

Prothrombin loading of vascular smooth muscle cell-derived exosomes regulates coagulation and calcification

Kapustin, Alexander; Schoppel, Michael; Schurgers, Leon; Reynolds, Joanne; McNair, Rosamund; Heiss, Alexander; Jahnen-Dechent, Willi; Hackeng, Tilman; Schlieper, Georg; Harrison, Paul; Shanahan, Catherine

License:

None: All rights reserved

Document Version

Peer reviewed version

Citation for published version (Harvard):

Kapustin, A, Schoppel, M, Schurgers, L, Reynolds, J, McNair, R, Heiss, A, Jahnen-Dechent, W, Hackeng, T, Schlieper, G, Harrison, P & Shanahan, C 2016, 'Prothrombin loading of vascular smooth muscle cell-derived exosomes regulates coagulation and calcification', *Arteriosclerosis Thrombosis and Vascular Biology*.

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

Eligibility for repository: Checked on 09/1/2017

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Prothrombin loading of vascular smooth muscle cell-derived exosomes regulates coagulation and calcification

Alexander N. Kapustin^{1*}, Michael Schoppet^{2*}, Leon J. Schurgers³, Joanne L. Reynolds¹, Rosamund McNair¹, Alexander Heiss⁴, Willi Jahnen-Dechent⁴, Tilman M. Hackeng³, Georg Schlieper⁵, Paul Harrison⁶ and Catherine M. Shanahan¹

¹*BHF Centre of Research Excellence, Cardiovascular Division, King's College London, UK*

²*Department of Internal Medicine and Cardiology,
Philipps-University, Marburg, Germany*

³*Department of Biochemistry, Cardiovascular Research Institute CARIM,
University of Maastricht, The Netherlands*

⁴*Department of Biomedical Engineering, RWTH Aachen University, Germany*

⁵*Department of Nephrology and Clinical Immunology,
RWTH Aachen University, Germany*

⁶*Institute of Inflammation and Ageing, College of Medical and Dental Sciences, University of
Birmingham, Birmingham, UK*

**These authors contributed equally to this work*

Keywords: Smooth muscle cells, vascular calcification, prothrombin, exosomes

Subject codes: Basic, Translational, and Clinical Research/ Vascular Biology

Word count: 6252

Number of figures and tables: 5 figures and 2 tables

TOC category: basic studies

TOC subcategory: vascular biology

Running title: Circulating Gla proteins inhibit calcification

Address for correspondence:

Catherine Shanahan, PhD
Cardiovascular Division
James Black Centre

King's College London
125 Coldharbour Lane
London SE5 9NU
Tel: ++44 20 7848 5221
Fax ++44 20 7848 5193
Email: cathy.shanahan@kcl.ac.uk

Abstract

Objective-The drug warfarin blocks carboxylation of vitamin K dependent proteins and acts as an anticoagulant and an accelerant of vascular calcification. The calcification inhibitor matrix Gla protein (MGP), produced by vascular smooth muscle cells (VSMCs), is a key target of warfarin action in promoting calcification, however it remains unclear whether proteins in the coagulation cascade also play a role in calcification.

Approach and Results-Vascular calcification is initiated by exosomes and proteomic analysis revealed that VSMC exosomes are loaded with Gla-containing coagulation factors; IX and X, prothrombin (PT) and proteins C and S. Tracing of Alexa488-labeled PT showed that exosome loading occurs by direct binding to externalized phosphatidylserine (PS) on the exosomal surface and by endocytosis and recycling via late endosomes/multivesicular bodies (LE/MVB). Notably, the PT Gla domain and a synthetic Gla-domain peptide inhibited exosome-mediated VSMC calcification by preventing nucleation site formation on the exosomal surface. PT was deposited in the calcified vasculature and there was a negative correlation between vascular calcification and the levels of circulating PT. In addition, we found that VSMC exosomes induced thrombogenesis in a tissue factor (TF)- and PS-dependent manner.

Conclusions- Gamma carboxylated coagulation proteins are potent inhibitors of vascular calcification suggesting warfarin action on these factors also contributes to accelerated calcification in patients receiving this drug. VSMC exosomes link calcification and coagulation acting as novel activators of the extrinsic coagulation pathway and inducers of calcification in the absence of Gla-containing inhibitors.

Graphic Abstract

Abbreviations

EV, extracellular vesicles

F1, prothrombin fragment 1

F1.2, prothrombin fragment 1+2

Gla, Carboxyglutamic acid

LE/MVB, late endosomes/multivesicular bodies

MGP, matrix Gla protein

PIVKA-II, protein induced by vitamin K absence or antagonism II

Pre2, prethrombin-2 (Pre-2)

PS, phosphatidylserine

PT, prothrombin

TF, tissue factor

VSMCs, vascular smooth muscle cells (VSMCs),

Introduction

Vascular calcification contributes to increased cardiovascular morbidity and mortality in ageing, atherosclerosis, diabetes mellitus and chronic kidney failure^{1, 2}. It occurs in the medial and intimal layers of arteries and is driven by vascular smooth muscle cells (VSMCs) which can undergo osteo-chondrogenic differentiation and secrete calcifying exosomes or apoptotic bodies that initiate deposition of calcium (Ca) phosphate (P) crystals³⁻⁵.

Under physiological conditions VSMCs are protected from calcification by expression of inhibitors including the vitamin K-dependent proteins; matrix Gla protein (MGP), bone Gla protein (BGP; osteocalcin), Gla rich protein (GRP) and growth arrest-specific gene-6 protein (Gas 6)⁶⁻⁹. These proteins all contain specific glutamate residues (Glu) necessary for their function that are carboxylated in a vitamin K-dependent manner. Increased accumulation of uncarboxylated, inactive forms of MGP, GRP and BGP have been observed in the calcified vasculature or the circulation of patients with vascular calcification stressing the importance of adequate vitamin K-status in the inhibition of calcification^{8, 10-12}.

