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Investigation of Cofactor Activities of Endothelial Microparticle-Thrombomodulin with Liposomal Surrogate

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Investigation of cofactor activities of endothelial microparticle-thrombomodulin with liposomal surrogate☆

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ABSTRACT

Thrombomodulin (TM) is a type I transmembrane glycoprotein mainly expressed on the endothelial cells, where it binds thrombin to form the thrombin-TM complex that can activate protein C and thrombin-activable fibrinolysis inhibitor (TAFI) and induce anticoagulant and anti-fibrinolytic reactions, respectively. Cell activation and injury often sheds microparticles that contain membrane TM, which circulate in biofluids like blood. However, the biological function of circulating microparticle-TM is still unknown even though it has been recognized as a biomarker of endothelial cell injury and damage. In comparison with cell membrane, different phospholipids are exposed on the microparticle surface due to cell membrane "flip-flop" upon cell activation and injury. Liposomes can be used as a microparticle mimetics. In this report, we prepared TM-containing liposomes with different phospholipids as surrogates of endothelial microparticle-TM and investigated their cofactor activities. We found that liposomal TM with phosphatidylethanolamine (PtEtn) showed increased protein C activation but decreased TAFI activation in comparison to liposomal TM with phosphatidylcholine (PtCho). In addition, we investigated whether protein C and TAFI compete for the thrombin/TM complex on the liposomes. We found that protein C and TAFI did not compete for the thrombin/TM complex on the liposomes with PtCho alone and with low concentration (5%) of PtEtn and phosphatidylserine (PtSer), but competed each other on the liposomes with higher concentration (10%) of PtEtn and PtSer. These results indicate that membrane lipids affect protein C and TAFI activation and microparticle-TM may have different cofactor activities in comparison to cell membrane TM.

1. Introduction

Thrombomodulin (TM) is a type I transmembrane glycoprotein that is mainly expressed on endothelium and serves as an essential cofactor for the activation of anticoagulant protein thus contributing to hemostatic balance [1]. Specifically, TM modulates the activity of thrombin from a procoagulant to an anticoagulant protease [2]. When bound to TM, thrombin is unable to generate fibrin or activate platelets but instead becomes a potent activator of protein C. Activated protein C (APC) is an anticoagulant protease that selectively inactivates coagulation factors Va and VIIIa, thus providing an essential feedback mechanism to prevent excessive coagulation [3]. In addition, TM also enhances thrombin-catalyzed proteolytic activation of thrombin activatable fibrinolysis inhibitor (TAFI) [4]. Activated form of TAFI (TAFIa) cleaves C-terminal lysine and arginine residues from partially degraded fibrin. The arginine removal suppresses the ability of fibrin to catalyze plasminogen activation thus delaying clot lysis [5]. Therefore, TM contributes to the balance of hemostasis by acting as both anticoagulant and procoagulant factor.

Apart from its cell membrane form, TM also exists in microparticles in biofluids like blood [6]. Microparticles are small membrane blebs that are released from cells in response to cellular activation, injury or apoptosis, which constitute reliable hallmarks of cell damage [7]. Early work by Satta and co-workers demonstrated that lipopolysaccharide (LPS) treatment increases TM activity on monocyte-derived microparticles by up to 80% [8]. TM shedding from activated endothelium *via* endothelial microparticles was also observed [9]. Endothelial microparticles presenting TM and other cell-specific surface antigens, such as tissue factor

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and endothelial protein C receptor have been used as biomarkers of sepsis-induced disseminated intravascular coagulation (DIC) [10]. It was found that endothelial microparticle-TM was increased significantly in severe sepsis patients versus those in healthy controls, suggesting that endothelial microparticle-TM may play a role in the progression of sepsis-induced DIC. In an *in vitro* study, significantly elevated levels of microparticle-TM were isolated from human umbilical vein endothelial cells (HUVECs) following physiologic cyclic strain [11]. Given the unique biological function of microparticles [12,13] and importance of TM to vascular homeostasis, a clearer understanding of the biological function of microparticle-TM related to physiological and pathological conditions is of significant interest. However, the biological activity of microparticle-TM remains unexplored.

