



## Lupus anticoagulants, thrombosis and the protein C system

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### ABSTRACT

Although lupus anticoagulants (LAs) are immunoglobulins that inhibit procoagulant reactions *in vitro*, these molecules are associated with thrombosis *in vivo*. We and others have hypothesized that this may be due to selective targeting of the activated protein C (APC) anticoagulant pathway. Populations of antibodies that interact with protein C or protein S in ways that inhibit their activity are obvious candidates for such pathological molecules. However, it is less clear how populations that appear to bind to membrane surfaces might target the APC anticoagulant complex selectively. Studies now show that the membrane requirements of the APC anticoagulant complex are significantly different from those of the procoagulant reactions. The most dramatic difference is the requirement for the presence of phosphatidylethanolamine (PE) in the membrane for optimal APC function. The inhibitory activity of at least some LAs is enhanced by the presence of PE, but the anti-APC activity is enhanced even more, resulting in the plasma from these patients clotting faster than normal when APC is present. Structure-function studies have been undertaken to understand the PE dependence of this reaction better. Chimeric proteins in which all or part of the Gla domain of protein C has been replaced by the homologous region of prothrombin have been prepared. Unexpectedly, the PE dependence resides primarily in the C-terminal half of the Gla domain. Using liposomes of various composition, we found both the presence of the PE head group and unsaturation of the fatty acid chains are required for optimal inactivation of factor Va. It is hoped that a better understanding of the biochemistry of these reactions, combined with the use of the chimeric proteins described, will permit us to design better assays for the identification of pathologic LAs.

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Key words: lupus anticoagulant, thrombosis, protein C system

Lupus anticoagulants (LAs), although defined according to their ability to block the phospholipid based coagulation cascade *in vitro*, are associated with thrombosis *in vivo*. Many diverse mechanisms have been proposed to account for this

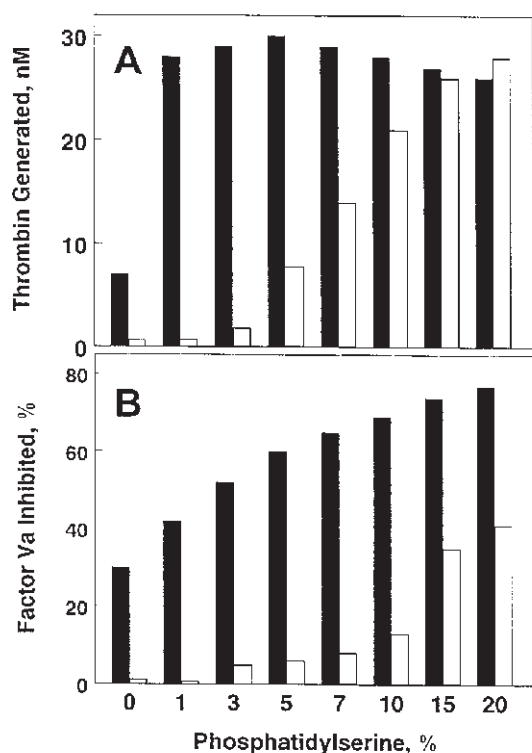
apparent dichotomy.<sup>1-3</sup> Based on what is known about the central importance played by the protein C anticoagulant pathway,<sup>4</sup> we and others<sup>5-7</sup> have proposed that selective inhibition of this system is a logical mechanism by which at least a population of LAs might be prothrombotic. It is readily apparent how inhibitory antibodies specific for the proteins involved would be prothrombotic. Some of these antibodies might be directed to neoepitopes that are expressed on the proteins only in the presence of phospholipid. However, it is less apparent how antibodies which show LA activity in an assay in which activated protein C (APC) is not functional (such as those used to define LA activity) or show reactivity with membrane or phospholipid surface would lead to hypercoagulability. In order to understand how selective inhibition might occur, it is necessary to understand the structure/function relationships of the anticoagulant reactions relative to those of the procoagulant reactions.