It is still unknown how calcification is inhibited by vitamin K-dependent proteins. Reduced Gas6 carboxylation results in inhibition of Axl-dependent anti-apoptotic pathways and subsequent VSMC apoptosis and calcification^{9, 13}. MGP has been implicated in BMP-2 binding and inhibition of BMP2-dependent osteogenic signalling¹⁴. MGP has also been detected in VSMC exosomes where it inhibits Ca/P precipitation although the precise mechanisms by which the Gla domain inhibits the nucleation of Ca salts remain unclear^{15, 16}. Interestingly, VSMCs internalise liver derived fetuin-A from the circulation and load it into exosomes where it acts as a potent inhibitor of calcification. Thus, both locally expressed as well as serum-derived proteins are involved in the prevention of exosome-mediated VSMC calcification⁵.

Circulating vitamin K-dependent coagulation factors including prothrombin (PT) and factors VII, IX and X and proteins C and S also harbour Gla residues which mediate Ca-dependent protein binding to negatively charged phospholipids, such as PS¹⁷⁻²¹. Activation of coagulation in the vasculature has been linked to PS-exposing extracellular vesicles (EVs) shed by apoptotic macrophages, endothelial cells, erythrocytes and VSMCs^{17, 22, 23, 24}. In particular, the coagulation cascade is triggered by VSMC EVs bearing tissue factor (TF) that can activate Factor VII with subsequent activation of the prothrombinase complex (consisting of Factor Va, Factor Xa and calcium) to catalyze PT conversion to thrombin^{17, 22, 25-28}. Anticoagulation therapy with warfarin causes elevation of circulating levels of liver-derived uncarboxylated PT (PIVKA-II) and results in the inhibition of thrombogenesis²⁹. Besides its effect on vitamin K-dependent coagulation proteins, warfarin increases circulating levels of undercarboxylated MGP and BGP

and causes vascular calcification in rodent models and accelerates both valve and vascular calcification in man³⁰⁻³³. However, despite the global use of anticoagulation therapies in clinical practice, the effects of warfarin on VSMC mediated calcification remain elusive.

Here we report that circulating vitamin K-dependent coagulation proteins, PT in particular, can bind to the surface of VSMC exosomes via PS and can also be loaded into exosomes by cellular internalization and recycling via the late endosome/multivesicular body (LE/MVB) compartment. We identify PT as a novel circulating calcification inhibitor that also acts to induce thrombin generation in a TF- and PS-dependent manner on the surface of exosomes. Thus, anticoagulation therapy with warfarin may enhance vascular calcification via impaired carboxylation of vitamin K-dependent coagulation factors delivered to VSMC exosomes, which play a dual role in calcification and coagulation.

Material and methods

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

Results

Circulating Gla proteins are abundant in VSMC exosomes

We previously demonstrated the recycling of the circulating serum protein, fetuin-A by VSMC exosomes.⁵ To determine whether other serum proteins are recycled in VSMC exosomes we interrogated a proteomic dataset of human VSMC-derived exosomes⁵, isolated under normal or calcifying conditions, for bovine proteins indicative of serum uptake. The most abundant bovine proteins in VSMC exosomes were albumin, fetuin-A and fibronectin (Table 1). Notably, circulating vitamin K-dependent coagulation proteins including factors II (PT), IX and X, and anticoagulation proteins C and S were also detected in VSMC-derived exosomes and their loading was increased under calcifying conditions (Table 1, *Number of Assigned Spectra*).

We compared the primary structure of circulating vitamin K-dependent coagulation proteins and MGP⁷. The alignment of the Gla-domain sequences revealed a similarity, of over 50% in the composition of the Gla-domain between vitamin K-dependent coagulation proteins (Supplementary Figs. S1A and S1B). Prominently, there were 4 conserved Glu-residues and one cysteine residue in all these proteins suggesting coagulation proteins may potentially modulate vascular calcification.

Gla proteins differentially inhibit VSMC calcification in vitro

VSMCs were treated with physiological doses of a number of Gla containing proteins to test their capacity to inhibit calcification *in vitro* (Fig. 1A). MGP is a potent local inhibitor of calcification however circulating MGP has no inhibitory properties *in vivo*³⁴ and is only effective *in vitro* at doses above physiological levels³⁵. As expected MGP1-53 (Supplementary Figs. S2A and C), osteopontin and osteocalcin had little effect on calcification when added exogenously to the media at circulating concentrations (Fig. 1A). In contrast PT, the most abundant circulating coagulation factor with physiological concentrations ~1.4 μ M was able to potently inhibit calcification in a manner similar to fetuin A (Fig. 1A).

PT expression was not observed in VSMCs either by western blotting or PCR (Supplementary Fig. S3A). However, incubation of VSMCs with PT resulted in its accumulation in exosomes, apoptotic bodies and whole cell lysates (Fig. 1B) with exosomes also enriched with PT intermediate activation products, F1 and F1.2 (Fig. 1B). In agreement with our proteomic data, calcifying conditions increased the levels of PT and F1 both in exosomes and VSMCs (Fig. 1C).

PT inhibits exosome-induced calcification and reduces VSMC apoptosis.

