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Phosphatidylethanolamine at the Luminal Endothelial Surface—Implications for Hemostasis and Thrombotic Autoimmunity

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Abstract

Objective—Accumulating evidence suggests that phosphatidylethanolamine (PE) is physically present at the luminal endothelial surface, where it tentatively functions as a critical anticoagulant. The goal of the current investigation was 3-fold: to characterize the distribution profile of PE at the luminal endothelial surface; to examine the immunoreactivity to the vascular endothelium by anti-PE (aPE) sera from patients presenting with thrombosis; and to discuss the potential mechanism of PE upregulation by endothelial cells.

Methods—The rat aortic arch was selected as major conduit vessel under significant hemodynamic burden. The presence of PE and the antigenic profile of aPE sera at the luminal endothelial surface were examined using duramycin as a PE-binding probe and immunohistochemistry. Phosphatidylethanolamine upregulation at endothelial cell surface was investigated using cultured monolayer subject to laminar shear stress or thrombin treatment.

Results—High levels of PE were detected at the luminal endothelial surface of aortic flow dividers, the ascending aorta, and the outer curvature of the aortic arch. The antigenic profiles of aPE sera, which are highly associated with elevated thrombotic risks in patients, are consistent with PE distribution along the endothelial surface. Finally, PE is upregulated at the surface of cultured endothelial cells in response to luminal shear stress but not thrombin.

Conclusions—The current data describe the physical distribution of vascular PE at the blood–endothelium interface. The luminal PE presents a vulnerability to anti-PE autoimmunity and is consistent with the association between aPE and elevated risk for idiopathic thrombosis.

Keywords

phosphatidylethanolamine; endothelium; duramycin; aPE; thrombosis

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Declaration of Conflicting Interests

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Introduction

Accumulating evidence in the past decades indicates that phosphatidylethanolamine (PE) is a critical anticoagulant in the vasculature. But the physical presence and distribution of PE at the luminal endothelial surface remain poorly characterized. Such knowledge will have important implications for hemostatic health and disorders.

The bulk of in vivo evidence on the presence and regulatory roles of PE in the cardiovascular system comes from its depletion or deficiency, which has devastating consequences.¹⁻⁴ Anti-PE (aPE) autoantibodies, also known as aPEs, are strongly associated with clinical cases of unexplained thrombosis.⁵⁻⁹ Additionally, aPEs are highly associated with recurring fetal loss among pregnancies in humans.¹⁰⁻¹² This scenario is supported by genetic knockout studies in mice, where a deficiency in ethanolamine kinase 2, which is a key enzyme in PE bio-synthesis, results in placental thrombosis and fetal loss.¹³

More direct evidence on the anticoagulant roles of PE is derived from in vitro experiments, where PE is found to be an essential cofactor for the protein C anticoagulant pathway. In a reconstituted coagulation system, the proteolytic inactivation of factor Va by activated protein C (APC) is amply enhanced by PE in a concentration-dependent manner.^{14,15} Such enhancement is specific to PE, and no amount of other phospholipid species is able to reproduce the effect.¹⁵ Additional experimental data demonstrate that, apart from the protein C pathway, PE is a suppressant of the factor Xa-prothrombin system.¹⁶

An inferred message from the current literature on PE is that its anticoagulant roles may be exerted at the blood–endothelium interface. A recent study documented the binding of duramycin, which is a PE-specific peptide, at the aortic flow bifurcations, providing clues that PE may indeed be present at the blood–endothelium interface.¹⁷ To expand and validate the prior observation, the goal of the current investigation was 3-fold: to characterize the distribution profile of PE at the luminal endothelial surface of the aorta; to examine the immunoreactivity to the vascular endothelium by aPE sera from patients presenting with thrombosis; and to discuss the potential mechanism of PE upregulation by endothelial cells. The data presented here will shed light on the distribution of PE at the blood–endothelium interface, which has implications for the modulation of hemostasis by the endothelium, and perhaps provide additional evidence on the correlation between aPE and an elevated risk for thrombosis.

Methods

To conduct the histological characterization of vascular PE, duramycin, which is a PE-specific peptide, was biotinylated as follows. Duramycin (2 mg) was incubated with sulfo-*N*-hydroxysulfosuccinimide ester biotin (NHS-biotin) at 1:1.2 molar ratio, in the presence of 1 equivalent of triethylamine. The reaction was carried out in dimethylformamide (DMF) overnight at room temperature. Duramycin-biotin was purified using high-performance liquid chromatography (HPLC) and confirmed using mass spectrometry (expected molecular weight [MW] = 2352.0 g/mole, actual MW = 2352.3 g/mole).

