Modulation of Protein C Activation by Histones, Platelet Factor 4, and Heparinoids

New Insights Into Activated Protein C Formation

M. Anna Kowalska, Guohua Zhao, Li Zhai, George David III, Stephen Marcus, Sriram Krishnaswamy, Mortimer Poncz

Objective—Histones are detrimental in late sepsis. Both activated protein C (aPC) and heparin can reverse their effect. Here, we investigated whether histones can modulate aPC generation in a manner similar to another positively charged molecule, platelet factor 4, and how heparinoids (unfractionated heparin or oxygen-desulfated unfractionated heparin with marked decrease anticoagulant activity) may modulate this effect.

Approach and Results—We measured in vitro and in vivo effects of histones, platelet factor 4, and heparinoids on aPC formation, activated partial thromboplastin time, and murine survival. In vitro, histones and platelet factor 4 both affect thrombin/thrombomodulin aPC generation following a bell-shaped curve, with a peak of >5-fold enhancement. Heparinoids shift these curves rightward. Murine aPC generation studies after infusions of histones, platelet factor 4, and heparinoids supported the in vitro data. Importantly, although unfractionated heparin and 2-O, 3-O desulfated heparin both reversed the lethality of high-dose histone infusions, only mice treated with 2-O, 3-O desulfated heparin demonstrated corrected activated partial thromboplastin times and had significant levels of aPC.

Conclusions—Our data provide a new contextual model of how histones affect aPC generation, and how heparinoid therapy may be beneficial in sepsis. These studies provide new insights into the complex interactions controlling aPC formation and suggest a novel therapeutic interventional strategy. (Arterioscler Thromb Vasc Biol. 2014;34:120-126.)

Key Words: activated protein C resistance ▪ heparin ▪ histones ▪ platelet factor 4

Sepsis is a significant clinical challenge associated with high morbidity and mortality.1 Key features include the development of both a coagulopathy and loss of vascular integrity. It has been shown that histones, released from leukocytes by endotoxin, are toxic and that activated protein C (aPC) reduces this cytotoxic effect.2 Therapeutic interventions to increase the level of aPC, but with limited success.3–5 Attempts to modulate the level of aPC, by IIa-TM that contains CS sidechains (TMCS) is accelerated, both in vitro and in vivo, by the platelet-derived chemokine CXCL4 (platelet factor 4 [PF4]).24,25 Binding studies using surface plasmon resonance have confirmed a strong interaction of PF4 with both the glycosaminoglycan moiety of TM and the Gla domain of PC.22 We have investigated previously the physiological implications of these findings. After IIa infusion, aPC generation correlated with platelet PF4 levels in different PF4 transgenic mice lines. Moreover, platelet PF4 was protective against lipopolysaccharide-induced endotoxemia.23 More recent data from our group show that the effect of PF4 on aPC generation by IIa-TMCS actually follows a bell-shaped curve,24 with a specific optimal PF4:TMCS molar ratio.25 High doses of PF4 that suppress aPC generation are well within the aPC demonstrated enhanced susceptibility to infection.18 The implications of this observation on enhancing aPC generation in sepsis need further elucidation.

TM is an anionic molecule because of posttranslational O-linked glycanation by chondroitin sulfate (CS).19 Initial studies suggested that generation of aPC by IIa complexed to TM that contains CS sidechains (TMCS) is accelerated, both in vitro and in vivo, by the platelet-derived chemokine CXCL4 (platelet factor 4 [PF4]).20,21

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBHA.113.302236/-/DC1. Correspondence to M. Anna Kowalska, Children’s Hospital of Philadelphia, 3615 Civic Center Blvd, Philadelphia, PA 19104. E-mail kowalska@email.chop.edu

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBHA.113.302236

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Results

Histones Modulate PC Activation

Previous studies have shown that positively charged molecules, such as PF4 and PRT, modulate aPC generation by IIa bound to TMCS following a bell-shaped response.24 Given the recently described deleterious role of histones in sepsis and aPC generation,2 we now tested whether histones, as another small positively charged protein, would have a similar effect on aPC generation from TMCS. In vitro, the rate of aPC formation increased with increasing concentrations of mixed histones, peaking at 10 to 20 µg/mL (Figure 1A), in the presence of either low (0.5 nmol/L) or high (40 nmol/L) concentrations of soluble (s)TMCS. Peak rates of aPC formation were 5-fold greater than that seen in the absence of histones. In contrast, enhanced aPC formation was not seen with TMCS (Figure 1B). Instead, histones had only a minor inhibitory effect, similar to that noted by Ammollo et al.30 Experiments using human recombinant individual histones H1 and H4 gave similar results (Figure 1 in the online-only Data Supplement).