To test further the effects of PT on vascular calcification we treated VSMCs in elevated Ca/P conditions and assessed calcification by ^{45}Ca incorporation and Alizarin red S staining (Figs. 2A and B). PT inhibited calcification in a dose-dependent manner at concentrations within the physiological range. The inhibitory effects of PT were more pronounced in Ca- rather than P-induced calcification (Supplementary Figs. S4A and S4B). Fetuin-A blocks calcification by binding to nascent Ca/P crystals preventing further growth and acting as a “mineral chaperone”.^{36,37} Fetuin-A and PT inhibited VSMC calcification in a synergistic manner indicating that PT acts via different mechanisms (Fig. 2C) and in agreement with this fetuin-A but not PT completely prevented Ca/P precipitation *in vitro* (Fig. 2D).

To study the effects of PT on exosome-initiated calcification, exosomes were isolated from VSMCs treated in the absence or presence of PT and their calcification potential was quantified *ex vivo*¹⁵. Exosomes isolated from Ca/P treated VSMCs displayed calcification (Fig. 2E). Direct addition of PT to exosomes isolated from VSMCs incubated in Ca/P media significantly reduced exosome calcification (Fig. 2E), suggesting PT may inhibit calcification by binding to PS externalized on the exosome surface thus preventing nucleation site formation. However, inhibition of exosome calcification was also observed when VSMCs were treated with PT for 24h and then washed to remove PT from the media before exosome isolation 24h later, suggesting PT is also loaded into exosomes via the LE/MVB pathway.

VSMC apoptosis also contributes to calcification and treatment of VSMCs with calcifying media resulted in apoptosis of approximately 20-25% of cells as assessed by TUNEL staining and time-lapse microscopy (Supplementary Figs. S4C and S4D). Addition of PT significantly reduced VSMC apoptosis induced by elevated Ca/P, but had no effect on apoptosis induced by serum withdrawal (data not shown), suggesting PT can prevent mineral overload induced apoptosis.

PT is recycled in VSMC exosomes in a Ca-dependent manner.

To test if PT can directly bind to VSMC exosomes we immobilised exosomes on latex beads, incubated with Alexa488-labelled PT and observed strong binding (Fig. 3A). The interaction of PT with PS is mediated by the N-terminal F1 domain harbouring Gla-residues²⁰ and, consistently, addition of annexin A5 or F1 blocked PT binding to exosomes (Fig. 3A). We also observed reciprocal competition for interactions with exosomal PS between PT and annexin A5 (Supplementary Fig. S3B).

Addition of alexa488-labelled PT to VSMCs resulted in endocytosis of full-length PT in a Ca-dependent manner (Fig. 3B). To test whether PT endocytosis is mediated by high-affinity

binding to a cellular receptor or fluid-phase uptake³⁸, we studied endocytosis of alexa488-labelled PT in the presence of unlabeled PT. Notably, endocytosis of alexa488-labelled PT was inhibited by 5-fold molar excess of unlabelled PT, indicating that uptake is mediated by high affinity binding of PT to the cell surface potentially via Gla/PS binding (Fig. 3C). Colocalization with EEA1 and CD63 showed that PT is endocytosed by VSMCs and delivered to early endosomal and LE/MVB compartments (Fig. 3D). Immunocytochemistry also confirmed that PT-Alexa488 uptake was further stimulated by elevated Ca and inhibited by unlabelled PT (Fig. 3D). To distinguish PT which binds to exosomes in the cell media, and PT which is endocytosed and recycled in exosomes via LE/MVB we incubated VSMCs with PT for 16h (“pulse”) and then washed cells with acidic buffer (pH 2.5) to remove all cell-surface bound PT and continued incubation in media without PT (“chase”). Exosomes isolated from VSMCs incubated in the presence of PT in the media, were enriched with full-length protein and fragments, F1.2 and F1 (Fig. 3E). In contrast, exosomes obtained after the chase treatment contained full length PT and its intermediate activation product with molecular weight ~34kDa, most likely Pre2, an inactive α -thrombin precursor consisting of the catalytic domain. (Fig. 3E and Supplementary Fig. S1C). Thus, PT is loaded on, and in, exosomes by direct surface binding and cellular endocytosis.

The Gla domain but not proteolytic activity is required for VSMC calcification inhibition by PT.

To identify the domain of PT responsible for blocking calcification we tested different PT forms (Fig. 4A). F1 which contains 10 Gla residues was the most potent inhibitor, even more effective than full length PT. F2, which does not contain a Gla domain, and uncarboxylated (Glu)-PT were also able to inhibit calcification although to a much lesser extent, indicating that several domains contribute to PT effects on calcification. In contrast, thrombin had no effect on VSMC calcification (Fig. 4A). To confirm that catalytic activity is not required for the PT inhibitory effect, we showed that addition of the thrombin inhibitor hirudin did not abrogate PT inhibitory effects on VSMC calcification (Fig. 4B). Finally, to investigate whether other Gla-containing coagulation proteins identified in exosomes may also affect VSMC calcification, we used a Gla peptide representing the N-terminal region of protein S containing 11 Gla residues which forms a stable conformation due to its intramolecular disulfide loop (Supplementary Figs. S2B and S2C). Addition of this Gla peptide completely abrogated VSMC calcification (Fig. 4B) confirming the utility of Gla residues in inhibiting calcification.

PT accumulates at sites of calcification in vivo and is reduced in patients with vascular calcification.

To confirm a relationship between calcification and PT we analysed the distribution of PT in the calcified vasculature using an F1-specific antibody. Immunohistochemistry showed that low levels of PT were deposited in the healthy vessel wall while in calcified vessels PT was significantly present in both intimal and medial layers (Fig. 4C). Moreover, we observed overlap between the distribution of PT and fetuin-A, suggesting that PT is deposited in the vessel wall most likely via exosomes in a manner similar to fetuin-A^{5, 36}.