For experiments involving animals, the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Institutional Animal Care and Use Committee. Experiments were performed to detect luminal PE in the aorta, given that the aortic hemodynamics pose a significant source of prothrombogenic risk factors.⁸ To detect PE on the luminal endothelial surface, rats (*n* = 6) were sacrificed and the aortas were freshly dissected and rinsed with Hanks Balanced Salt Solution (HBSS) to remove residual blood. A solution of duramycin-

biotin (1.2 μ M) in HBSS was infused into the aorta using a syringe pump for 3 minutes at 3 mL/min. After washing with HBSS, avidin–fluorescein isothiocyanate (FITC; Sigma St. Louis, Mo) was infused into the vessel at 0.25 μ M. The aorta was washed again to remove nonbound avidin-FITC. Longitudinal cryosections (10 μ m) of the aorta were prepared and examined using a confocal microscope at 490/515 nm. Negative controls (n = 3) were omission of the duramycin-biotin.

Immunohistochemistry was used to validate the duramycin binding results. Specifically, rat aortas (n = 3) were dissected and rinsed in HBSS as described above. A mouse aPE monoclonal immunoglobulin G (IgG) was infused into the rat aorta, followed by wash to remove nonbound antibody. The aortic tissue was fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) and incubated with a goat antimouse IgG secondary antibody conjugated to horse radish peroxidase (HRP) at 4°C overnight with gentle stirring. Cryosections of 10 μ m thick were prepared, and bound antibody was visualized using diaminobenzidine (DAB). A negative control was included by using a non-PE binding monoclonal mouse IgG. A second negative control consisted of omission of the primary antibody.

To examine whether the vascular PE is a target for aPE auto-antibodies, we investigated the immunogenicity of the rat aorta using aPE-positive human sera collected from patients with thrombotic disorders. The use of human sera conforms with the principles outlined in the Declaration of Helsinki for use of human tissue or participants and was approved by the institutional ethics review board. The sera tested were shown previously to contain either an IgG that reacts directly with PE or an immunoglobulin M (IgM) that binds PE in a kininogen-dependent manner. For the dependent aPE, adult bovine plasma, at 1:10 dilution in HBSS, was infused into the aorta as a source of PE-binding cofactors (kininogens) for the IgM. The human aPE sera were diluted 1:10 in a HBSS containing 1% bovine serum albumin and slowly infused into the aortas (n = 3 for aPE IgG, n = 3 for aPE IgM). This was followed by washing and fixation using paraformaldehyde in PBS. The aortas were incubated overnight with a goat antihuman IgG-HRP or a goat antihuman IgM-HRP. Cryosections were prepared and developed with DAB staining. Negative controls (n = 2) were performed identically except that the primary human serum was from an aPE-negative individual. In a second control (n = 2), the primary serum was omitted.

To examine whether the immunoreactivity against PE takes place at the endothelial surface or intracellular components, transmission electron microscopy (trans EM) was used to characterize the immunohistochemistry on an ultrastructural level. After infusion of the primary aPE serum and washing, the aorta was incubated with goat antihuman IgG-gold colloids (6 nm) overnight at 4°C. Tissue samples were processed with standard preparation for EM and examined in a JEOL JEM2100 electron microscope.¹⁸

Because it has been established that PE and phosphatidylserine (PS) are asymmetrically distributed in the plasma membrane of a typical, viable mammalian cell, and are known substrates for aminophospholipid translocases, flippases, floppases, and scramblases,¹⁹ additional immunohistochemical staining was performed to probe for the presence of PS using an anti-PS serum, using an identical protocol for immunohistochemistry described above.

Finally, in vitro experiments were conducted to explore the nature of PE upregulation at the endothelial surface, under the premise that the PE level at the blood–endothelium interface may be modulated in response to flow stimulations. Bovine aortic endothelial cells were grown to confluence on gelatin-coated cover slips and transferred to unidirectional laminar flow chambers. The cells were subject to laminar shear stress at 15 dyne/cm² for 3 hours at

37°C. Radiolabeled duramycin, in the form of ^{99m}Tc -duramycin (Technetium-99m-labelled duramycin), was synthesized as described previously for the quantitative binding measurements.²⁰ At the end of shear stress treatment, the treated ($n = 8$) and control cells ($n = 6$) were incubated for 5 minutes with 15 μCi of ^{99m}Tc -duramycin. After stringent washing, the binding of ^{99m}Tc -duramycin to the endothelial monolayer was measured using a gamma counter at an energy level of 140 ± 15 keV. To validate the specificity of the binding data, the procedure was repeated in the presence of an aPE monoclonal antibody ($n = 4$). Furthermore, to investigate whether PE upregulation at the endothelial surface is mediated by coagulation, the cells were treated with 0.5 ($n = 3$) and 50 U/ml ($n = 3$) of thrombin for 20 minutes at 37°C without shear stress. The binding of ^{99m}Tc -duramycin to the monolayer was assayed as described above.