Membrane lipid asymmetry associated with defined lipid leaflet composition is a well-known phenomenon [14]. In quiescent cells, phosphatidylcholine (PtCho) is abundant on the outer surface, whereas phosphatidylserine (PtSer) and phosphatidylethanolamine (PtEtn) are mainly sequestered to the inner leaflet [15]. However, cell activation or cellular trauma in certain environment causes a collapse of lipid asymmetry, resulting in externalization of PtSer and PtEtn and exposure of positive and negative phospholipids on the cell surface [15]. Cell activation and injury accompanied by a membrane "flip-flop" is assumed to yield microparticle formation [16]. Microparticles vary in size, phospholipid and protein composition, which may explain that they can support either procoagulant or anticoagulant activity [17]. It is generally accepted that microparticles released from activated cells are enriched in PtSer and PtEtn lipid compared with their parent cells [18,19]. It is also known that aminophospholipids are exposed on microparticle surface, which are involved in blood coagulation. Thus, they are considered as biomarkers reflecting prothrombotic state in many diseases [20,21].

Cell membrane mimetic systems provide useful tools for studying and understanding the biological functions of membrane proteins and lipids and provide enormous opportunities in developing products for biomedical research and applications [22]. Liposome, in which lipid composition closely resembles that of cell membranes, has been extensively studied as model of cell membrane applications. Reconstitution of membrane proteins into liposomes is a useful technique to study the functions of the membrane proteins [23]. On the other hand, liposomes with different ratios of PtEtn and PtSer have been used as microparticle mimics [24,25]. Earlier work demonstrated that native TM incorporated into lipid vesicles resulted in substantially enhanced protein C activation [26,27]. These results raise a very important question of whether lipid membrane also affects TAFI activation *via* thrombin/TM complex and if so, whether protein C and TAFI activation processes favor the same or different lipid environment. To our knowledge, there are no comparison studies on the effects of phospholipids in generating APC and TAFIa by the TM-thrombin complex so far. In this study, we prepared liposomal TM conjugates with different phospholipids to mimic microparticle-TM and investigated their cofactor activities. Specifically, liposomal TM conjugates consisting of PtCho alone, 5 and 10% molar percentages of PtEtn and PtSer in PtCho were prepared and their protein C and TAFI activation activities were investigated, respectively. Subsequently, fibrinolysis time of different TM-liposome formulations were assessed as well (Fig. 1).

2. Materials and methods

2.1. Materials

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Dialysis was performed using Amicon Ultra Centrifugal Filters (Millipore). Bovine Serum Albumin (BSA), heparin, hippuric acid, 3-methylhippuric acid, hippuryl-arginine, sephadex G-75, triton x100 were obtained from Sigma-Aldrich (St. Louis, MO). BIOPHEN CS-01 substrate was obtained from Aniara (West Chester, OH). Human Protein C, human APC, human prothrombin and α-thrombin, commercial full rabbit TM, human antithrombin III, human fibrinogen, plasminogen, TAFI and α2-antiplasmin were from Haematologic Technologies (Essex Junction, VT). Tissue plasminogen activator (tPA) was obtained from Innovative Research (Novi, MI). Phospholipids (16:0 PtCho, 16:0 PtEtn, 16:0 PtSer (L-isomer)) were obtained from Avanti Polar Lipids (Alabaster, AL). Human plasma was from Sigma-Aldrich. UV–vis absorbance measurements were performed on SpectraMax Plus 384 Microplate reader and Varian Bio50 UV–vis spectrophotometer. TAFIa activity samples were analyzed using Shimadzu 20AD HPLC system with Waters Xselect HSS T3 column (3.5 μm, 2.1 × 100 mm). Fluorescence was measured using Hitachi F-7000 instrument. Gel permeation chromatography (GPC) was performed using AKTA FPLC system. Dynamic light scattering (DLS) measurements to measure particle size were performed on Brookhaven Instruments analyzer. Statistical analysis was performed by using SPSS Statistics software (IBM) *via* a paired-samples *t*-test.

