ABT-737 treatment caused rapid cytochrome c release, eventually followed by caspase-dependent IMM disruption that again closely coincided with PSer exposure. A non-mitochondrial and integrin-mediated mechanism has been implicated in the formation of a novel PSer-externalizing platelet subpopulation. Using image cytometry, this subpopulation is demonstrated to be the result of the interaction of an aggregatory platelet and a procoagulant platelet rather than indicative of a novel intracellular mechanism regulating platelet PSer externalization. Using electron microscopy, similar interactions between aggregatory and procoagulant platelets are demonstrated in vitro and in vivo within a mesenteric vein hemostatic thrombus.

Conclusions—Platelet PSer externalization is closely associated with the mitochondrial event of IMM disruption identifying a common pathway in PSer externalizing platelets. The limited interaction of procoagulant platelets and integrin-active aggregatory platelets identifies a potential mechanism for procoagulant platelet retention within the hemostatic thrombus.

Keywords
platelet; cell death; inner mitochondrial membrane; phosphatidylserine

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INTRODUCTION

Phosphatidylserine (PSer) externalization facilitates coagulation and acts as a signal for cell clearance. Increased PSer externalization on the platelet surface allows assembly of coagulation factor complexes, increased thrombin generation, and fibrin formation. In platelets, distinct apoptotic and agonist-initiated mechanisms have been implicated in the regulation of PSer externalization. Common to both of these mechanisms is the central role of the mitochondrion and mitochondrially-mediated events.

When platelets are strongly stimulated, a subpopulation of activated platelets exposes high levels of PSer on their surface. In these agonist-initiated procoagulant platelets, sustained calcium elevation and cyclophilin-D (CypD) mediated mitochondrial permeability transition pore (mPTP) formation play key roles in the regulation of PSer externalization. The mPTP is an inducible, large and non-selective pore that is formed within the inner mitochondrial membrane (IMM). Sustained mPTP formation results in IMM disruption, loss of mitochondrial transmembrane potential ($\Delta \Psi_m$), and mitochondrial swelling. As this process is morphologically analogous to that observed in cells undergoing necrosis, these agonist-initiated procoagulant platelets have also been referred to as necrotic platelets.

Treatment of platelets with a BcL-xL inhibitor, such as ABT-737, also results in PSer externalization. Inhibition of BcL-xL results in Bax/Bak mediated caspase activation, PSer externalization, and in vivo platelet clearance. Apoptosome formation in Bax/Bak mediated apoptosis is dependent on disruption of the outer mitochondrial membrane (OMM) and cytochrome c release from the intermembrane space to the cytoplasm. In contrast to agonist-initiated PSer externalization, the relationship of $\Delta \Psi_m$ with apoptotic PSer externalization is less well characterized with varying reports regarding whether $\Delta \Psi_m$ is lost or maintained in the apoptotic platelet.

An integrin-mediated pathway of platelet PSer externalization has been characterized in platelets stimulated with a high concentration of thrombin. Unlike apoptotic platelets or platelets stimulated with agonists, PSer externalization in these integrin-dependent procoagulant platelets was associated neither with increased concentrations of cytoplasmic calcium nor with mitochondrial membrane depolarization. Thus, this pathway would represent a novel mechanism of agonist-initiated PSer externalization not associated with a necrotic mechanism of cell death.

Here we dissect the mitochondrial events occurring in platelets with regulated externalization of PSer. Novel platelet flow cytometric assays were developed to allow a detailed kinetic and mechanistic examination of how and when mitochondrial events participate in platelet PSer externalization. Using these novel assays and through the kinetic examination of mitochondrial events in platelets, IMM disruption is shown to be closely and temporally associated with PSer externalization in each of these disparate pathways. Delineation of integrin-mediated PSer externalization revealed a close interaction of procoagulant platelets with activated and non-PSer externalized platelets providing insights...
into a potential cellular mechanism of procoagulant platelet retention within a hemostatic thrombus.