Results

The physical presence of PE at the luminal endothelial surface was independently cross-validated using 2 different probes, duramycin and aPE monoclonal IgG. An anatomical sketch of the rat aorta with major branches is illustrated in Figure 1A. Figure 1B shows that duramycin binds strongly at the aortic flow dividers. The binding is the most prominent at the apex of the flow divider and gradually declines toward the periphery. The binding of duramycin, presumably to surface-exposed PE at the luminal endothelial lining, was confirmed by immunohistochemistry using a purified aPE monoclonal IgG (Figure 1C). The relative level of immunoreactivity, as reflected by the deposition of DAB, was measured semiquantitatively using laser scanning microscopy (Figure 1D). In contrast, neither of the negative controls, including the non-PE binding IgG and primary antibody omission, showed detectable reactivity at the region (Figure 1E). On an ultrastructural level seen using trans EM, significant deposition of gold colloids was detected on the luminal surface of the endothelial membrane at the apex of flow dividers, with few internalized to intracellular compartments (Figure 1F).

According to duramycin binding and immunohistochemistry, apart from the flow dividers, a significant presence of PE was detected at the luminal endothelial surface of other regions that are likely associated with hemodynamic burden in the aorta. These include the ascending aorta and the outer curvature of the aortic arch (Figure 2). In contrast, the level of PE was substantially diminished at the periphery of the flow dividers and the inner curvature of the aortic arch.

With the aPE sera, distinctly positive immunoreactivity was consistently identified at the endothelial lining of the flow dividers and other regions that appears to be under hemodynamic burden (Figure 3A). The pattern of distribution is consistent with the binding of duramycin and aPE monoclonal IgG. In contrast, no immunoreactivity was observed at these regions with the serum from the individual who is aPE negative (Figure 3B). The pattern of immunoreactivity from the patient serum containing aPE IgM is similarly distributed. These results further substantiated a prominent presence of PE at the luminal endothelial surface and, importantly, underscored the susceptibility of vascular PE to aPE autoimmunity.

Although the staining procedure was performed identically, unlike the aPE serum or aPE monoclonal IgG, the anti-PS serum had no consistent immunoreactivity at the apex of aortic flow dividers or other regions of the luminal surface. This result is consistent with the fluorescent microscopy study using Annexin V-FITC, which binds specifically PS. Thus, apart from the PS, the transposition of PE at the luminal endothelial surface may be modulated by a distinct, yet to be established mechanism.

In vitro, laminar shear stress of 15 dyne/cm², which is typical of the values found in the human aorta, resulted in a 62.0% ± 11.2% elevation in ^{99m}Tc-duramycin binding to the endothelial monolayer compared to nonshear-stressed control cells (Figure 4). In the presence of aPE antibody, the binding of ^{99m}Tc-duramycin to shear-stressed cells was blocked (Figure 4). In contrast, no change in ^{99m}Tc-duramycin binding was detected in endothelial cells treated with thrombin at 2 different dosages (0.5 and 50 U/mL; Figure 4). There was an absence of elevated cellular damage or cell death associated with any of the above experimental conditions compared to nontreated control cells.

Discussion

Main findings from this investigation are as follows. (1) Using PE-specific probes, including duramycin and a purified aPE monoclonal IgG, we discovered and validated a significant level of PE at the luminal endothelial surface of the aorta. The distribution pattern of vascular PE tends to correlate with the level of hemodynamic stress (flow dividers, ascending aorta, and outer curvature of the aortic arch). (2) The aPE-positive sera procured from patients with idiopathic thrombosis have a strong immunoreactivity against the aforementioned aortic regions. (3) Laminar shear stress, but not thrombin treatment, promotes an upregulation of PE at the surface of endothelial cells. Overall, these data provide the spatial evidence to support a physical presence of PE at the blood–endothelium interface. These findings, in turn, have implications for an underlying regulatory mechanism that modulates the coagulation potential at the endothelial surface and the pathological vulnerability toward thrombotic risks in patients who are positive for aPE autoimmunity.

The protein C anticoagulant pathway is essential for maintaining the blood in a fluid state by downregulating prothrombogenic factors.²¹ In prior studies, the PE dependency of protein C activities has been demonstrated using coagulation assays. It was subsequently shown that the substitution of the Gla domain in protein C abolishes its PE-dependent proteolytic activities, substantiating the role of the Gla domain in conferring a binding specificity toward PE.^{14,15} A physical presence of PE at the luminal endothelial surface enables a direct access to PE-containing membrane surface by circulating protein C. The highly regionalized distribution profile of vascular PE at surfaces under high hemodynamic stress is indicative of a regulatory mechanism in PE-dependent protein C activation in response to hemodynamics.