2.2. Liposomal TM preparation

Lipids were dissolved in $CHCl_3$ or $CHCl_3$: MeOH mixture (1:1 vol:vol for PtSer and PtEtn containing liposomes) followed by sonication for 2 min and solvent evaporation under vacuum overnight. Afterwards, lipids were combined with Tris-HCl buffer (20 mM, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$) and swelled using freeze-thaw cycles with liquid nitrogen and a 65 °C water bath ten times. Subsequently, lipid stock solutions were stored at room temperature under nitrogen gas atmosphere. Immediately before use, lipid solutions were sonicated for 45 min. Rabbit TM was incubated with 500 μL of 10 mg/mL of swelled lipid solution (PtSer and PtEtn percent molar fractions were incorporated in PtCho) for 1 h at room temperature at 1 to 174 000 of TM to lipid ratio (mol:mol). Liposomes were extruded at 50 °C three times through 200 nm, then ten times through 100 nm polycarbonate membranes using pressurized nitrogen gas. Liposomes were then characterized by DLS. The mean liposome sizes were around 130 nm.

2.3. Protein C activation assay

Human alpha thrombin (1 nM final conc.) was combined with different types and formulations of TM (10 nM final conc.) and Protein C (960 nM final conc.) in a final volume of 50 μL Tris-HCl buffer. BSA (0.01 mg/mL final conc.) was added and the solution was incubated for 1 h at room temperature. Antithrombin III (20 nM final conc.) and heparin (0.1 nM final conc.) were added to stop the reaction. After 10 min, the solution was completed to 165 μL with Tris-HCl buffer followed by 35 μL addition of BIOPHEN CS-01 substrate solution (1 mg/mL stock, 0.064 mM final conc.). Absorbance change at 405 nm was measured for 10 min at 0.1 min intervals using a microplate reader at room temperature. The slope of the initial linear change in absorbance was compared to a calibration curve with APC and the hydrolysis rate of substrate BIOPHEN CS-01 (conc. range 0–4.1 × 10^{-11} mol) (Supporting Information Fig. S3).

2.4. TAFI activation assay

TAFI activation and thrombin quenching conditions as well as all

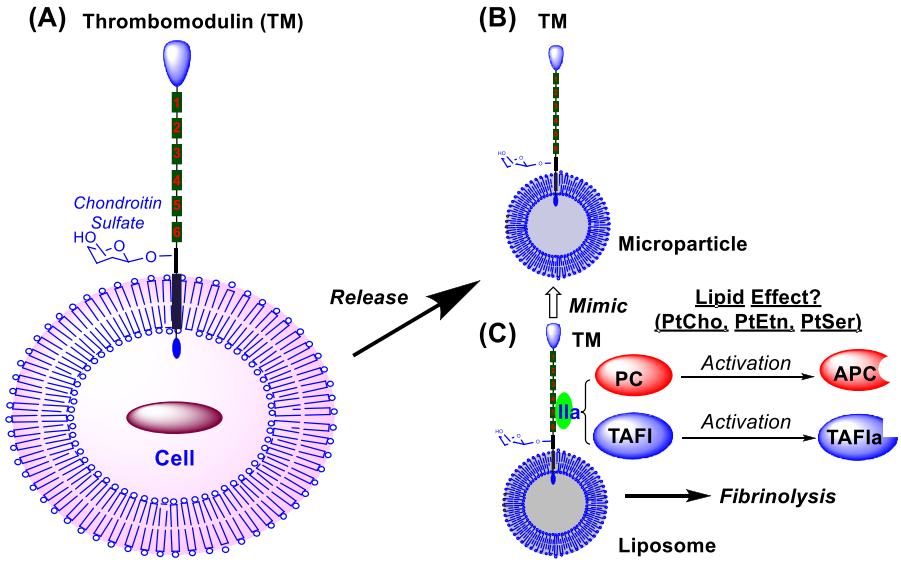

Fig. 1. Thrombomodulin (TM) in the cell (Cellular TM, **A**), microparticle (microparticle-TM, **B**) and liposome (liposomal TM, **C**) as microparticle-TM mimetics for studying microparticle-TM function and phospholipid effects on TM's cofactor activities, protein C (PC) activation, thrombin-activable fibrinolysis inhibitor (TAFI) activation, and fibrinolysis, respectively. IIa: Thrombin.