MATERIALS AND METHODS

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

RESULTS

Inner and outer mitochondrial membrane disruption and PSer externalization in platelets

A key event in apoptotic cell death is the release of cytochrome c from the mitochondrial intermembrane space to the cytosol.\textsuperscript{17} Controlled digitonin permeabilization of the plasma membrane can be utilized to examine retention of cytochrome c within the mitochondrial intermembrane space.\textsuperscript{18} Cytochrome c release was examined in platelets following treatment with ABT-737 for 30 and 90 minutes. In untreated platelets cytochrome c is retained as measured by staining with a labeled anti-cytochrome c antibody. In contrast, cytochrome c in ABT-737 treated platelets is not retained following digitonin permeabilization (Figure 1A). This can be attributed to mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release to the cytosol. To directly examine IMM disruption, a calcein-cobalt assay was utilized.\textsuperscript{19} Cobalt is normally excluded from the mitochondrial matrix. IMM disruption in calcein and cobalt loaded platelets results in cobalt entry into the mitochondrial matrix and a quenching of the remaining calcein fluorescence. Increased IMM disruption, as assessed by quenching of the calcein fluorescence, was observed in ABT-737 treated platelets (Figure 1B).

Utilizing these cytometric assays, a detailed kinetic analysis of mitochondrial events and PSer externalization was performed in platelets following exposure to ABT-737 (Figure 1C) and to the agonists thrombin and convulxin (Figure 1D). Treatment of platelets with ABT-737 resulted in rapid loss of cytochrome c consistent with Bcl-2 protein mediated MOMP. Cytochrome c release occurred much more rapidly than PSer externalization in ABT-737 treated platelets. Whereas MOMP (as assessed by cytochrome c loss) was at a maximum within ten minutes, maximal PSer externalization was not observed until sixty minutes following treatment. Investigation of IMM disruption demonstrated that, unlike MOMP, IMM disruption temporally coincided with PSer externalization. Dual agonist-stimulation resulted in rapid IMM disruption (Figure 1D) consistent with the established importance of mitochondrial permeability transition (mPTP) formation in the regulation of agonist-initiated PSer externalization. Notably, similar to ABT-737 treated platelets IMM disruption and PSer externalization were temporally correlated. Interestingly, loss of cytochrome c was not observed in agonist-stimulated platelets over the examined time frame. This is consistent with the known minimal role of Bcl-2 proteins in agonist-mediated PSer externalization.\textsuperscript{6}

Mitochondrial mechanisms regulating PSer externalization in apoptotic platelets

Mitochondrial events were examined in Bax/Bak double knockout (Bax/Bak dKO; apoptosis resistant) platelets and caspase inhibitor (10 µM Q-VD-Oph) pre-treated platelets following ABT-737 treatment. PSer externalization, IMM disruption, and cytochrome c release were
all completely inhibited in Bax/Bak dKO platelets consistent with ABT-737’s mechanism of action and dependence on Bax/Bak-mediated MOMP (Figure 2A, C). Caspase activity mediates events downstream of cytochrome c-initiated apoptosome formation. Accordingly, MOMP formation was unaltered by caspase inhibition (Figure 2B, C). As has been previously shown, caspase inhibition markedly abrogated ABT-737 initiated PsSer externalization in platelets. IMM disruption was similarly blunted in caspase-inhibited platelets. Notably, here, as in uninhibited apoptotic platelets and agonist-stimulated platelets, the mitochondrial event of IMM disruption occurred coincident with PsSer externalization.

Visualization of mitochondrial disruption in PsSer externalizing platelets

IMM disruption results in mitochondrial swelling due to influx of water into the mitochondrial matrix through the formerly impermeable IMM. In contrast, the presence of an intact IMM limits swelling in mitochondria that have recently undergone MOMP. MitoTracker stained mitochondria were visualized in platelets. Unlike TMRM, MitoTracker undergoes covalent binding to the thiol groups of mitochondrial proteins and is retained after fixation or within depolarized mitochondria. When stimulated by agonists that cause minimal PsSer externalization, no change in mitochondrial area per platelet was noted by confocal microscopy. Small punctate organelles were stained in unstimulated and thrombin-stimulated platelets (Figure 3A, B, F). Similarly, the mitochondrial depolarizing agent CCCP (10 µM) had no effect on mitochondrial area consistent with the presence of structurally intact mitochondria (Figure 3D, F). To allow simultaneous examination of annexin V negative and positive populations, threshold conditions were used to stimulate high-level PsSer externalization using the agonists thrombin and convulxin and the apoptotic stimulus ABT-737. Significant mitochondrial swelling was seen in platelets with high levels of PsSer externalization as measured by annexin V binding (Figure 3 C, E, F). In annexin V negative platelets no increase in mitochondrial area was noted. Together these results are consistent with those obtained using calcein-cobalt demonstrating the close correlation of IMM disruption, as assessed here by mitochondrial swelling, with high-level PsSer externalization in both agonist-stimulated and apoptotic platelets.