PF4 and Heparin Influence Histone-Mediated aPC Generation

In sepsis, platelets are activated and release their α-granules content, including PF4.23,29 We therefore tested the possibility that PF4 and histones act in concert to regulate PC activation. We have shown24 that PF4 affects aPC formation following a bell-shaped curve with an optimal concentration of ~25 µg/mL in vitro, a concentration that can be achieved readily in vivo in the vicinity of a thrombus.26 We show that in the absence of histones (Figure 2A), inclusion of PF4 at a suboptimal concentration (3 µg/mL) increased the rate of aPC formation ≈3-fold and at a near-peak PF4 concentration of 25 µg/mL by ≈7-fold (Figure 2A). Coaddition of variable amounts of histones with different fixed concentrations of PF4 yielded peak aPC generation at lower histone concentrations as the PF4 concentration increased (Figure 2A). These findings are consistent with the idea that histones and PF4 additively

Materials and Methods

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

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>aPC</td>
<td>activated protein C</td>
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<tr>
<td>aPTT</td>
<td>activated partial thromboplastin time</td>
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<tr>
<td>CS</td>
<td>chondroitin sulfate</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>EPCR</td>
<td>endothelial protein C receptor</td>
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<tr>
<td>ODSH</td>
<td>2-O, 3-O desulfated heparin</td>
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<tr>
<td>PF4</td>
<td>platelet factor 4 (CXCL4)</td>
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<tr>
<td>PRT</td>
<td>protamine sulfate</td>
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<tr>
<td>TM</td>
<td>thrombomodulin</td>
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<tr>
<td>UFH</td>
<td>unfractionated heparin</td>
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range that can be achieved locally at sites of platelet activation.26 Moreover, other small positive proteins, such as protamine sulfate (PRT), can modulate aPC generation with a similar bell-shaped profile.28 We have also shown that unfractionated heparin (UFH), which binds PF4 with greater affinity than does CS, effectively reduces available PF4, shifting the aPC generation bell-shaped curve to the right. Thus, added UFH enhances aPC formation at high PF4 concentrations while inhibiting at low PF4 concentrations.28 A similar effect of UFH was seen for the modulation of PRT on aPC.

Chromatin has been shown to be released from activated neutrophils by a process called NETosis and can become a source for free histones27 that, in turn, can worsen the outcome in sepsis in humans.28 In murine models, infused histones alone can simulate many of the outcomes seen in endotoxemic lethal shock.26 Infused aPC2 or UFH29 reverse histone-induced lethality in mice. Relevant to our studies, histones were shown to inhibit aPC generation in vitro in the presence of TM and IIa.30 However, because histones are positively charged small proteins, such as PF4 and PRT, we hypothesized that they would modulate aPC generation by IIa complexed to TMCS along a bell-shaped curve, enhancing aPC generation at low concentrations and only inhibiting aPC generation at high concentrations. We reasoned that in severe sepsis, large amounts of both histones and PF4 are released and inhibit aPC generation. The amount of UFH needed to reverse these effects and again enhance aPC generation off of TMCS may markedly exceed typical heparinization doses, resulting in a marked anticoagulant effect. However, partially desulfated 2-O, 3-O desulfated heparin (ODSH) has been noted to bind well to PF4,31 yet it has ~60-fold less antithrombin activity.32 We reasoned that ODSH at an optimal level may bind and neutralize much of the free histones and PF4, allowing maximum aPC generation without causing undue anticoagulant effects. We now confirm our hypotheses that histones affect aPC generation from TMCS along a bell-shaped curve, that this effect is additive with the effect of PF4, and that ODSH can improve aPC generation in vivo without prolonging the activated partial thromboplastin time (aPTT) in a murine histone infusion model. The potential clinical implications of these findings in sepsis are discussed.