enzyme concentrations were identical to APC assay for results to be comparable. After activation, the solution was completed to 200 μL with Tris-HCl buffer containing hippuryl-arginine (0.4 mM final conc.). The reaction was allowed to run for 10 min at room temperature, subsequently, stopped by adding 100 μL of 1 M HCl. Ethyl acetate (300 μL) was added to the solution and vortexed for 10 min. 200 μL of the organic (top) layer was extracted, air dried, and reconstituted to 66.7 μL of H_2O. An internal standard of methyl-hippuric acid was added to the sample (final conc. of 3.45×10^{-8} M).

2.5. Hippuric acid measurements for TAFIa activity

An HPLC methodology for hippuric acid measurement was derived from previous reports [28,29]. The separation of analytes was carried out on a Waters Xselect HSS T3 column with a binary gradient elution using mobile phase composed of 85% of 10 mM phosphate (pH 3.0) and 15% acetonitrile (A), and acetonitrile (B) at a flow rate of 0.3 mL/min. The gradient elution started with 10% B, increased linearly to 60% B from 2 to 8 min, then stepped to 90% B and held for 7 min, and lastly returned to the initial condition with a total run time of 20 min. Hippuric acid was evident at 3.5 min and methyl-hippuric acid was evident at 5.5 min. Hippuryl-arginine was not detected (Supporting Information Fig. S4). TAFIa activation activity was determined from hippuric acid concentration using a calibration curve constructed with standard hippuric acid solutions (Supporting Information Fig. S5).

2.6. Fibrinolysis assay

A previously published protocol was followed with slight modifications for fibrinolysis determination [30]. A solution (100 μL) containing 40% (by vol.) human plasma, 7.34 nM tPA, and 20 nM TM in Tris-HCl buffer without Ca^{2+} was added to each well of a 96-well plate. The plate was equilibrated at 37 °C in a microplate scanner along with the activation solution. Subsequently, 100 μL of activation solution containing 12 nM human alpha thrombin and 20 mM Ca^{2+} was added to the initial solution to initiate fibrin clot formation. Turbidity at 405 nm was measured to determine clot formation and subsequent fibrinolysis (clearing of solution). Measurements were performed for 60 min at 37 °C. Fibrinolysis time was defined as the halfway point between initial peak absorbance (clot formation) and the first trough absorbance (clot lysis).

3. Results and discussion

Microparticles are enriched in PtSer and PtEtn lipid compared with the cell surface of their parent cells [18,19], however, their density is still unknow. In this study, PtCho was chosen as the primary lipid as it represents the major component of cellular membranes and provides a net-neutral charge to a cell at rest. PtSer and PtEtn were chosen to represent the major groups of charged phospholipids, typically sequestered in the inner leaflet of cell membrane at rest, yet highly present on microparticles. Therefore, incorporation of these phospholipids into liposomes aimed to mimic their exposure in microparticles [24,25]. Commercial full rabbit TM was reconstituted into unilamellar phospholipid vesicles using an extrusion method in which TM was added to a swelled phospholipid solution prior to extrusion [27]. The lipid contents in the liposomes were verified by semiquantitative TLC methodology (Supporting Information S1). The incorporation of TM into liposomes was determined by fluorescent labeling and indirect Bradford protein concentration measurements, which indicated over 90% incorporation of TM into liposomes (Supporting Information S2), which is in concord with the previous report [27]. Results from gel permeation chromatography (GPC) analysis further confirmed a high percentage of TM incorporation into liposomes due to the absence of free TM elution peak during chromatography (data not shown).