Mechanisms regulating integrin-dependent PsSer externalization

Although loss of mitochondrial integrity, as demonstrated here (Figure 1) and previously, is strongly correlated with PsSer externalization in both apoptotic and agonist-stimulated (necrotic) platelets, integrin-dependent PsSer externalization has not been correlated with changes in mitochondrial integrity. First, we validated integrin-dependent PsSer exposure in murine platelets using similar platelet concentrations and similarly high levels of stimulation with thrombin. As has been demonstrated two distinct PsSer externalizing subpopulations could be distinguished by flow cytometry; 1) PsSer externalizing platelets with high intracellular calcium (loss of FuraRed staining ((i) in Figure 4A)) or low intracellular calcium (maintenance of FuraRed staining ((ii) in Figure 4A)); 2) PsSer externalizing platelets with loss of mitochondrial transmembrane potential (ΔΨm) (low TMRM staining ((i) in Figure 4B)) or maintenance of mitochondrial transmembrane potential (ΔΨm) (high TMRM staining ((ii) in Figure 4B)); and 3) PsSer externalizing platelets with low (i) or high (ii) JON/A staining, consistent with the absence or presence of activated integrin αIIbβ3, as recognized by the activation-specific antibody JON/A, respectively (Figure 4C). Of note, the
characteristics of platelet subpopulation \( i \), such as high cytosolic calcium level and loss of mitochondrial integrity, correspond closely with the classically described agonist-initiated PSer externalizing subpopulation.\(^{21}\) Population \( ii \) corresponds with the integrin-dependent PSer externalizing platelet subpopulation, of which the described characteristics include a normal cytosolic calcium level and intact mitochondria.\(^{16}\)

To investigate the role of mitochondrially-regulated cell death mechanisms in regulating PSer externalization in these two distinct subpopulations, platelets were obtained from Bax/Bak dKO mice (apoptosis-resistant mice due to impaired MOMP formation)\(^2\) and CypD KO mice, which have markedly abrogated mPTP formation and PSer externalization.\(^{10}\) As apoptosis in platelets occurs independent of large changes in calcium flux,\(^{22, 23}\) it was initially hypothesized that formation of the novel cytometric subpopulation (population \( ii \)) would be driven by Bax/Bak and that formation of the high calcium platelets with loss of \( \Delta \Psi_m \) (population \( i \)) would be driven by CypD. However, deletion of Bax/Bak had no effect on the formation of the integrin-mediated PSer externalizing cytometric subpopulation (population \( ii \)) (Figure 4D–F, population \( ii \), gray bar). Rather, CypD deletion prevented formation of population \( ii \) (Figure 4D–F, population \( ii \), black bar), even though loss of TMRM staining, a well-established marker of mPTP formation, was not observed in population \( ii \) of wild type mice (Figure 4B, \( ii \)). This surprising finding initially suggested a potentially novel role for CypD distinct from its well-characterized role in regulating mPTP formation. As expected, the formation of platelets with the characteristics of population \( i \), the classic PSer externalizing platelet, was dependent on CypD (Figure 4D–F, population \( i \), black bar).

Classical PSer externalizing platelets have distinct forward (FSC) and side scatter characteristics (SSC). Examination of the integrin-dependent cytometric subpopulation demonstrated that they had FSC and SSC characteristics (Figure 5A, population \( ii \), black) distinct from classical PSer externalizing platelets (Figure 5A, population \( i \), gray) and also had slightly larger FSC and SSC characteristics than the subpopulation of ann V negative activated platelets (Figure 5A, red). This pattern, together with the previously demonstrated integrin-dependence of the novel cytometric subpopulation and its dependence on CypD, suggested an alternative explanation for the formation of population \( ii \). To investigate the possibility that the novel subpopulation might be a consequence of small aggregates (>1 platelet) consisting of platelets with different characteristics, Cell Tracker Violet (CTV) or Cell Tracker Green (CTG)-labeled platelets were mixed and stimulated with 100 nM thrombin (Figure 5B). A substantial fraction of the platelet events at higher platelet concentrations were positive for both stains consistent with the formation of small platelet aggregates as the platelet concentration increased. Also consistent with this hypothesis, abrogation of aggregation utilizing an integrin \( \alpha_{IIb}\beta_3 \) antagonist (eptifibatide -2 µg/mL) approximately halved the formation of population \( ii \) as had been previously demonstrated by Topalov et al (Supplemental figure I).\(^{16}\)