Fetuin-A levels are reduced in patients with vascular calcification, thus we tested whether there is also a correlation between vascular calcification and PT concentration. Vascular calcification of the iliac arteries was assessed by plain X-ray of the pelvis (Table 2). Patients with vascular calcification had lower levels of PT ($p < 0.01$) when compared to patients without calcification. Univariate logistic regression confirmed this finding ($p = 0.012$; hazard ratio 0.971; 95% confidence interval 0.948–0.993) and after adjustment for age, gender, and diabetes mellitus the finding remained significant ($p = 0.004$; hazard ratio 0.905; 95% confidence interval 0.845–0.969). Addition of fetuin-A levels to the multivariable model did not change the results (data not shown).

VSMC exosomes display TF and PS-dependent procoagulant activity.

The accumulation of PT activation products, F1 and F1.2 in exosomes (Fig. 1B) suggested that exosomes may play a role in thrombin activation (Supplementary Fig. S1C) so we tested if VSMC exosomes contain TF and found it was enriched in exosomes but not apoptotic bodies (Fig. 5A). The addition of VSMC exosomes to plasma potently stimulated thrombogenesis, which was abrogated in the presence of annexin A5 (Fig. 5B) confirming thrombogenic activity is via PS exposure. Once PT is activated, F1 and F1.2 bound to exosomes may prevent further activation of PT by blocking PS sites. Indeed, exosomes isolated from VSMCs pre-incubated with PT demonstrated reduced thrombogenic activity (Fig. 5C).

Notably, no thrombin activation was observed in PT-deficient plasma upon addition of exosomes with bound PT (data not shown). PT binding to exosomes may prevent prothrombinase formation by blocking PS, so we tested if exosome-associated PT can be activated by a pre-activated prothrombinase complex. First, we incubated PT and factors Va/Xa with exosomes isolated from VSMCs incubated in the absence of PT and analysed the supernatant and exosome-associated products by western blotting using antibodies specific for either F1 or catalytic domain (Fig. 5D). No PT was detected in the exosomes isolated from VSMCs incubated in PT-free conditions (Fig. 5D, *lane 1*). Upon addition of PT we observed soluble and exosome-associated full-length PT (Fig. 5D, *lane 2*). Addition of activated

prothrombinase (Va/Xa) along with PT resulted in the release of thrombin heavy chain (B chain) with molecular weight ~31kDa in the supernatant phase (Fig 5D, *lane 3*). We also observed B chain and activation product F1 associated with exosomes (Fig 5D, *lane 3*). Next, we analysed exosomes isolated from VSMCs treated in the presence of PT. Using anti-F1 antibodies we detected PT and PT intermediate activation products, F1, F1.2 associated with exosomes (Fig. 5D, *lane 4*). Using an anti-catalytic domain antibody we detected PT and an intermediate activation product with molecular weight ~34kDa (Fig. 5C, *lane 4*), again most likely Pre2 as compared to the position of B chain (Fig 5D, *lane 3*). Addition of prothrombinase had no effect on exosome-associated PT and PT activation products (Fig. 5D, *lane 5*), indicating that there is a pool of exosome-bound PT and PT intermediate activation products not available to prothrombinase. Thus, VSMC exosomes are thrombogenic however accumulation of PT and PT intermediate activation products reduces their pro-thrombogenic properties in a negative-feedback loop.

Altogether these data show that VSMC exosomes activate coagulation in a TF- and PS-dependent manner and accumulation of PT and PT activation products diminish both PS-mediated pro-calcific and pro-coagulant exosomal activities in a negative-feedback manner (Fig. 6).

Discussion

PT is loaded on VSMC exosomes and protects against calcification in a Gla-dependant manner.

Vitamin K-antagonist treatment aimed at the prevention of thromboembolic complications causes accelerated vascular calcification in both animal models and patients^{32, 33} and this effect is thought to be associated with accumulation of uncarboxylated endogenously expressed Gla proteins such as MGP and GRP^{7, 8, 10, 30}. In the present study we show for the first time that the Gla-residues in circulating coagulation factors, PT in particular, also contribute to inhibition of vascular calcification. As opposed to fetuin-A, a circulating calcification inhibitor which acts by direct binding to nascent growing calcium phosphate crystals to limit growth^{5, 37}, PT inhibits exosome-mediated calcification via a PS/Gla interaction, thus disrupting nucleation site formation. ‘Pulse-chase’ experiments indicated that there are two mechanisms of PT loading into exosomes – direct binding to the exosomal membrane and calcium-dependent endocytosis and secretion via the LE/MVB/exosome pathway. The contribution of the latter pathway of PT loading into exosomes on calcification is currently unclear. This PT pool may be loaded inside

exosomes via unknown mechanisms described previously for fetuin-A^{5, 39} where it can shield luminal PS thus enhancing the overall protective effects against calcification. Interestingly, PT endocytosis and exosomal loading were stimulated by calcium, which is required for the PS/Gla interaction⁴⁰ and elevated calcium conditions have been observed in atherosclerotic plaques⁴¹ and in CKD patients suggesting that the internal loading pathway may be important under conditions of mineral imbalance. Accumulating evidence demonstrates the toxicity of crystalline Ca/P particles thus inhibition of crystal nucleation by PS/Gla interactions could also limit VSMC apoptosis^{42, 43}. Interestingly, fragment F2 also displayed a modest protective effect indicating that the F2 kringle 2 domain could contribute to the regulation of VSMC viability in calcifying conditions by modulating cell signalling but this hypothesis is yet to be tested.