Our initial measurements determined the extent of protein C activation by free TM and liposomal TM conjugates with different lipids in a previous reported method [31]. Briefly, all protein C activation assays were performed with thrombin and, protein C for 60 min in a buffer solution (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% BSA) containing 5 mM Ca^{2+} at room temperature, followed by stopping the reaction with antithrombin III and heparin. The APC generation was measured by monitoring the hydrolysis of substrate BIOPHEN CS-01 at 405 nm absorbance (Supporting Information

Fig. S3). Next, TAFI activation by free TM and liposomal TM conjugates with same lipid composition were conducted with thrombin and TAFI. All TM, lipids and enzyme concentrations and quenching conditions were kept identical to APC assay for result comparability. The TAFIa generation was determined by measuring TAFIa activity on hippuryl-arginine hydrolysis followed by HPLC analysis of hippuric acid concentration (Supporting Information Figs. S4 and S5). Previous studies reported approximately 50% distribution of TM on the outer leaflet of liposomes [32] by comparing TM activity before and after treatment with a detergent in order to transfer protein to the liposome surface [26]. Other work utilized anti-TM antibodies to determine total concentration of TM without localization distinction [27]. Our data agrees with previous reports evidenced by an approximate doubling of APC activity after Triton detergent treatment of the liposomal TM with different lipids (Supporting Information Fig. S6).

After establishing TM incorporation and localization in liposomes, we determined protein C activation by different microparticle-TM conjugates. We also co-incubated free TM with different liposomes (without TM) to determine if any direct lipid effects on protein C activation. Consistent with previous studies, we observed an approximate tripling in APC generation followed by TM incorporation into PtCho liposomes (Fig. 2C) compared to free TM (Fig. 2B). More than five-fold increase in APC generation was evident in 5% PE liposomal system (Fig. 2D), which correlates with a previous report of PtEtn positive influence on APC generation by TM [26]. However, similar APC generation in 5% and 10% PtSer systems (Fig. 2E) were observed as in PtCho liposomes.

Next, we investigated TAFI activation activity of the microparticle-TM with the same liposome preparations (Fig. 3). After activation, TAFIa containing solution was diluted 12-fold and hippuryl-arginine was added followed by incubation for 10 min. Then, the reaction was quenched, free hippuric acid was extracted and measured using an HPLC method. TAFI activation approximately doubled in PtCho-TM liposomes (Fig. 3C) in comparison to free TM (Fig. 3B). However, compared to PtCho-TM, reduction in TAFI activation was observed in liposomal TM with 5% PtEtn or 5% PtSer, followed by much greater reduction in TAFI activation in liposomal TM with 10% PtEt or 10% PtSer (Fig. 3D and E). We estimate that 11.6 μM of hippuric acid generated under PtCho-TM liposome conditions resulted from approximately 4.4 nM of TAFIa (given the k_{cat} of 52.4 ± 4 s^{-1} for hippuric acid generation by TAFIa [33] and assuming catalysis near V_{max}). This is significantly lower than the amount of APC generated (175.2 nM) under the same conditions. However, TAFIa is a very potent fibrinolysis attenuator at low nanomolar concentration [30]. Estimated 4.4 nM of TAFIa is greater than the threshold concentration for antifibrinolytic effect.

Protein C and TAFI are concurrently activated in a TM-dependent manner. It is an important question whether protein C and TAFI compete for the thrombin/TM complex in physiological and pathological conditions. In this study, we included co-incubation of protein C and TAFI to simulate competitive activation of protein C and TAFI by the thrombin/TM complex on the liposomes. A mixed results for protein C activation and TAFI activation on TM-liposomes with different lipids were observed when co-incubated with TAFI and protein C. No significant effect from TAFI on APC generation or from protein C on TAFI activation on the PtCho-TM liposomes (Figs. 2C and 3C), 5% PtEtn TM liposomes (Figs. 2D, 3D) and 5% PtSer TM liposomes (Figs. 2E and 3E) were observed under competitive conditions, indicating that TAFI and protein C do not compete each other on these liposomes. These results are in consistence with an *in vitro* study that TAFI and protein C do not compete for their activation on endothelial cells [34]. However, significant effect on protein C and TAFI activation on 10% PtEtn liposomes (Figs. 2D, 3D) and 10% PtSer TM liposomes (Figs. 2E and 3E) were observed under competitive conditions, indicating that TAFI and protein C compete each other on these liposomes. An earlier study with soluble TM in solution found that protein C and TAFI act as competitive inhibitors for the thrombin/TM complex [35]. Our results show a lipid-dependent competition between protein C and TAFI, which raises the possibility that distinct thrombin/TM complexes on the liposomes and activate protein C and TAFI differently. It is possible that when anchored to different lipid membranes, thrombin/TM complexes are stabilized in different conformations that favor the binding of PC or TAFI because each substrate binds to distinct residues on TM. Overall, these results indicate that protein C and TAFI may compete for the thrombin/TM complex on endothelial cells under pathological conditions, which deserve further investigation.