**Image cytometry of activated platelets in suspension**

Image cytometry was utilized to visualize the PSer externalizing platelet subpopulations. Platelets separately labeled with Cell Tracker Violet (CTV) and Cell Tracker Green (CTG)
were mixed, stimulated with 100 nM thrombin, and assessed with the platelet activation markers JON/A and ann V. Figure 5C demonstrates the distribution of CTV+CTG+ events in thrombin-activated platelets using the conditions described by Topalov et al. Few CTV+CTG+ events (Figure 5C, green-colored population) could be observed among JON/A low and ann V+ events (classical PSer+ platelet, population i) consistent with their decreased adhesiveness and their decreased recognition by the activation-dependent integrin αIIbβ3 antibody JON/A. In contrast CTV+CTG+ events comprised a large proportion of JON/A high and ann V+ events (integrin-dependent PSer+ platelet, population ii) consistent with a large majority of these events being the result of the formation of small aggregates. A small proportion of CTV+CTG+ events also occurred among JON/A high, ann V− events in a distribution consistent with a small aggregate. Visual examination of sequential events in each cytometric subpopulation directly confirmed the implications of the mixing studies (Figure 5D, E). Nearly all events consistent with the characteristics of the novel integrin-dependent subpopulation consisted of small platelet aggregates with one PSer+ platelet and one or more PSer− platelet(s). >95% of JON/A high and ann V+ events could be clearly distinguished as a small platelet aggregate (>1 platelet). In contrast, in less than 5% of JON/A low and Ann V+ events could an aggregate be distinguished. A similar pattern of aggregate formation was observed when TMRM and ann V were utilized (Figure 5E, bottom panel). This same analysis using image cytometry of human platelets using PAC-1 and ann V revealed almost identical results (Supplemental Figure II).

**Procoagulant platelets participate in aggregates in vitro and in vivo**

Transmission electron microscopy studies were utilized to investigate whether similar interactions between activated and procoagulant platelets as observed in population ii might occur in vivo. Thrombin and convulxin stimulated platelets in suspension were examined by transmission electron microscopy one minute and seven minutes after stimulation (Figure 6A). One minute following stimulation, a distinct procoagulant platelet subpopulation could not be identified, a result that is consistent with delayed formation of the procoagulant platelet subpopulation relative to other earlier platelet activation events, such as granule release or integrin activation. Seven minutes after initial stimulation, two subpopulations of platelets could be clearly identified based on their distinct appearances and electron density characteristics. These consisted of a population of platelets with similar characteristics to that observed one minute following stimulation and an electron-lucent balloon-like population. The electron-lucent balloon-like platelets could be identified as the procoagulant platelet subpopulation based on their greatly decreased formation in platelets obtained from CypD KO mice (Figure 6A, bottom panel), and their limited formation among platelets stimulated with thrombin alone (data not shown). Here among WT platelets, interaction of procoagulant and activated platelets was occasionally seen consistent with the results obtained using image cytometry (Figure 6B). Similarly, examination of the periphery of murine hemostatic plugs obtained five minutes following injury of a mesenteric venule demonstrated interaction of interrupted populations of peripheral electron-lucent platelets with a central core of more electron-dense platelets (Figure 6C). Together, these results indicate that activated platelets interact with procoagulant platelets and indicate a potential mechanism for the retention of procoagulant platelets within the forming hemostatic plug.
DISCUSSION

In this study the roles of specific mitochondrial events in PSer exposure were carefully dissected. Intriguingly, high-level PSer externalization induced both by platelet agonist(s) and by apoptotic stimuli was mechanistically and kinetically associated with IMM disruption, evidenced by alteration in the permeability of the normally IMM-impermeant ion cobalt and by the presence of mitochondrial swelling, an indicator of free water entry into the highly osmotic mitochondrial matrix. In contrast, MOMP could be dissociated from PSer externalization in both apoptotic and agonist-stimulated platelets. No correlation of MOMP with PSer externalization was noted in agonist-stimulated platelets, and in apoptotic platelets MOMP was not temporally correlated with PSer exposure, but instead preceded PSer externalization. Furthermore, caspase inhibition, while markedly abrogating PSer externalization, had minimal effect on MOMP.