Importantly, extensive calcification of elastic fibres is commonly observed in patients with pseudoxanthoma elasticum harbouring mutations in γ -glutamyl carboxylase which causes multiple coagulation factor deficiency⁴⁴ providing further evidence that Gla-containing coagulation factors may regulate vascular calcification *in vivo*. PTs role in calcification is also supported independently by previous reports showing its presence in association with calcified kidney stones⁴⁵. In this study we also detected PT in close association with calcified regions in human arteries where its distribution overlapped with fetuin-A deposits and the reduction of circulating PT levels negatively correlated with vascular calcification. These data mirror previous clinical data with fetuin-A showing reduced levels in dialysis patients who exhibit enhanced vascular calcification and mineral imbalance⁴⁶. Interestingly, as opposed to MGP clinical data, the appearance of minute levels of under-carboxylated PT in the circulation (>2ng/ml which corresponds to $\approx 0.00003\mu\text{mol/L}$) is not associated with vascular calcification⁴⁷ as long as the total circulating PT levels remain high ($\approx 1.4\mu\text{mol/L}$). Although the mechanisms of fetuin-A and PT loss in the circulation in dialysis patients are yet to be investigated it is tempting to speculate that the major cause is the formation of fetuin-mineral complexes consisting of Ca/P and fetuin-A initially identified in the serum of rats treated with bisphosphonates⁴⁸. Formation of complexes between fetuin-A and nascent mineral nuclei into primary calciprotein complexes is thought to inhibit further mineral growth and may facilitate clearance from the blood⁴⁸ and these complexes have been recently associated with coronary artery calcification scores and aortic aortic pulse wave velocity in CKD patients^{49, 50}. However, the exact composition of these complexes remains elusive and PT and fetuin-A-loaded exosomes may contribute by providing the membrane surface to form the fetuin-A-mineral complex core at early phases of calciprotein complex formation. *In vivo* these complexes are yet to be tested for the presence of PT and exosomal

markers such as CD63 or Tsg101 and it would also be interesting to test how warfarin treatment affects the composition of these complexes.

VSMC exosomes are thrombogenic

Recent studies suggest that thrombogenic activity in the atherosclerotic plaque is associated with EVs shed by apoptotic cells, especially VSMCs.^{17, 22, 25, 26} Although VSMC EV thrombogenic activity exceeds the potency of platelet-derived EVs, most likely due to the presence of TF and PS, the mechanisms of thrombogenic EV secretion by VSMCs remained unknown²². TF has recently been shown to be secreted in exosomes in various cell types and was detected in VSMCs in cytoplasmic MVB-like structures and in VSMC culture media in small particles of an unknown nature^{24, 51, 52}. Here we report that apart from triggering calcification, PS externalized on VSMC exosomes, acts as a catalytic surface for thrombogenesis. Interestingly, activation of PT on apoptotic cells or synthetic PS/PC vesicles occurs via the initial cleavage of PT at R320 yielding meizothrombin, a potent activator of anti-coagulant pathways^{53, 54}. In contrast, we detected Pre2 and F1.2 on VSMC exosomes indicating that activation of PT on VSMC exosomes occurs by the PT cleavage at R271 generating catalytically inactive Pre2 and F1.2 and Pre2 can be instantly cleaved at R320 generating procoagulant α -thrombin⁵⁵. This specificity may be defined by specific membrane proteins, such as integrins as reported earlier for platelets⁵⁶. Unexpectedly, we observed a pool of exosome-associated PT and Pre2 that was not available for activation by factor Xa and these exosomes displayed reduced thrombogenic activity. There are two possible explanations for this pool of PT that cannot be activated. Firstly, PT may be loaded inside the exosomal lumen as discussed above. Secondly, activation of PT by factor Xa, by cleavage of the R320 bond in particular, requires membrane binding of factor Xa suggesting sterical hindrance may prevent activation of PT and Pre2²⁸.

In conclusion, PT is a novel circulating vascular calcification inhibitor acting via Gla/PS binding to exosomes. Binding of PT to exosomes also activates coagulation pathways whilst gradual loading of exosomes with PT and PT activation products reduces both pro-calcification and pro-coagulant activities by Gla/PS interactions. Hence, vascular protection in patients at high risk for vascular calcification may be achieved by using direct oral anticoagulants instead of warfarin or by supplementation with small Gla peptides e.g. Gla peptide derivative from protein S. Modulation of exosome biogenesis pathways, such as SMPD3 which is activated in vascular pathologies^{57, 58} may also provide alternative therapeutic approaches to reduce plaque thrombogenicity causing late stage thrombotic events in patients after myocardial infarction or coronary artery stenting⁵⁹.

Acknowledgments

Sources of funding

This work was supported by grants from the British Heart Foundation (BHF) to C.M.S and by a fellowship grant from Prof. Dr. A. Schmidtman-Stiftung, Germany to M.S.

Conflict of Interest Disclosures

None.