It is important to note that the ability of TAFIa to cleave terminal lysines and arginines of fibrin may be different from its ability to cleave a small molecule substrate used in the assay. To assess the differences in TAFI activation in different lipid environments translated to its physiological function, we further investigated whether fibrinolysis rates are affected by the presence of different lipids in liposomal TM. As a result, about 25% increase in fibrinolysis

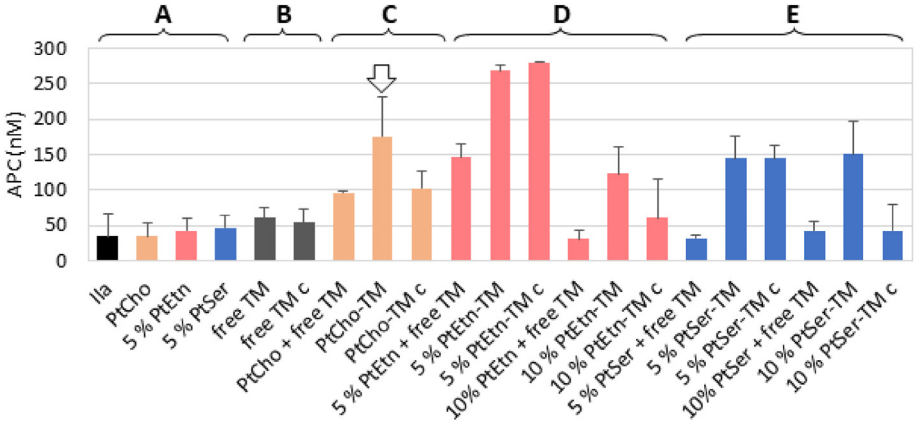

Fig. 2. APC generation activities of liposomal TM with different lipids. (**A**) Controls (IIa, PtCho liposome, 5% PtCho and 5%PtSer liposome), (**B**) free TM, (**C**) TM in/and PtCho liposome, (**D**) TM in/and PtCho/PtEtn liposome, (**E**) TM in/and PtCho/PtSer liposome. "c" at the end of sample name (i.e. free TM c) indicates co-incubation of protein C (960 nM) and TAFI (960 nM) in the same reaction mixture. Lipid + free TM indicates free TM added to the PtCho liposomes containing PtEtn or PtSer lipid. Data were presented as average ± SD (n = 3), with three independent repeats of the experiment. All samples are compared to PtCho TM-liposomes (arrow).