Materials and Methods

Materials and Methods are available in the online-only Supplement.
modulate the rate of aPC formation. The response to histones H1 and H4 was also similarly affected by PF4 (Figure I in the online-only Data Supplement). In the presence of UFH (5 µg/mL), just as seen with histones (Figure 2B) or PF4 alone,21 peak aPC generation modulated by histones plus PF4 was shifted to the right, requiring higher levels of the positively-charged proteins (Figure 2C).

We also studied the effect of various concentrations of ODSH on the rate of aPC formation in the presence of various concentrations of histones and in the absence or presence of PF4 (Figure 3A and 3B, respectively). Increased concentrations of ODSH shifted optimal aPC formation to the right, requiring increased levels of histones (Figure 3A) to reach the peak of aPC generation. The same was observed in the presence of UFH (Figure IIA in the online-only Data Supplement). Again, PF4 and histones were additive so that at each concentration of ODSH or UFH tested, less histones were needed to reach optimal aPC generation in the presence of PF4 than without it (compare Figure 3B to 3A and compare Figure IIB to IIA in the online-only Data Supplement).

In Vivo Effects of Histones and PF4 on aPC Generation in Mice
To establish the physiological correlates for our observations in vivo, we examined the ability of intravenously injected histones and PF4 to modulate aPC formation in mice. Previously, we had shown that infusion of IIa (80 U/kg) into wild-type (WT) or PF4KO mice increases aPC formation in a manner that could be further augmented by the intravenous infusion of PF4 (1–5 mg/kg).23,24 and the same was observed for lower concentrations of IIa (8–40 U/kg; Kowalska et al, unpublished data 2010). Our present studies were pursued using PF4KO mice to eliminate the confounding effects of histone-dependent platelet activation that could lead to variable PF4 release from platelets29 (Figure III in the online-only Data Supplement). Injection of histones alone at concentrations of 1 to 20 mg/kg did not produce a significant increase in in vivo aPC generation (data not shown). Histones coinfected with low-dose IIa (8 U/kg equivalent to ≈1 pmol of IIa/20 g mouse) increased aPC detected in plasma in a histone concentration-dependent manner (Figure 4, open circles). Concurrent injection of PF4...
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Histones and PF4 were intravenously injected into the jugular vein, and aPC was measured in blood obtained from the vena cava. n=4 to 5 per data point. Mean±1 SD are shown. *P<0.01 as compared with histone alone.

Histones, PF4, and Heparinoids and aPC Formation

Histones stimulate activated protein C (aPC) generation in vivo in mice after thrombin (IIa) injection. Mouse aPC measured in plasma 10 minutes after coinjection of IIa and increasing concentrations of histones in the absence (open circles) or presence of platelet factor 4 (PF4; black circles) in PF4KO mice. Histones and PF4 were intravenously injected into the jugular vein, and aPC was measured in blood obtained from the vena cava. n=4 to 5 per data point. Mean±1 SD are shown. *P<0.01 as compared with histone alone. By Kowalska et al.
monitoring and therapies. Septic patients have activation of blood coagulation, consumption of anticoagulant factors, and increased platelet activation. In late-stage sepsis, EC dysfunction leads to decreased TM and EPCR levels on the surface of ECs and impaired aPC generation. Recombinant human sTM (ART-123) has been used for the treatment of disseminated intravascular coagulation associated with hematologic malignancy or infection and with sepsis. TMCS and TM−CS are expressed to different extents in various vascular beds and in different species. It has been shown that the TMCS form has a higher affinity for IIa than TM−CS and is more effective in inhibiting clotting. Mouse studies have also shown that the efficacy of aPC is linked to the ability of aPC to activate cytoprotective effects by signaling through EPCR and protease-activated receptor-1. Thus, it is possible that therapies that enhance local levels of aPC generation would be particularly efficacious in sepsis, but only if the capacity to generate aPC is intact, suggesting that early institution of monitoring and adjusting heparinoid infusions would be necessary.