References

1. Sage AP, Tintut Y, Demer LL. Regulatory mechanisms in vascular calcification. *Nat Rev Cardiol.* 2010;7:528-536.
2. Shanahan CM, Crouthamel MH, Kapustin A, Giachelli CM. Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. *Circ Res.* 2011;109:697-711.
3. Shanahan CM, Cary NR, Salisbury JR, Proudfoot D, Weissberg PL, Edmonds ME. Medial localization of mineralization-regulating proteins in association with Monckeberg's sclerosis: evidence for smooth muscle cell-mediated vascular calcification. *Circulation.* 1999;100:2168-2176.
4. Proudfoot D, Skepper JN, Hegyi L, Bennett MR, Shanahan CM, Weissberg PL. Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. *Circ Res.* 2000;87:1055-1062.
5. Kapustin AN, Chatrou ML, Drozdov I, Zheng Y, Davidson SM, Soong D, Furmanik M, Sanchis P, De Rosales RT, Alvarez-Hernandez D, Shroff R, Yin X, Muller K, Skepper JN, Mayr M, Reutelingsperger CP, Chester A, Bertazzo S, Schurgers LJ, Shanahan CM. Vascular smooth muscle cell calcification is mediated by regulated exosome secretion. *Circ Res.* 2015;116:1312-1323.
6. Luo G, Ducey P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature.* 1997;386:78-81.
7. Shanahan CM, Proudfoot D, Tyson KL, Cary NR, Edmonds M, Weissberg PL. Expression of mineralisation-regulating proteins in association with human vascular calcification. *Z Kardiol.* 2000;89 Suppl 2:63-68.
8. Viegas CS, Rafael MS, Enriquez JL, Teixeira A, Vitorino R, Luis IM, Costa RM, Santos S, Cavaco S, Neves J, Macedo AL, Willems BA, Vermeer C, Simes DC. Gla-rich protein acts as a calcification inhibitor in the human cardiovascular system. *Arterioscler Thromb Vasc Biol.* 2015;35:399-408.
9. Collett GD, Sage AP, Kirton JP, Alexander MY, Gilmore AP, Canfield AE. Axl/phosphatidylinositol 3-kinase signaling inhibits mineral deposition by vascular smooth muscle cells. *Circ Res.* 2007;100:502-509.
10. Schurgers LJ, Teunissen KJ, Knapen MH, Kwaijtaal M, van Diest R, Appels A, Reutelingsperger CP, Cleutjens JP, Vermeer C. Novel conformation-specific antibodies against matrix gamma-carboxyglutamic acid (Gla) protein: undercarboxylated matrix Gla

- protein as marker for vascular calcification. *Arterioscler Thromb Vasc Biol.* 2005;25:1629-1633.
11. Cranenburg EC, Vermeer C, Koos R, Boumans ML, Hackeng TM, Bouwman FG, Kwaijtaal M, Brandenburg VM, Ketteler M, Schurgers LJ. The circulating inactive form of matrix Gla Protein (ucMGP) as a biomarker for cardiovascular calcification. *J Vasc Res.* 2008;45:427-436.
 12. Szulc P, Chapuy MC, Meunier PJ, Delmas PD. Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *J Clin Invest.* 1993;91:1769-1774.
 13. Nakano T, Kawamoto K, Kishino J, Nomura K, Higashino K, Arita H. Requirement of gamma-carboxyglutamic acid residues for the biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular smooth muscle cells. *Biochem J.* 1997;323 (Pt 2):387-392.
 14. Zebboudj AF, Imura M, Bostrom K. Matrix GLA protein, a regulatory protein for bone morphogenetic protein-2. *J Biol Chem.* 2002;277:4388-4394.
 15. Reynolds JL, Joannides AJ, Skepper JN, McNair R, Schurgers LJ, Proudfoot D, Jahnen-Dechent W, Weissberg PL, Shanahan CM. Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. *J Am Soc Nephrol.* 2004;15:2857-2867.
 16. Kapustin AN, Davies JD, Reynolds JL, McNair R, Jones GT, Sidibe A, Schurgers LJ, Skepper JN, Proudfoot D, Mayr M, Shanahan CM. Calcium regulates key components of vascular smooth muscle cell-derived matrix vesicles to enhance mineralization. *Circ Res.* 2011;109:e1-e12.
 17. Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. *Circulation.* 1999;99:348-353.
 18. Bombeli T, Karsan A, Tait JF, Harlan JM. Apoptotic vascular endothelial cells become procoagulant. *Blood.* 1997;89:2429-2442.
 19. Flynn PD, Byrne CD, Baglin TP, Weissberg PL, Bennett MR. Thrombin generation by apoptotic vascular smooth muscle cells. *Blood.* 1997;89:4378-4384.
 20. Gitel SN, Owen WG, Esmon CT, Jackson CM. A polypeptide region of bovine prothrombin specific for binding to phospholipids. *Proc Natl Acad Sci U S A.* 1973;70:1344-1348.
 21. Nesheim ME, Taswell JB, Mann KG. The contribution of bovine Factor V and Factor Va to the activity of prothrombinase. *J Biol Chem.* 1979;254:10952-10962.
 22. Leroyer AS, Isobe H, Leseche G, Castier Y, Wassef M, Mallat Z, Binder BR, Tedgui A, Boulanger CM. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. *J Am Coll Cardiol.* 2007;49:772-777.
 23. Schecter AD, Giesen PL, Taby O, Rosenfield CL, Rossikhina M, Fyfe BS, Kohtz DS, Fallon JT, Nemerson Y, Taubman MB. Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools after growth factor stimulation. *J Clin Invest.* 1997;100:2276-2285.
 24. Schecter AD, Spirn B, Rossikhina M, Giesen PL, Bogdanov V, Fallon JT, Fisher EA, Schnapp LM, Nemerson Y, Taubman MB. Release of active tissue factor by human arterial smooth muscle cells. *Circ Res.* 2000;87:126-132.
 25. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Natl Acad Sci U S A.* 1989;86:2839-2843.
 26. Toschi V, Gallo R, Lettino M, Fallon JT, Gertz SD, Fernandez-Ortiz A, Chesebro JH, Badimon L, Nemerson Y, Fuster V, Badimon JJ. Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation.* 1997;95:594-599.