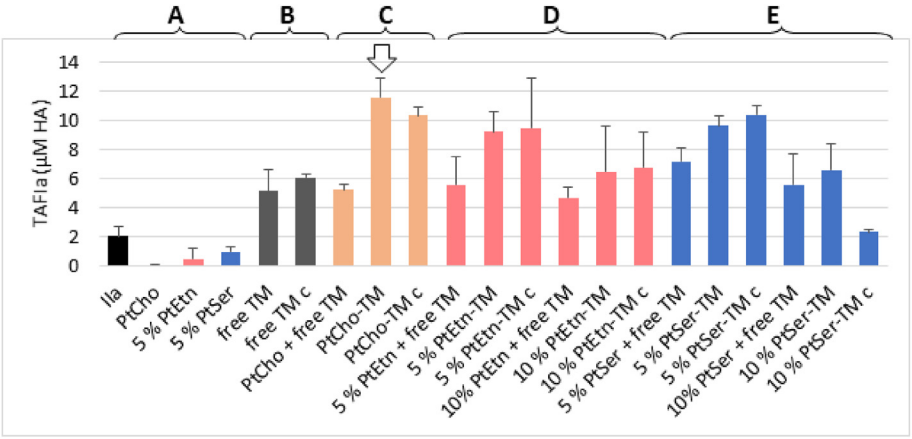

Fig. 3. TAFIa generation activities of liposomal TM with different lipids. (**A**) Controls (IIa, PtCho liposome, 5% PtCho and 5%PtSer liposome), (**B**) free TM, (**C**) TM and PtCho liposome, (**D**) TM and PtCho/PtEtn liposome, (**E**) TM and PtCho/PtSer liposome. "c" at the end of sample name (i.e. free TM c) indicates co-incubation of protein C (960 nM) and TAFI (960 nM) in the same reaction mixture. Lipid + free TM indicates free TM added to the mixture containing liposomes of different lipid composition. Data were presented as average ± SD (n = 3), with three independent repeats of the experiment. All samples are compared to PtCho TM-liposomes (arrow).

time of liposomal TM with 5% PtEtn and 5% PtSer (Fig. 4), which are correlated with their lower TAFI activation activity compared to liposomal TM with PtCho (Fig. 3). However, approximately about 25% reduction in fibrinolysis time in 10% PtEtn and 10% PtSer compared to liposomal TM with PtCho (Fig. 4) even though their TAFI activation activities are much lower than liposomal TM with PtCho. The difference between TAFIa activity and fibrinolysis time in liposomal TM with 10% PtEtn and 10% PtSer may be due to phospholipid effect on plasminogen and prothrombin in the plasma, which deserves additional study.

4. Conclusion

This study demonstrates the preparation, characterization, and cofactor activity studies of liposomal TM as a surrogate of endothelial TM-microparticles. It also investigates cofactor activities of liposomal TM on protein C and TAFI activation. This study confirmed increased protein C activation in 5% PtEtn lipid environment while observing reduction in TAFI activation in 5% PtSer and 10% PtEtn and PtSer lipid environment in comparison to PtCho liposomes. In addition, lipid-dependent competition between TAFI and protein C was observed on the liposomes. Further, fibrinolysis time at physiological enzyme concentrations was reduced in high PtSer lipid environments. This study indicates that PtEtn and PtSer lipids appear to influence TM's cofactor activity for both protein C and TAFI. This effect is lipid- and concentration-dependent and additional studies including wider range of lipid components are warranted. Overall, these results indicate that microparticle-TM may have different cofactor activities in comparison to cell membrane TM. The liposomal protein conjugate preparation outlined in this study can be used to incorporate other protein and lipid components into a microparticle surrogate and study the activities of interest.

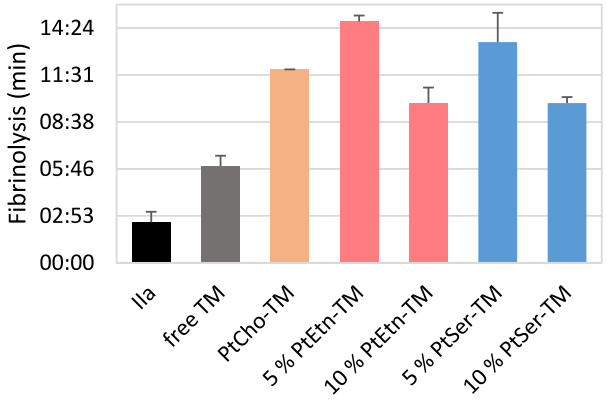

Fig. 4. Lipid effects on free TM and liposomal TM fibrinolysis rates. Average rates of fibrinolysis of liposomal TM with different lipids. Data were presented as average ± SD (n = 3), with three independent repeats of the experiment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.02.024.

Supplementary data to this article can be found online.

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