### Membrane requirements of the coagulation complexes and lupus anticoagulants

For many years, it was believed that the membrane requirements for all of the coagulation reaction complexes were the same. That is, they all required negatively charged phospholipids and phosphatidylserine (PS) was the preferred phospholipid for these membranes.<sup>8</sup> However, this was mostly a self-fulfilling prophecy, as the majority of the studies were performed with the prothrombinase complex and then generalized to the other complexes of interest. In addition, some of the molecules of potential interest, such as phosphatidylethanolamine (PE) do not behave ideally, making some of the studies difficult. This is of some importance, as PE has been implicated as part of the antigenic specificity of clinically relevant LAs.<sup>9-12</sup> We therefore asked two basic questions. First, are the membrane requirements of the protein C anticoagulant pathway really the same as those for the procoagulant complexes? Secondly, if they are not the same, do the characteristics of the anticoagulant active membranes more closely mimic those of prothrombotic LAs?

PE was added to liposomes containing 20% PS with the remainder made up with phosphatidylcholine (PC). The ability of these vesicles to support prothrombin activation or factor Va inactivation by activated protein C was determined using purified com-

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**Figure 1.** The dependence of the prothrombinase and the APC complex activities on the phosphatidylserine content of vesicles containing 50% phosphatidylethanolamine. **A.** Prothrombin was activated using vesicles containing the mole % PS indicated in the presence (solid bars) or absence (open bars) of 50% PE. Reaction conditions were: 1.4  $\mu$ M prothrombin, 0.2 nM factor Va, 2 nM factor Xa and 14  $\mu$ g/mL phospholipid. **B.** Factor Va was inactivated essentially as described<sup>13</sup> using vesicles containing the mole % PS indicated in the presence (solid bars) or absence (open bars) of 50% PE. Reaction conditions were: 0.2 nM factor Va, 4 pM APC, 70 nM protein S, 20  $\mu$ g/mL phospholipid. Residual Va activity was measured in a prothrombinase assay as in part A after inhibition of the APC using 2 nM factor Xa, 1.4  $\mu$ M prothrombin, 20  $\mu$ g/mL 20%PS:PC vesicles. Thrombin generated in both cases was determined using a chromogenic assay. (From Esmon NL. *Thrombogenic mechanisms of antiphospholipid antibodies*. *Thromb Haemost* 1997; 78:79-82. ©1997 F.K.Schattauer Verlagsgesellschaft mbH.)

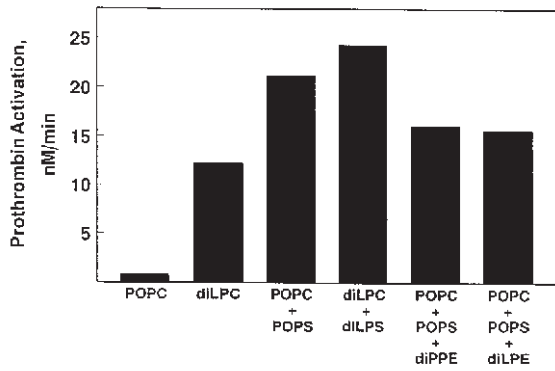
ponents.<sup>13,14</sup> A dramatic difference was observed. Although the rate of prothrombin activation was only slightly affected by the addition of PE, the ability of APC to inactivate factor Va was essentially dependent upon the presence of PE in the vesicles. In contrast, the addition of cholesterol to these liposomes, which would affect overall fluidity, greatly inhibited the inactivation of factor Va.<sup>14</sup> Others have observed negligible effects of the addition of cholesterol to similar vesicles on prothrombin activation.<sup>15</sup> The degree of factor Va inhibition was linearly dependent on the amount of PE present up to the maximum tested, 40%.<sup>14</sup> Vesicles containing 40% PE were also tested

for their activity in plasma clotting assays by a modification of the dilute Russell's viper venom assay.<sup>5</sup> As was observed with purified components, although these vesicles enhanced the clotting time in the absence of APC somewhat, the ability of APC to anticoagulate the plasma effectively was dependent on the presence of PE.<sup>5,14</sup>

The presence of PE in the vesicles also changed how much PS was necessary for optimal activity in the two reactions (Figure 1). When PE was present at 50%, 1% PS was sufficient for the maximal rate of prothrombin activation observed with 20% PS in the absence of PE. However, the maximal rate obtained was unaltered.<sup>16</sup> This is in general agreement with other researchers' results.<sup>15</sup> PE has also been found to have similar effects on other procoagulant reactions.<sup>17,18</sup> In the case of APC activity, however, although the presence of PE decreased the amount of PS required for maximal activity, the effect was not as dramatic as that observed for prothrombin activation. More importantly, there was no concentration of PS that resulted in an equivalent rate of factor Va inactivation as that observed when both PE and PS were present in the membranes.