The studies here point to a link between physical disruption of the IMM and PSer exposure. IMM disruption, unless corrected, invariably results in a loss of $\Delta \Psi_m$ due to disruption of the physical barrier to ion movement. That the effect of IMM disruption is not mediated through its effects on $\Delta \Psi_m$ is evidenced by studies here and by others. In the absence of evidence of physical disruption of the IMM, as when induced by using a membrane uncoupler, loss of $\Delta \Psi_m$ has no association with immediate PSer externalization. The mechanism by which IMM disruption might cause scramblase activation remains to be identified. Calcium-dependent and caspase-dependent components of scramblase activity have recently been elucidated. TMEM16F is a calcium-dependent membrane protein required for normal PSer externalization in response to agonist-stimulation. Increased calcium elevation has been suggested as the mechanism regulating scramblase activation and TMEM16F function. Whether cytoplasmic calcium elevation is the sole regulator of agonist-induced PSer externalization has been questioned. Detailed studies examining loss of $\Delta \Psi_m$ and calcium transients in agonist-stimulated platelets have demonstrated close association of mPTP formation and PSer externalization. In marked contrast, the level of cytoplasmic calcium elevation could be readily dissociated from PSer externalization. Together, these data point to an essential role for a mPTP-regulated mediator as an upstream signal regulating scramblase activation, most likely in coordination with an essential regulatory function mediated by cytosolic calcium elevation.

In apoptotic cells Xkr8 has been identified as a novel caspase-dependent component of the scramblase machinery. Caspase-dependent cleavage of Xkr8 potentiates PSer externalization in apoptotic cells. The data presented here suggest that coordinated caspase-dependent permeabilization of the IMM, distinct from CypD-mediated IMM permeabilization, might function to regulate PSer exposure in the apoptotic context. Such a potential mediator is suggested by the close link between IMM disruption and PSer externalization in both WT and caspase-inhibited platelets. Although the nature of such a potential mediator is not identified here, the concept that mitochondrial proteins or factors can be co-opted to act as a signal mediating apoptotic or necrotic events has clear precedent.
In addition to its role in the regulation of classic agonist-stimulated procoagulant platelet formation, cyclophilin D is demonstrated here to play an important role in mediating integrin-regulated procoagulant platelet formation. Distinct from classical agonist-initiated procoagulant platelet formation, this pathway of PSer externalization had been demonstrated using cytometric assays to be associated with neither elevated levels of cytoplasmic calcium nor with loss of ΔΨm. Other unique characteristics of this PSer externalizing subpopulation included maintenance of integrin αIIbβ3 in an activated state and its dependence on a high platelet concentration and integrin function. It is demonstrated here that it is highly probable that the flow cytometry event identified by Topalov et al., in fact, consists of an aggregate of an activated, aggregatory platelet and a classical procoagulant platelet. Consistent with their results, activation of washed platelets resulted in the formation of two distinct PSer positive events, one of which had the characteristics of agonist-induced procoagulant platelets and a second with the characteristics described by Topalov et al. Mixing studies and image cytometry clearly demonstrate that the unique characteristics of this cytometric subpopulation could be accounted for by the formation of an aggregate consisting of an activated PSer negative platelet and a procoagulant PSer positive platelet (Table 1). The dependence of the unique subpopulation on integrin αIIbβ3 activation and its inhibition by eptifibatide is consistent with a role for the activated integrin in formation of these cytometric aggregates. Although the site of interaction between these two platelet types is not clearly demonstrated in this study, one can readily envision a mechanism in which activated integrin αIIbβ3 on the PSer negative platelet interacts with the fibrin(ogen) coat, or cap, on the PSer positive platelet generating the unique cytometric population. Multiple mechanisms may limit aggregate size in this in vitro setting, including the absence of stirring and the utilization of washed platelets, which limits extracellular fibrin(ogen) to that released by the activated platelet. In addition, the incorporation of the procoagulant platelet may function to limit aggregate propagation. Notably, in cytometric assays, few if any aggregates were observed consisting of two PSer positive events, a finding consistent with the decreased aggregatory and adhesive ability of the procoagulant platelet.