27. Mann KG, Nesheim ME, Church WR, Haley P, Krishnaswamy S. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood*. 1990;76:1-16.
28. Bradford HN, Orcutt SJ, Krishnaswamy S. Membrane binding by prothrombin mediates its constrained presentation to prothrombinase for cleavage. *J Biol Chem*. 2013;288:27789-27800.
29. Suttie JW, Mummah-Schendel LL, Shah DV, Lyle BJ, Greger JL. Vitamin K deficiency from dietary vitamin K restriction in humans. *Am J Clin Nutr*. 1988;47:475-480.
30. Schurgers LJ, Barreto DV, Barreto FC, Liabeuf S, Renard C, Magdeleyns EJ, Vermeer C, Choukroun G, Massy ZA. The circulating inactive form of matrix gla protein is a surrogate marker for vascular calcification in chronic kidney disease: a preliminary report. *Clin J Am Soc Nephrol*. 2010;5:568-575.
31. Menon RK, Gill DS, Thomas M, Kernoff PB, Dandona P. Impaired carboxylation of osteocalcin in warfarin-treated patients. *J Clin Endocrinol Metab*. 1987;64:59-61.
32. Price PA, Faus SA, Williamson MK. Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler Thromb Vasc Biol*. 1998;18:1400-1407.
33. Wahlqvist ML, Tanaka K, Tzeng BH. Clinical decision-making for vitamin K-1 and K-2 deficiency and coronary artery calcification with warfarin therapy: are diet, factor Xa inhibitors or both the answer? *Asia Pac J Clin Nutr*. 2013;22:492-496.
34. Murshed M, Schinke T, McKee MD, Karsenty G. Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins. *J Cell Biol*. 2004;165:625-630.
35. Schurgers LJ, Spronk HM, Skepper JN, Hackeng TM, Shanahan CM, Vermeer C, Weissberg PL, Proudfoot D. Post-translational modifications regulate matrix Gla protein function: importance for inhibition of vascular smooth muscle cell calcification. *J Thromb Haemost*. 2007;5:2503-2511.
36. Reynolds JL, Skepper JN, McNair R, Kasama T, Gupta K, Weissberg PL, Jahnen-Dechent W, Shanahan CM. Multifunctional roles for serum protein fetuin-a in inhibition of human vascular smooth muscle cell calcification. *J Am Soc Nephrol*. 2005;16:2920-2930.
37. Heiss A, DuChesne A, Denecke B, Grotzinger J, Yamamoto K, Renne T, Jahnen-Dechent W. Structural basis of calcification inhibition by alpha 2-HS glycoprotein/fetuin-A. Formation of colloidal calciprotein particles. *J Biol Chem*. 2003;278:13333-13341.
38. Goldstein JL, Brown MS. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J Biol Chem*. 1974;249:5153-5162.
39. Schlieper G, Aretz A, Verberckmoes SC, Kruger T, Behets GJ, Ghadimi R, Weirich TE, Rohrmann D, Langer S, Tordoir JH, Amann K, Westenfeld R, Brandenburg VM, D'Haese PC, Mayer J, Ketteler M, McKee MD, Floege J. Ultrastructural analysis of vascular calcifications in uremia. *J Am Soc Nephrol*. 2010;21:689-696.
40. Huang M, Rigby AC, Morelli X, Grant MA, Huang G, Furie B, Seaton B, Furie BC. Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins. *Nat Struct Biol*. 2003;10:751-756.
41. Olszak IT, Poznansky MC, Evans RH, Olson D, Kos C, Pollak MR, Brown EM, Scadden DT. Extracellular calcium elicits a chemokinetic response from monocytes in vitro and in vivo. *J Clin Invest*. 2000;105:1299-1305.
42. Ewence AE, Bootman M, Roderick HL, Skepper JN, McCarthy G, Epple M, Neumann M, Shanahan CM, Proudfoot D. Calcium phosphate crystals induce cell death in human vascular smooth muscle cells: a potential mechanism in atherosclerotic plaque destabilization. *Circ Res*. 2008;103:e28-34.

43. Dautova Y, Kozlova D, Skepper JN, Eppele M, Bootman MD, Proudfoot D. Fetuin-A and albumin alter cytotoxic effects of calcium phosphate nanoparticles on human vascular smooth muscle cells. *PLoS One*. 2014;9:e97565.
44. De Vilder EY, Vanakker OM. From variome to phenome: Pathogenesis, diagnosis and management of ectopic mineralization disorders. *World J Clin Cases*. 2015;3:556-574.
45. Stapleton AM, Simpson RJ, Ryall RL. Crystal matrix protein is related to human prothrombin. *Biochem Biophys Res Commun*. 1993;195:1199-1203.
46. Shroff RC, Shah V, Hiorns MP, Schoppet M, Hofbauer LC, Hawa G, Schurgers LJ, Singhal A, Merryweather I, Brogan P, Shanahan C, Deanfield J, Rees L. The circulating calcification inhibitors, fetuin-A and osteoprotegerin, but not matrix Gla protein, are associated with vascular stiffness and calcification in children on dialysis. *Nephrol Dial Transplant*. 2008;23:3263-3271.
47. Danziger J, Young RL, Shea KM, Duprez DA, Jacobs DR, Tracy RP, Ix JH, Jenny NS, Mukamal KJ. Circulating Des-gamma-carboxy prothrombin is not associated with cardiovascular calcification or stiffness: The Multi-Ethnic Study of Atherosclerosis (MESA). *Atherosclerosis*. 2016;252:68-74.
48. Price PA, Nguyen TM, Williamson MK. Biochemical characterization of the serum fetuin-mineral complex. *J Biol Chem*. 2003;278:22153-22160.
49. Hamano T, Matsui I, Mikami S, Tomida K, Fujii N, Imai E, Rakugi H, Isaka Y. Fetuin-mineral complex reflects extraosseous calcification stress in CKD. *J Am Soc Nephrol*. 2010;21:1998-2007.
50. Smith ER, Ford ML, Tomlinson LA, Rajkumar C, McMahon LP, Holt SG. Phosphorylated fetuin-A-containing calciprotein particles are associated with aortic stiffness and a procalcific milieu in patients with pre-dialysis CKD. *Nephrol Dial Transplant*. 2012;27:1957-1966.
51. Gardiner C, Harrison P, Belting M, Boing A, Campello E, Carter BS, Collier ME, Coumans F, Ettelaie C, van Es N, Hochberg FH, Mackman N, Rennert RC, Thaler J, Rak J, Nieuwland R. Extracellular vesicles, tissue factor, cancer and thrombosis - discussion themes of the ISEV 2014 Educational Day. *J Extracell Vesicles*. 2015;4:26901.
52. Mandal SK, Pendurthi UR, Rao LV. Cellular localization and trafficking of tissue factor. *Blood*. 2006;107:4746-4753.
53. Krishnaswamy S, Mann KG, Nesheim ME. The prothrombinase-catalyzed activation of prothrombin proceeds through the intermediate meizothrombin in an ordered, sequential reaction. *J Biol Chem*. 1986;261:8977-8984.
54. Hackeng TM, Tans G, Koppelman SJ, de Groot PG, Rosing J, Bouma BN. Protein C activation on endothelial cells by prothrombin activation products generated in situ: meizothrombin is a better protein C activator than alpha-thrombin. *Biochem J*. 1996;319 (Pt 2):399-405.
55. Haynes LM, Bouchard BA, Tracy PB, Mann KG. Prothrombin activation by platelet-associated prothrombinase proceeds through the prethrombin-2 pathway via a concerted mechanism. *J Biol Chem*. 2012;287:38647-38655.
56. Fager AM, Wood JP, Bouchard BA, Feng P, Tracy PB. Properties of procoagulant platelets: defining and characterizing the subpopulation binding a functional prothrombinase. *Arterioscler Thromb Vasc Biol*. 2010;30:2400-2407.
57. Galvani S, Trayssac M, Auge N, Thiers JC, Calise D, Krell HW, Sallusto F, Kamar N, Rostaing L, Thomsen M, Negre-Salvayre A, Salvayre R. A key role for matrix metalloproteinases and neutral sphingomyelinase-2 in transplant vasculopathy triggered by anti-HLA antibody. *Circulation*. 2011;124:2725-2734.