The presence of PE in the membrane affects the structure beyond the mere presence of a different head group. PE is known to induce the formation of hexagonal phase II structures in the lipid bilayer and some have reported that it is this hexagonal phase II structure of the membrane which is required for binding of LAs.<sup>19</sup> The different degrees of saturation present in the PE component used here and by others also affects membrane fluidity and/or the hexagonal phase-forming properties of the PE. In the case of prothrombin activation, once some degree of fluidity is present<sup>20</sup> due to the presence of unsaturated fatty acids on the phospholipids or the presence of cholesterol, additional fluidity or promotion of the hexagonal phase is not required for enhanced activation.<sup>15,21</sup> Gilbert and Arena also concluded that the enhanced factor VIII binding they observed when PE was present was not related to the hexagonal phase properties of PE.<sup>18</sup> It was therefore of interest to know whether the PE effects observed in the case of APC function were due to the PE head group *per se*, or to the greater degree of fatty acid unsaturation present in this phospholipid.

When the traditional palmitoyl (C16:0) oleoyl (C18:1) phosphatidyl choline (POPC) was replaced with dilinoleoyl (diL; C18:2 in both fatty acid positions) PC, there was a significant increase in prothrombin activation (Figure 2) consistent with that which has been observed previously.<sup>21</sup> Addition of POPS had the expected effect. The addition of PE, no matter what its degree of unsaturation (dipalmitoyl-PE (diPPE) being fully saturated), had no additional enhancing effect. If anything, the presence of PE inhibited the rate of prothrombin activation. The situation is very different in the case of APC inactivation of fac-



**Figure 2. Prothrombinase does not require the PE head group or polyunsaturation for optimal activity.** Prothrombin was activated using 10 µg/mL phospholipid of the compositions indicated. When present, PS was at 20% and PE was at 40%. Reaction conditions were: 0.2 nM factor Va, 2.0 nM factor Xa and 1.4 µM prothrombin. Abbreviations: PO, palmitoyl, oleoyl; diL, dilinoleoyl; diP, dipalmitoyl.

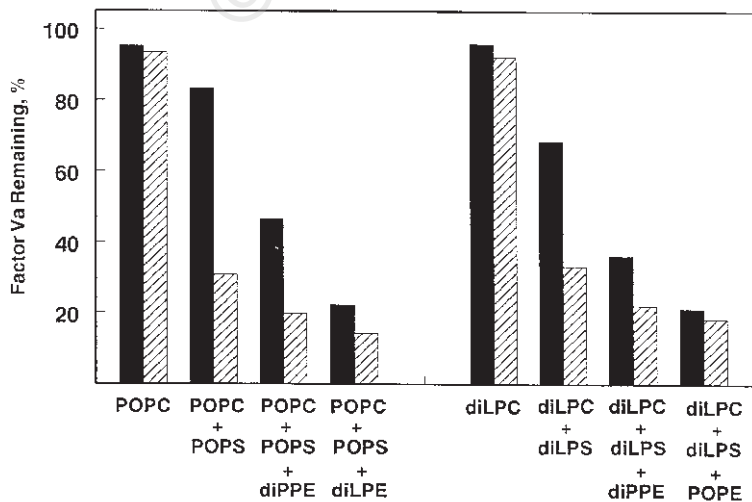
tor Va. As expected, the addition of POPS to POPC had very little effect (Figure 3, solid bars). Addition of fully saturated diPPE greatly increased the rate of inactivation, but the polyunsaturated form, diLPE, was required for maximal stimulation. As can be seen on the right side of the figure, just the presence of the diunsaturated diLPC:diLPS improved inactivation a small amount, possibly indicating increased fluidity alone has some enhancing effect. Again, the addition of diPPE had a significant effect but the presence of some degree of unsaturation in the PE moiety was required for maximal effect. We would conclude that both the PE head group *per se* and polyunsaturation in some membrane component is required for optimal inactivation of factor Va by APC. Although both features can affect the observed rate of prothrombin acti-

vation, neither is required for optimal activity. Similarly, although the presence of the PE head group and/or polyunsaturation or other molecules that alter membrane fluidity can affect the activity of other procoagulant reactions, neither is required for achieving near optimal activity.