The decreased adhesive qualities of the procoagulant platelet are well described. Procoagulant platelet formation is associated with a decrease in integrin αIIbβ3 function and decreased interaction of multiple platelet adhesive receptors with the platelet cytoskeleton. On the other hand, retention of fibrin(ogen) is increased on the procoagulant platelet surface. The association of procoagulant platelets with small aggregates of activated platelets demonstrated here suggests a model in which procoagulant platelets can participate in, but not propagate aggregate formation. Procoagulant platelet participation in the aggregate can be mediated through the interaction of activated platelets with the fibrin(ogen) cap on the procoagulant platelet surface. However, the decreased integrin function of the procoagulant platelet would limit subsequent propagation of the aggregate. In vivo similar mechanisms could function within a forming hemostatic plug to mediate retention of a limited layer of procoagulant platelets observed here and in previous electron microscopy studies of the hemostatic plug.

Understanding of events occurring within individual platelets following agonist stimulation has been greatly enhanced by the use of cytometric techniques. By using flow cytometry, together with novel labeling and imaging techniques, IMM disruption is demonstrated to be
a distinct mitochondrial event that is temporally associated with PSer exposure in both agonist-stimulated (mPTP-mediated) and apoptotic (apoptosome/caspase-mediated) platelets. An interesting and outstanding question is whether a common mediator released from the mitochondrial matrix contributes to scramblase activation and PSer externalization in the platelet. Continued delineation of the intracellular mechanisms regulating PSer externalization is expected to provide novel insights allowing the therapeutic manipulation of procoagulant platelet formation in hemostatic and thrombotic conditions.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**ABBREVIATIONS**

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ΔΨ_m</td>
<td>mitochondrial membrane potential</td>
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<tr>
<td>ann V</td>
<td>annexin V</td>
</tr>
<tr>
<td>CVX</td>
<td>convulxin</td>
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<tr>
<td>CypD −/−</td>
<td>cyclophilin D knock out</td>
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<tr>
<td>dKO</td>
<td>double knock out</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>IMM</td>
<td>inner mitochondrial membrane</td>
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<td>MOMP</td>
<td>mitochondrial outer membrane permeabilization</td>
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<td>mPTP</td>
<td>mitochondrial permeability transition pore</td>
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<tr>
<td>PSer</td>
<td>phosphatidylserine</td>
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<td>THR</td>
<td>thrombin</td>
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<tr>
<td>TMRM</td>
<td>tetramethyl rhodamine methylester</td>
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<tr>
<td>WT, +/+</td>
<td>wild type</td>
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References


18. Campos CB, Paim BA, Cosso RG, Castilho RF, Rottenberg H, Vercesi AE. Method for monitoring of mitochondrial cytochrome c release during cell death: Immunodetection of cytochrome c by


HIGHLIGHTS

- Platelet phosphatidylserine externalization is closely linked with disruption of the inner mitochondrial membrane both in platelets treated with apoptotic stimuli and with physiological agonist(s). This suggests a common mitochondrial mechanism released by mitochondrial disruption may regulate phosphatidylserine externalization in both apoptotic and agonist-stimulated platelets.

- Like classical procoagulant platelet formation, integrin-mediated procoagulant platelet formation as described by Topalov et al is mediated by CypD-mediated formation of the mitochondrial permeability transition pore.

- Despite their decreased adhesiveness, procoagulant platelets participate in limited aggregate formation both in vitro, when platelets are stimulated at near physiologic platelet concentrations, and in vivo, within the forming hemostatic plug.
Figure 1. Assessment of inner and outer mitochondrial membrane integrity in apoptotic and agonist-stimulated platelets

A) Cytochrome c release and MOMP in ABT-737 treated platelets. Murine platelets were treated with ABT-737 (1 µM) as indicated and cytochrome c retention assessed in digitonin-permeabilized platelets. B) IMM disruption in ABT-737 treated murine platelets. A calcein-cobalt assay was used to assess IMM integrity in ABT-737 treated (1 µM) platelets at the indicated time points. C, D) Kinetic analysis of ABT-737-treated (C) and thrombin and convulxin stimulated (D) platelets. MOMP (dashed line - % with loss of cytochrome c),
IMM disruption (gray line - % with loss of calcein-staining), and PSer externalization (solid line - % ann V⁺) were assessed. *p<0.01 versus unstim (time 0).
Figure 2. Mechanisms regulating mitochondrial membrane integrity and PSer externalization in apoptotic platelets