Our previous work has shown that aPC generation from TMCS can be upregulated several fold by several different positively charged small proteins, specifically by PF4 released from activated platelets, and iatrogenically by infused PF4 or PRT. This modulation of the rate of aPC formation follows a bell-shaped curve and can be neutralized by large negatively charged molecules, such as heparin. We now extend our data in a critical way by showing that histones regulate aPC formation in a manner similar to PF4. Like other highly positive small proteins, histones interact with the glycosaminoglycan sidechain of TM and enhance IIa/TM aPC generation activity multiple fold. This positive charge effect also follows a bell-shaped curve and likely peaks at a 1:1 molar ratio of histones and TMCS. Thus, our observations provide a more nuanced interpretation of the effect of histones on aPC generation in sepsis with a positive arm and a detrimental arm. Because there is growing evidence of the role of histones in inflammation and coagulopathy and its importance in sepsis-related life-threatening conditions in small animal models of endotoxemia, baboons, and sepsis patients, we think that our observation may have relevance as sepsis evolves in a patient, both for monitoring the changing pathobiology in patients and for offering potential new therapeutic strategies.

In Figure 6, we present a model of the interplay between released PF4 and histones and infused heparinoids in affecting the rate of aPC formation at various stages of sepsis. In mild sepsis, histones levels are low or absent (Figure 6, left) and PF4 may be released from platelets activated by lipopolysaccharide or by the low amount of histones. Depending on the platelet count and PF4 content, both of which can vary significantly, a variable amount of increased aPC generation would occur on intact EC TMCS. Infused UFH or ODSH in this setting is likely to diminish aPC generation, because the patient would likely be on the left arm of the bell-shaped curve of the effect of positive-charged small proteins on aPC generation. If the sepsis progresses, then release of neutrophil-extruded traps (NETs) will form with increased histone availability and more platelet activation and degranulation. Higher levels of PF4 and histones may lead to near-optimal aPC formation by IIa/TMCS complex (Figure 6, middle). In this case, UFH or ODSH doses may either increase or decrease aPC generation depending on the aggregate dose of positively charged small proteins present. In severe sepsis, large amounts of histones are circulating, as well as free PF4, and the result is suppressed aPC generation (Figure 6, right). In this setting, added typical clinical doses of UFH for its anticoagulant properties may have a negligible effect on aPC generation; however, ODSH with its little anticoagulant effects may be administered at higher doses and neutralize this high level of PF4 plus histones, and may lead to detectable aPC generation. At this optimal level of heparinoid infused, the effective concentration of positively charged proteins is lowered to the point of enhanced aPC generation by IIa/TMCS complex without significant aPTT prolongation.

These studies and their interpretation come with significant caveats regarding their applicability to patient care. The level of TMCS on individual human vascular beds has

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**Figure 6.** Proposed model of histones affecting activated protein C (aPC) generation in sepsis. Dashed-line arrows refer to a variable degree of expected response. The size and direction of the vertical arrows refer to the relative degree and direct of the expected effect on aPC generation. The size of the circle refers to the relative level of released platelet factor 4 (PF4) or histones. Left. In mild sepsis, low levels of positively charged histones and released PF4 increase aPC formation by thrombin/thrombomodulin that contains chondroitin sulfate sidechains complexes (upward gray arrow). Infused heparinoids decrease aPC generation (downward green arrow). Middle. At moderate sepsis, release of more free histones and PF4 enhances aPC generation and, depending on the level of free histones and PF4, infusion of heparinoids may either further increase or decrease aPC generation. Right. In severe sepsis, released histones and PF4 are excessive and aPC generation is inhibited. High doses of heparinoids would neutralize this double dose of positively charged small proteins and enhance aPC generation.
not been completely resolved. Studies suggest that different EC beds differ in the amount of TM and its proportion of CS sidechain modification. We have shown that PF4 affects aPC generation in the presence of primary human aorta ECs as a source of TM, consistent with the presence of TMcs. Overall levels of functional TM in sepsis are diminished, which we think only further supports the need to define strategies that enhance aPC generation. In addition, the studies presented in this article focus on aPC generation as a surrogate marker of good things occurring in sepsis, but the effects of histones are likely pleiotropic and observed changes in aPC generation and its correlation with good clinical outcome need further study, as emphasized by recent data showing a negative correlation of outcome and aPC levels in 1 model of sepsis. However, we propose that the described effects of histones and PF4 and heparinoids on aPC generation modeled in Figure 6 also apply to other downstream targets of heparinoids, and we anticipate that neutralization of more downstream targets, especially of histones, would be beneficial overall. We also think that the model in Figure 6 along with the data shown in Figures 5 may explain why heparin has been reported to be of variable use in sepsis, and that concurrent measurements of aPTT and aPC levels in sepsis to titer the effects of heparinoids on outcome may improve clinical outcome. Our studies also suggest that ODSSH with its lower anticoagulants effects offers the possibility of maximizing aPC while avoiding bleeding complications and contributes to better outcome in sepsis.