58. Kolmakova A, Kwiterovich P, Virgil D, Alaupovic P, Knight-Gibson C, Martin SF, Chatterjee S. Apolipoprotein C-I induces apoptosis in human aortic smooth muscle cells via recruiting neutral sphingomyelinase. *Arterioscler Thromb Vasc Biol.* 2004;24:264-269.
59. Yano M, Natsuaki M, Morimoto T, Nakagawa Y, Kawai K, Miyazaki S, Muramatsu T, Shiode N, Namura M, Sone T, Oshima S, Nishikawa H, Hiasa Y, Hayashi Y, Nobuyoshi M, Mitsudo K, Kimura T. Antiplatelet therapy discontinuation and stent thrombosis after sirolimus-eluting stent implantation: Five-year outcome of the j-Cypher Registry. *Int J Cardiol.* 2015;199:296-301.

Highlights.

1. Circulating Gla proteins are abundant in VSMC exosomes.
2. PT is a novel circulating vascular calcification inhibitor.
3. Gla domain but not proteolytic activity is required for VSMC calcification inhibition by PT.
4. PT accumulates at sites of calcification and PT circulation level is reduced in patients with vascular calcification.
5. VSMC exosomes display TF and PS-dependent thrombogenic activity.

Table 1. Bovine proteins in human VSMC exosomes detected by mass spectrometry.

VSMCs were incubated in DMEM supplemented with 0.1% BSA (CTRL, control) and 2.7mM Ca/2.5mM P (Ca/P) for 16h and exosomes isolated from cell media by differential centrifugation,

**proteins with Gla domain.*

Protein	Molecular Weight	Number of Assigned Spectra		Number of Unique Peptides		Percent Coverage	
		CTRL	Ca/P	CTRL	Ca/P	CTRL	Ca/P
<i>Calcification inhibitors</i>							
a-2-HS-glycoprotein	38	383	469	9	11	31	31
<i>Transport Proteins</i>							
Serum albumin	69	2834	2824	46	46	65	66
<i>Matrix proteins</i>							
Fibronectin	272	372	362	26	24	14	12
<i>Coagulation proteins</i>							
Prothrombin*	71	14	19	4	4	7.5	6.9
Factor X*	55	4	16	3	1	9.3	2.4
Factor V	249	10	12	4	3	1.9	1.5
Factor IX*	47	0	4	0	2	0	7.5
Protein C*	51	9	6	1	2	2.4	4.2
Protein S*	75	0	9	0	4	0	5.2

Table 2. Characterization of a patient cohort with chronic kidney disease (CKD), which were studied for their plasma concentrations of PT in relation to the presence of vascular calcification at the iliac/femoral arteries detected by plain X-ray of the pelvis.

	All	With vascular calcification	Without vascular calcification
Number	81	44	37
Gender (male/female)	40/41	25/19	15/22
Age (years)	55.38±9.33	56.43±9.46	54.14±9.15
BMI (kg/m ²)	23.47±3.40	23.32±3.03	23.66±3.82
Arterial hypertension	68 (84%)	35 (80%)	33 (89%)
Smoking	25 (31%)	17 (39%)	8 (22%)
Diabetes mellitus	7 (9%)	6 (14%)	1 (3%)
Years on dialysis	8.72±4.40	9.59±4.17	7.68±4.49
Cholesterol (mg/dl)	5.35±1.22	5.13±0.81	5.60±1.55
Triglycerides (mg/dl)	2.31±1.36	2.18±1.04	2.46±1.67
PT (%)	88.36±23.31	82.20±23.09	95.70±21.66*
			*p=0.0086 (Student's T-test); PT, prothrombin.