A, B) Kinetic analysis of Bax/Bak dKO (A) and caspase-inhibited (10 µM Q-VD-Oph 10 minutes pre-treated) (B) platelets following treatment with ABT-737 (1 µM). MOMP (dashed line - % with loss of cytochrome c, IMM disruption (gray line - % with loss of calcein-staining), and PSer externalization (solid line - % ann V^+) were assessed. C) Quantification of IMM disruption, MOMP formation, and PSer externalization at 90 minutes with ABT-737 treatment in WT, Q-VD-Oph treated, or Bax/Bak double knock-out platelets. *p<0.01 versus unstim (time 0) (A, B) or wild type (C).
Figure 3. Mitochondrial swelling in apoptotic and agonist-stimulated platelets

A–E) Anti-CD41 (blue), MitoTracker (purple), and annexin V (green) were used to stain human platelets treated/stimulated as indicated, which were visualized using confocal microscopy. Scale bar indicates 2 um. 

F) Relative mitochondrial area/platelet was assessed in annexin V+ and annexin V− platelets (n ≥3). *p<0.05 versus unstim.
Figure 4. Mechanisms regulating procoagulant platelet subpopulation formation in agonist-stimulated platelets

Murine platelets (2×10^8/mL) were stimulated with thrombin (100 nM). PSer externalization was evaluated using annexin V. Cytoplasmic calcium was evaluated using Fura Red (A, D); ΔΨm was assessed using TMRM (B, E); and integrin-activation state was interrogated using JON/A (C, F). Arrows are drawn to identify cytometric subpopulation i (classical procoagulant platelet) and cytometric subpopulation ii (integrin-dependent procoagulant platelet). (A–C) Dot plot of thrombin-stimulated platelets investigated with the indicated fluorescent markers. (D–F) Quantitation of the % of type i and type ii procoagulant platelets.
using the corresponding fluorescent marker in WT, Bax/Bak dKO, and CypD KO platelets. (n=4). *p<0.01 versus WT.
Figure 5. Cytometric characterization of the integrin-dependent procoagulant platelet subpopulation

A) FSC and SSC characteristics of classical (i) and integrin-dependent (ii) procoagulant platelet subpopulations among thrombin-stimulated platelets as defined using JON/A and annexin V. Pop. i (ann V+ JON/A low) (gray), pop. ii (ann V+ JON/A high) (black), other platelets (red).

B) Platelets were labeled with Cell tracker violet (CTV) or Cell tracker green (CTG) separately. Each stained platelet was mixed and stimulated with Thrombin to quantify platelet aggregates (CTV+/CTG+ events indicating platelet>1) *p<0.01 versus 0.1 x 10^8 platelets.

C) Platelets were labeled separately with CTV or CTG, mixed, and stimulated with TMRM to quantify platelet aggregates. *p<0.001 versus 0.1 x 10^8 platelets.
thrombin. CTV, CTG, ann V and JON/A were utilized for analysis. The CTV\(^+\)/CTG\(^+\) platelet subpopulation was indicated in green. **D**) Quantitation of visual analysis of stimulated murine platelets using image cytometry of individual cytometric events. 1000 images were analyzed per mouse. \(n=3\). **E**) Representative events obtained using image cytometry.
Figure 6. Transmission electron microscopy of murine procoagulant platelets
A) Platelets obtained from WT or CypD KO mice were stimulated with thrombin and convulxin and analyzed by TEM at the indicated time points. Scale bar indicates 2 µm. B, C) Representative micrographs of an (B) electron-lucent procoagulant platelet (arrows in B and C) closely associated with a more electron-dense platelet, and of a (C) cross-section of the periphery of a hemostatic plug obtained following puncture injury of the mouse mesentery.
# Table 1

Properties of cytometric subpopulations

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<tr>
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<th>Classical procoagulant platelet (subpopulation i)</th>
<th>Integrin-dependent procoagulant platelet (subpopulation ii)</th>
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<tr>
<td>Annexin V</td>
<td>−</td>
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<td>+</td>
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<td>Fura-Red high (Low calcium)</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Pac-1 or JON/A</td>
<td>+</td>
<td>−</td>
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<td>TMRM high</td>
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