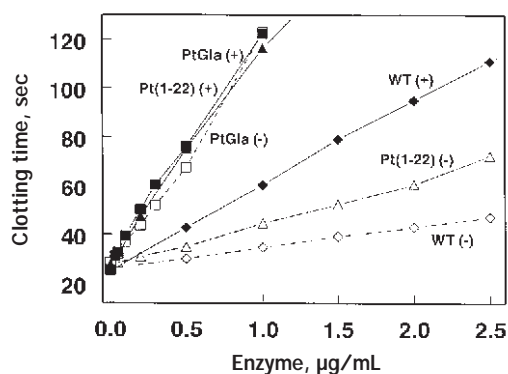
As we have shown previously, PE is required to observe the anti-APC activity of a variety of LA plasmas.<sup>5</sup> PE also enhances LA activity in the absence of APC. However, when purified reagents were employed, no conditions were found in which the prothrombinase reaction could be inhibited more than 50% by this particular immunoglobulin studied in detail.<sup>14</sup> This is probably not sufficient inhibition for an individual to be effectively "anticoagulated" by the antibody.<sup>22</sup> In contrast, the APC activity could be inhibited >90%, thus leading to a potential overall hypercoagulable state. Interestingly, the titer of this immunoglobulin was improved by inclusion of more PS in the liposomes (manuscript in preparation), just as the activity of the APC complex was improved by this inclusion (Figure 1). Thus, not only are the membrane requirements of the anticoagulant complex different from those of the procoagulant complexes, these requirements are also similar to those of at least a population of LAs associated with thrombosis and may be the basis for a link between the protein C pathway, lupus anticoagulants and thrombosis.

**Structure-function studies of protein C: what is important for LA activity?**

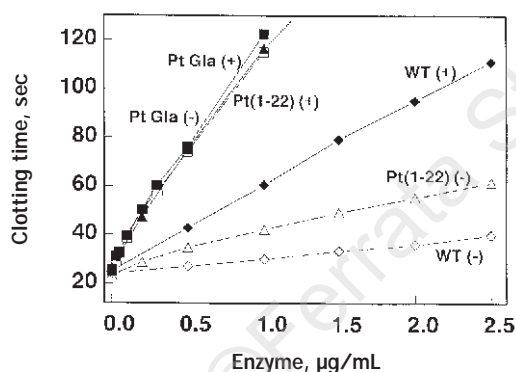
In order to understand the protein C-phospholipid interaction better, protein structure studies were undertaken. It was hoped that these studies might also lead to a better understanding of the LA inhibitory activity and possibly to useful reagents. Protein C is a vitamin K dependent protein, and contains the amino terminal Gla domain characteristic of such



**Figure 3. Both the PE head group and polyunsaturation are required for optimal APC activity of native APC but not the APC-Pt Gla chimera.** Factor Va was inactivated essentially as described<sup>24</sup> using 10 µg/mL phospholipid of the compositions indicated. When present, PS was at 20% and PE was at 40%. Abbreviations are defined in the legend to Figure 2. Reaction conditions were: 0.2 nM factor Va, 5 pM APC (solid bars) or APC-Pt Gla (hatched bars).



**Figure 4.** The effect of PE in liposomes on the anticoagulant activity of native or chimeric APC. Vesicles containing 20% PS, 80% PC (-) at 24  $\mu\text{g}/\text{mL}$  or 40% PE, 20% PS, 40% PC (+) at 8  $\mu\text{g}/\text{mL}$  were used in a one-stage clotting assay in the presence of APC, APC-Pt Gla or APC-Pt(1-22). Different phospholipid concentrations were necessary for the starting clotting times in the absence of APC to be similar. Mixtures contained 2.5 ng/mL factor X-activating enzyme from Russell's viper venom and a standard human plasma pool. Clotting was initiated by the addition of  $\text{CaCl}_2$ . (From Smirnov MD. *A Chimeric Protein C Containing the Prothrombin Gla Domain Exhibits Increased Anticoagulant Activity and Altered Phospholipid Specificity*. *J Biol Chem* 1998; in press. Copyright © 1998 the American Society for Biochemistry and Molecular Biology, Inc.)