The PE being at the blood–endothelium interface presents a potential vulnerability to aPE autoimmunity and supports an underlying immunological cause for aPE-associated thrombotic risks. In a reconstituted coagulation system with PE-containing membranes, aPE isolated from patients with idiopathic thrombosis potentially inhibits the APC anticoagulant activities.^{14,22} The current data provide the first spatial characterization of the antigenic target on a tissue level for aPE sera. The data revealed a prominent immunoreactivity by aPE sera at the aortic endothelial lining, particularly at regions under hemodynamic burden. This pattern of distribution is strikingly consistent with that of vascular PE detected using duramycin and a purified aPE monoclonal IgG. These observations are in keeping with the notion that aPE-positive autoimmune disorders likely result in the masking of endothelial PE, whereby impedes its participation in anticoagulation pathways at the vascular luminal surface. Such an interference may shift the coagulation potential sufficiently to promote a greater thrombotic risk.

Mechanotransduction pathways have been implicated in the regulation of endothelial morphology and functions in response to vascular hemodynamic changes,^{23–27} but to what extent mechanotransduction modulates the endothelial anticoagulant mechanisms remains unknown. However, evidence on the correlation between the vascular architecture and the

expression of anticoagulant factors at the endothelium suggests an adaptive plasticity that responds to hemodynamic stimuli. For instance, Weiler-Guettler et al discovered that thrombomodulin is highly expressed in endothelial cells at the flow dividers in major arteries.²⁸ In addition, the presence of EPCR is prominent at the endothelium of major blood vessels but not in the capillaries.²⁹ The data from the current investigation are consistent in that shear stress may be involved in an upregulation of endothelial surface PE and that the distribution patterns of endothelium-mediated anticoagulants may reflect a degree of regional response to vascular hemodynamics. The underpinning regulatory mechanism, which is likely mediated by the endothelial mechanotransduction pathways, warrants further investigations.

In conclusion, our findings are significant in several respects. (1) The physical presence of PE is detected by 2 independent methods at the luminal endothelial surface, in particular, at vascular regions under hemodynamic stress. (2) The PE level at the endothelial surface is sensitive to shear stress but not to thrombin stimulation. (3) Phosphatidylethanolamine at the endothelial surface is susceptible to aPE binding, which is consistent with an association between aPE and idiopathic thrombosis.

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Figure 1.

Phosphatidylethanolamine (PE) at the endothelial surface. A, An anatomical diagram of the rat aorta, where the flow dividers are highlighted in red. B, Fluorescent confocal micrograph of duramycin binding at the aortic flow divider. C, Immunohistochemistry staining using a purified mouse anti-PE (aPE) monoclonal immunoglobulin G (IgG) as primary antibody. Positive staining at the apex of the flow divider is marked by arrows. D, Relative signal intensity, in arbitrary units, of diaminobenzidine (DAB) deposition along the flow divider after immunohistochemical reactions using the aPE monoclonal IgG. E, Negative control using a non-PE-binding mouse IgG. F, Transmission electron microscopy (trans EM) micrograph demonstrating the deposition of gold colloids at the luminal endothelial surface without significant binding to the intracellular components.

Figure 2.

Presence of phosphatidylethanolamine (PE) at the luminal endothelial lining in the rat aorta by immunohistochemistry using the anti-PE (aPE) monoclonal immunoglobulin G (IgG). Representative micrographs of the regions marked 1 to 5 in (A) are shown in panels (B) to (F), respectively. The presence of significant immunoreactivity is marked by arrows. Apart from the flow dividers (shown previously), PE is detected in the ascending aorta and the outer curvature.

Figure 3. Phosphatidylethanolamine (PE) at the aortic luminal endothelial surface. A, Immunohistochemistry reactivity using the serum from an anti-PE (aPE)-positive patient. The presence of prominent aPE binding is marked by arrows. B, Control staining using the serum from an individual who is aPE negative.

Figure 4.

^{99m}Tc-duramycin binding to cultured endothelial cells. The binding is expressed as percentage change in treated over control cells. The treatments include (from left to right) thrombin low—0.5 U/mL thrombin; thrombin high—50 U/mL thrombin; shear—laminar shear stress at 15 dyne/cm²; shear and anti-PE (aPE)—shear stress at 15 dyne/cm² in the presence of aPE monoclonal antibody.