Acknowledgments

We thank Drs C.T. Esmon and G. Furrel at the Oklahoma Medical Research Foundation for supplies to perform mouse aPC ELISA.

Sources of Funding

This work was supported by AHA grants 2251065 (M.A. Kowalska) and National Institutes of Health grants P01HL074124 (S. Krishnaswamy), P01HL40387 (M. Poncz), and P01HL110860 (M. Poncz).

Disclosures

ODSSH was provided by ParinGenix.

References

Severe sepsis remains a major clinical challenge. Histones appear in the plasma of patients with severe sepsis and contribute to poor outcomes. Previous studies suggested that histones could be detrimental, partially by inhibiting activated protein C generation, a product thought to improve survival by enhancing vascular integrity. We show that histones and another protein, platelet factor 4, both affect activated protein C generation from thrombomodulin following a bell-shaped curve. This effect is further modulated by heparinoids, such as the anticoagulant heparin, and oxygen-desulfated heparin with markedly decreased anticoagulant properties. We show that these heparinoids can improve activated protein C generation in vitro. In vivo, oxygen-desulfated heparin can improve activated protein C generation while not causing a bleeding state. Our studies provide a more nuanced appreciation of histones in sepsis and suggest how patients may be better monitored and how oxygen-desulfated heparin can be used as a novel therapeutic intervention.
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Arterioscler Thromb Vasc Biol. 2014;34:120-126; originally published online October 31, 2013; doi: 10.1161/ATVBAHA.113.302236

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/1/120

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Figure Supplement I. Effect of PF4 on aPC generation in the presence of histones. aPC generation was monitored as in Figure 2 but in the presence of various histones. Histones were added in the absence (open symbols) or presence (grey symbols) of PF4 (25 µg/ml). Circles denote addition of mixed histones (used through the paper). Squares represent pure histone H1 (M.W. 20.7 kD) and diamonds histone H4 (M.W. 11.2 kD). N = 3, each done in duplicate. Mean ± 1 SD shown.
Figure Supplement II. UFH affects aPC generation modulated by histones.

Generation of aPC as in Figure 2. Increasing concentrations of histones were added to the assay mix containing control buffer (open symbols) or UFH: 5 µg/ml (light gray) 10 µg/ml (dark gray) or 20 µg/ml (black): (A) Histones alone or (B) Histones in the presence of 25 µg/ml of PF4. N = 3, each done in duplicate. Mean ± 1 SD shown.
Figure Supplement III. Histones modulate aPC generation in vivo in mice (IIa injection).

Increasing concentrations of histones were IV injected into the jugular vein of WT mice in the absence (open diamonds) or presence of 5 mg/ml of PF4 (closed diamonds). IIa (20 U/kg) was injected into opposite jugular vein. Blood was obtained from the vena cava 10 minutes later, and mouse aPC was measured in plasma. N = 3-5 per data point. Mean ± 1 SD are shown. *p<0.003 as compared to no PF4 consistent with prior publication23.

NOTE: The direct comparison between this figure and Figure 5 in the paper cannot be made since WT and PF4KO mice used in both studies were not related.
Figure Supplement IV. Histones and PF4 affect additively aPC generation in vivo (no IIa injection).

Mouse aPC measured in plasma 10 minutes after co-injection of PF4 (5 mg/kg) and histones (50 mg/kg) in PF4KO mice. Histones and PF4 were IV injected into the jugular vein and aPC was measured in blood obtained from the vena cava. N = 5-8 per data point. Mean ± 1 SD are shown. *p<0.01 as compared to histone alone.
Figure Supplement V. Effect of UFH or ODSH on aPTT.