**Figure 5.** The effect of protein S on the anticoagulant activity of native or chimeric APC. Plasma clotting was initiated as described in Figure 4 using 8  $\mu\text{g}/\text{mL}$  vesicles containing 40% PE, 20% PS, 40% PC in the presence or absence of an inhibitory monoclonal antibody to protein S (S155, 300  $\mu\text{g}/\text{mL}$ ). (-), no protein S activity present; (+) protein S activity present. (From Smirnov MD. *A Chimeric Protein C Containing the Prothrombin Gla Domain Exhibits Increased Anticoagulant Activity and Altered Phospholipid Specificity*. *J Biol Chem* 1998; 273:9031-40. ©1998 the American Society for Biochemistry and Molecular Biology, Inc.)

proteins.<sup>23</sup> This highly homologous domain has been implicated in membrane binding. It seemed likely that the small differences within this region between protein C and prothrombin might be responsible for the differences in the PE dependent behavior. Two

chimeras were therefore constructed; one in which the entire Gla domain and hydrophobic stack of pro-tein C was replaced with that of prothrombin, called PC-Pt Gla, and a second in which only residues 1-22 representing the amino terminal half of this domain was swapped, called PC-Pt(1-22). The properties of these mutants were compared with those of the wild type protein.<sup>24</sup> As predicted, the activated PC-Pt Gla (APC-Pt Gla) was no longer significantly influenced by the presence of PE in the liposomes (Figure 4). This mutant was also only slightly influenced by the fatty acid composition of the phospholipids used (Figure 3, hatched bars). However, there were two other, unexpected results. First, the APC-Pt(1-22) protein retained the sensitivity to the presence of PE in the membrane, indicating that it is the C-terminal half of the Gla domain that is responsive to this property of the membrane. Most studies have focused on the amino terminal of the Gla domain<sup>25</sup> for insights into the protein-membrane interactions of this class of proteins. However, examination of the structure of this region as modeled from the crystal structures of prothrombin<sup>26</sup> and factor VII<sup>27</sup> indicates how this region may be involved in membrane surface interaction (see ref. #24 for discussion). The second unexpected result was the significantly greater anticoagulant activity of both of the chimeric proteins. This property is not fully understood. Preliminary results suggest that prothrombin can inhibit the activity of the wild type protein more potently than the APC-Pt Gla chimera.<sup>28</sup> Other factors that may be inhibiting the native protein more than the chimera are not known, but these chimeras may be useful probes to identify these putative inhibitors. These properties of the PC-Pt Gla chimera may be very useful both clinically and diagnostically. Clinically, the chimera may be useful as an anticoagulant in patients with strong PE dependent, prothrombotic LAs. Diagnostically, it may be a useful reagent for the identification of PE dependent LAs that inhibit the APC complex.

These chimeras were also used to investigate the interaction of APC with its cofactor, protein S, in factor Va inactivation. These studies revealed yet another interesting feature about protein C and a potential use for the chimera. As can be seen in Figure 5, not only is the APC-Pt Gla insensitive to the presence of PE in the liposomes, it is also insensitive to the presence of protein S in the plasma. In contrast, the APC-Pt(1-22) retains protein S dependence. Again this indicates that it is the C-terminal region of the Gla domain of protein C that is required for proper interaction with another component of the complex. The APC-Pt Gla should also be a useful therapeutic agent in patients with protein S deficiency, whether chronic or acute due to an inflammatory state.<sup>29</sup> This would include those patients who are protein S deficient due to anti-protein S antibodies, a situation observed in a significant number of LA patients with thrombosis.<sup>6,30-32</sup>



## Summary

In conclusion, the membrane requirements for the activated protein C anticoagulant complex differ significantly from those of the procoagulant complexes. These properties, in particular the requirement for PE in the membrane, mimic the lipid requirements for at least a population of LAs associated with thrombosis. In addition, in studies designed solely to investigate the basic biochemistry of the molecules involved, a reagent was developed which may have significant diagnostic and clinical potential.

A most satisfying result indeed.

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All the authors contributed to design the study.

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## Disclosures

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

## Manuscript processing

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