Blood was collected from cut tails 20 minutes after injection of heparinoids into the jugular vein, and aPTT measured in plasma of blood collected from tail. WT mice were injected with control buffer (at 0 mg/kg heparinoid concentrations, open circles), ODSH (gray circles) or UFH (black squares) at the concentrations indicated. N = 5 mice per data point, * = p<0.01 vs. UFH.
Materials and Methods

Reagents
All reagents, including high molecular-weight, porcine UFH, sodium salt (specific activity 196 U/mg), unless specified otherwise, were from Sigma-Aldrich. Heparinoid ODSH (specific activity 150-160 U/mg) was a gift from ParinGenix Inc. Chromogenic substrate S2366 was from Chromogenix/diaPharma, recombinant hirudin from Calbiochem, and PRT was from American Pharmaceutical Partners. Mixed histones for in vitro studies were from Roche Diagnostic and those used for studies in vivo from Sigma-Aldrich. Histones H1 and H4 were obtained from New England Biolabs. Human soluble (s) TM was purified from TM-expressing HEK293 cells by anion-exchange chromatography and affinity chromatography on IIa-sepharose. Non-glycosylated, low molecular weight TM was separated from high molecular weight TM by high performance anion exchange chromatography to yield TMCS. The different forms of TM were validated by Western blot as described. Rabbit TM was purchased from Hematologic Technologies. Human protein C, was isolated from plasma as described, and further purified by immunoaffinity chromatography using the Ca2+-dependent monoclonal antibody HPC4, provided as a gift from Dr. C.T. Esmon, Oklahoma Medical Research Foundation.

In vitro aPC assay
Generation of aPC was assayed in 96-well plates as described. Briefly, rabbit or human soluble (s) TMCS or sTMCS, was mixed with various amounts of histones, PF4 and/or heparinoid for 10 minutes in assay buffer (final concentration, 20 mM Tris, 100 mM NaCl, 1 mM CaCl2, 0.1% BSA, pH 7.5). Final concentrations of TMs were 0.5-40 nM. PC (final concentration, 500 nM) was added for an additional 10 minutes followed by addition of IIa at a final concentration of 0.2 or 2 nM and then incubated for 15 minutes. All incubations were done at 37°C. The reaction was quenched with 1 mM EDTA and 100 nM hirudin. Concentrations of aPC formed in the quenched samples were inferred from initial rate measurements after the addition of 0.5 mM S2366. Initial rates of chromogenic substrate cleavage were determined by measuring absorbance at 405 nm using a Thermomax Microtiter V max plate reader (Molecular Devices).

In vivo aPC measurements
PF4 knockout (PF4KO) mice have been previously generated and characterized by us. WT mice were also on C57Bl6 background. All mice had been backcrossed ≥10 times onto a C57Bl6 background. Mice studied were 8-12 weeks of age. All animal experiments were approved by the Children’s Hospital of Philadelphia’s institutional animal care and use committee.

After mice were anesthetized with intraperitoneal injection of pentobarbital (100 mg/kg), in vivo aPC generation was assayed in plasma of mice injected via the jugular vein over 2 minutes with murine IIa at 8 U/kg (~1 pmol/20 g mouse) concurrently with histones (0-20 mg/kg) in the absence or presence of 5 mg/kg of PF4. After 10 minutes, blood was drawn from vena cava into sodium citrate/benzamidine (final concentration 5 mM/50 mM, respectively), centrifuged for 10 minutes at 5,000 rpm at 4°C and plasma frozen. In some experiments, animals were pre-injected with UFH or ODSH or buffer 10 minutes before injection of histones. Histones at 20, 50 or 75 mg/kg, was injected and blood was drawn after 30 minutes in surviving animals. Plasma aPC levels were measured by capture ELISA using an anti-mouse aPC antibody and recombinant mouse aPC as a standard (both kindly supplied by Dr. Esmon) and chromogenic substrate Spectrozyme PCa (American Diagnostica).

Activated partial thromboplastin time (aPTT) was measured in plasma of blood collected from tails of mice infused with heparinoids, followed 10 minutes later by various concentrations of histones, infused via a jugular vein. Time to clot formation was measured after mixing plasma with TriniCLOT Automated aPTT reagent (Tcoag).

Statistical analysis
Differences between groups were compared using a two-tailed Student’s t-test. Statistical analyses were performed using Microsoft Excel (Microsoft). Differences were considered significant when p values were < 0.05.

REFERENCES:


