Prothrombin Loading of Vascular Smooth Muscle Cell–Derived Exosomes Regulates Coagulation and Calcification

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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 MGP (matrix Gla [carboxyglutamic acid] protein), 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, PT (prothrombin), 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. 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–dependent 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.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:e22-e32. DOI: 10.1161/ATVBAHA.116.308886.)

Key Words: anticoagulants • exosomes • myocytes, smooth muscle • prothrombin • vascular calcification

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 osteochondrogenic differentiation and secrete calcifying exosomes or apoptotic bodies that initiate deposition of calcium (Ca) phosphate (P) crystals.3–5

Under physiological conditions, VSMCs are protected from calcification by expression of inhibitors including the vitamin K–dependent proteins; MGP (matrix Gla [carboxyglutamic acid] protein), BGP (bone Gla protein; osteocalcin), GRP (Gla-rich protein), and Gas6 (growth arrest-specific gene-6 protein).6–9 All these proteins 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 has 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 antiapoptotic pathways and subsequent VSMC apoptosis and calcification.9,13 MGP has been implicated in BMP-2 (bone morphogenic protein 2) binding and inhibition of BMP-2–dependent osteogenic signaling.14 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

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remain unclear.\textsuperscript{15,16} Interestingly, VSMCs internalize 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 and serum-derived proteins are involved in the prevention of exosome-mediated VSMC calcification.\textsuperscript{5}

Circulating vitamin K–dependent coagulation factors, including PT (prothrombin) and factors VII, IX, and X and proteins C and S, also harbor Gla residues which mediate Ca-dependent protein binding to negatively charged phospholipids, such as phosphatidylserine (PS).\textsuperscript{17–21} Activation of coagulation in the vasculature has been linked to PS-exposing extracellular vesicles (EVs) shed by apoptotic macrophages, endothelial cells, erythrocytes, and VSMCs.\textsuperscript{17,22–24} 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.\textsuperscript{17,22,25–28} Anticoagulation therapy with warfarin causes elevation of circulating levels of liver-derived uncleared PT (PIVKA-II [protein induced by vitamin K absence or antagonism II]) and results in the inhibition of thrombogenesis.\textsuperscript{29} Besides its effect on vitamin K–dependent coagulation factors, warfarin increases circulating levels of undercarboxylated MGP and BGP and causes vascular calcification in rodent models and accelerates both valve and vascular calcification in man.\textsuperscript{30–33} 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.

\textbf{Materials and Methods}

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

\textbf{Results}

\textbf{Circulating Gla Proteins Are Abundant in VSMC Exosomes}

We previously demonstrated the recycling of the circulating serum protein, fetuin-A by VSMC exosomes.\textsuperscript{5} To determine whether other serum proteins are recycled in VSMC exosomes, we interrogated a proteomic data set of human VSMC-derived exosomes\textsuperscript{5} 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

\begin{table}[h]
\centering
\caption{Bovine Proteins in Human VSMC Exosomes Detected by Mass Spectrometry}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
Protein & Molecular Weight & Number of Assigned Spectra & & Number of Unique Peptides & & Percent Coverage & \\
& & CTRL & Ca/P & CTRL & Ca/P & CTRL & Ca/P & \\
\hline
Calcification inhibitors & & & & & & & & \\
a-2-HS-glycoprotein & 38 & 383 & 469 & 9 & 11 & 31 & 31 & \\
\hline
Transport proteins & & & & & & & & \\
Serum albumin & 69 & 2834 & 2824 & 46 & 46 & 65 & 66 & \\
\hline
Matrix proteins & & & & & & & & \\
Fibronectin & 272 & 372 & 362 & 26 & 24 & 14 & 12 & \\
\hline
Coagulation proteins & & & & & & & & \\
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 & \\
\hline
\end{tabular}
\end{table}

Vascular smooth muscle cells (VSMCs) were incubated in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 0.1% bovine serum albumin (control [CTRL]) and 2.7 mmol/L Ca/2.5 mmol/L P (Ca/P) for 16 h and exosomes isolated from cell media by differential centrifugation.

*Proteins with Gla domain.
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 MGP.7 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 (Figure IA and IB in the online-only Data Supplement). Prominently, there were 4 conserved Glu residues and 1 cysteine residue in all these proteins, suggesting that coagulation proteins may potentially modulate vascular calcification.

**Gla Proteins Differentially Inhibit VSMC Calcification In Vitro**

VSMCs were treated with physiological doses of several Gla-containing proteins to test their capacity to inhibit calcification in vitro (Figure 1A). MGP is a potent local inhibitor of calcification; however, circulating MGP has no inhibitory properties in vivo24 and is only effective in vitro at doses above physiological levels.35 As expected, MGP1-53 (Figure IIA and IIC in the online-only Data Supplement), osteopontin, and osteocalcin had little effect on calcification when added exogenously to the media at circulating concentrations (Figure 1A). In contrast, PT, the most abundant circulating coagulation factor with physiological concentrations \(\approx 1.4 \, \mu\text{mol/L}\), was able to potently inhibit calcification in a manner similar to fetuin A (Figure 1A).

PT expression was not observed in VSMCs either by Western blotting or polymerase chain reaction (Figure IIIA in the online-only Data Supplement). However, incubation of VSMCs with PT resulted in its accumulation in exosomes, apoptotic bodies, and whole cell lysates (Figure 1B), with exosomes also enriched with PT intermediate activation products, F1 and F1.2 (Figure 1B). In agreement with our proteomic data, calcifying conditions increased the levels of PT and F1 both in exosomes and in VSMCs (Figure 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 \(^{45}\text{Ca}\) incorporation and alizarin red S staining (Figure 2A and Figure IVA in the online-only Data Supplement). PT inhibited calcification in a dose–dependent manner at concentrations within the physiological range. The inhibitory effects of PT were more pronounced in Ca-induced rather than P-induced calcification (Figure IVB and IVC in the online-only Data Supplement). 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 (Figure 2B) and in agreement with this fetuin-A but not PT completely prevented Ca/P precipitation in vitro (Figure 2C).

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
Exosomes isolated from Ca-/P-treated VSMCs displayed calcification (Figure 2D). Direct addition of PT to exosomes isolated from VSMCs incubated in Ca/P media significantly reduced exosome calcification (Figure 2D), suggesting that 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 24 hours and then washed to remove PT from the media before exosome isolation 24 hours later, suggesting that 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 ≈20% to 25% of cells as assessed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) staining and time-lapse microscopy (Figure IVD and IVE in the online-only Data Supplement). 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 whether PT can directly bind to VSMC exosomes, we immobilized exosomes on latex beads, incubated with Alexa488-labeled PT and observed strong binding (Figure 3A). The interaction of PT with PS is mediated by the N-terminal F1 domain harboring Gla-residues,20 and consistently, addition of annexin A5 or F1 blocked PT binding to exosomes (Figure 3A). We also observed reciprocal competition for interactions with exosomal PS between PT and annexin A5 (Figure IIIB in the online-only Data Supplement).

Addition of alexa488-labeled PT to VSMCs resulted in endocytosis of full-length PT in a Ca-dependent manner (Figure 3B). To test whether PT endocytosis is mediated by high-affinity binding to a cellular receptor or fluid-phase uptake,38 we studied endocytosis of alexa488-labeled PT in the presence of unlabeled PT. Notably, endocytosis of alexa488-labeled PT was inhibited by 5-fold molar excess of unlabeled PT, indicating that uptake is mediated by high-affinity binding of PT to the cell surface potentially via Gla/PS binding (Figure 3C). Colocalization with EEA1 (early endosome antigen 1) and CD63 showed that PT is endocytosed by VSMCs and delivered to early endosomal and LE/MVB compartments (Figure 3D). Immunocytochemistry also confirmed that PT-Alexa488 uptake was further stimulated by elevated Ca and inhibited by unlabeled PT (Figure 3D).

Figure 2. PT (prothrombin) inhibits vascular smooth muscle cell (VSMC) calcification in vitro by blocking exosome nucleation activity. A, PT inhibits VSMC calcification induced by elevated Ca/P (2.7 mmol/L Ca/2.5 mmol/L P) in a dose-dependent (Continued) manner. Mean±SD, N=3, Student t test, *P<0.05, **P<0.001. B, PT acts synergistically with fetuin-A. VSMCs were incubated with Ca/P (2.7 mmol/L/2.5 mmol/L) in the presence or absence of fetuin-A (0.2 µmol/L). Student t test, N=3. C, Crystal precipitation in solution is efficiently blocked by fetuin-A but not by PT. D, PT inhibits calcification of VSMC-derived exosomes assessed using 45Ca uptake. VSMCs were incubated in calcifying conditions (2.7 mmol/L Ca/2.5 mmol/L P) in the absence ([CaP]) or presence of 1.25 µmol/L PT ([CaP+PT]) and calcifying exosomes were isolated by ultracentrifugation. PT was also added directly to calcifying exosomes ([CaP]+PT). Hydroxyapatite (HA) was used as a positive control for mineralization. Mean±SD, N=3, ANOVA, ***P<0.001.
Figure 3. PT (prothrombin) accumulates in vascular smooth muscle cell (VSMC) exosomes in vitro. A, Phosphatidylserine-dependent binding of Alexa488-labeled PT to VSMC exosomes. Alexa488-labeled PT binding to VSMC-derived exosomes immobilized to beads was analyzed by flow cytometry. Left, Flow cytometry histogram. Right, Binding quantification shows that PT, AnxA5 (annexin A5), and F1 (PT fragment 1) reduced PT binding to exosomes. N=3, ANOVA, ***P<0.001. B, Ca-dependent uptake of full-length PT by VSMCs. Cells were incubated with PT (0.73 µmol/L) for 6 h, washed with 0.1 mol/L glycine (pH2.5) to remove surface-bound PT, and lysed and analyzed by Western blotting using antibody specific for F1. Representative image, N=3. C, Quantification of PT uptake by VSMCs by flow (Continued)
To distinguish PT that binds to exosomes in the cell media, and PT that is endocytosed and recycled in exosomes via LE/MVB, we incubated VSMCs with PT for 16 hours (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 (Figure 3E).

In contrast, exosomes obtained after the chase treatment contained full-length PT and its intermediate activation product with molecular weight ≈34 kDa, most likely Pre2 (prothrombin-2), an inactive α-thrombin precursor consisting of the catalytic domain. (Figure 3E and Figure IC in the online-only Data Supplement). Thus, PT is loaded on, and in, exosomes by direct surface binding and cellular endocytosis.

**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 (Figure 4A). F1 that 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 (Figure 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 (Figure 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 because of its intramolecular disulfide loop (Figure IIB and IIC in the online-only Data Supplement). Addition of this Gla peptide completely abrogated VSMC calcification (Figure 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 analyzed 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, whereas in calcified vessels PT was significantly present in both intimal and medial layers (Figure 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 radiograph of the pelvis (Table 2). Patients with vascular calcification had lower levels of PT (P<0.01) when compared with patients without calcification. Univariate logistic regression analysis of the presence of vascular calcification (Figure 5A) showed a significant inverse association of PT with age, sex, and diabetes mellitus. Furthermore, patients without vascular calcification had lower levels of PT (<0.01) when compared with patients with vascular calcification. On adjustment for age, sex, 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).

Table 2. Characterization of a Patient Cohort With Chronic Kidney Disease, 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 Radiograph of the Pelvis

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>With Vascular Calcification</th>
<th>Without Vascular Calcification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>81</td>
<td>25/19</td>
<td>37</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>40/41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>55.38±9.33</td>
<td>56.43±9.46</td>
<td>54.14±9.15</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.47±3.40</td>
<td>23.32±3.03</td>
<td>23.66±3.82</td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>68 (84%)</td>
<td>35 (80%)</td>
<td>33 (89%)</td>
</tr>
<tr>
<td>Smoking</td>
<td>25 (31%)</td>
<td>17 (39%)</td>
<td>8 (22%)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>7 (9%)</td>
<td>6 (14%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>Years on dialysis</td>
<td>8.72±4.40</td>
<td>9.59±4.17</td>
<td>7.68±4.49</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>5.35±1.22</td>
<td>5.13±0.81</td>
<td>5.60±1.55</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>2.31±1.36</td>
<td>2.18±1.04</td>
<td>2.46±1.67</td>
</tr>
<tr>
<td>PT, %</td>
<td>88.36±23.31</td>
<td>82.20±23.09</td>
<td>95.70±21.66</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; and PT, prothrombin. *P=0.0086 (Student t test).

Notably, no thrombin activation was observed in PT-deficient plasma on addition of exosomes with bound PT (data not shown). PT binding to exosomes may prevent prothrombinase formation by blocking PS, so we tested whether exosome-associated PT can be activated by a preactivated prothrombinase complex. First, we incubated PT and factors Va/Xa with exosomes isolated from VSMCs incubated in the presence of PT and analyzed the supernatant and exosome-associated products by Western blotting using antibodies specific for either F1 or catalytic domain (Figure 5D). No PT was detected in the supernatant of VSMCs incubated in PT-free conditions (Figure 5D, lane 1). On addition of PT, we observed soluble and exosome-associated full-length PT (Figure 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 ≈31 kDa in the supernatant phase (Figure 5D, lane 3). We also observed B chain and activation product F1 associated with exosomes (Figure 5D, lane 3). Next, we analyzed exosomes isolated from VSMCs treated in the presence of PT. Using anti-F1 antibodies, we detected PT and PT intermediate activation products, F1 and F1.2 associated with exosomes (Figure 5D, lane 4). Using an anticalytic domain antibody, we detected PT and an intermediate activation product with molecular weight ≈34 kDa (Figure 5C, line 4), again most likely Pre2 compared with the position of B chain (Figure 5D, lane 3). Addition of prothrombinase had no effect on exosome-associated PT and PT activation products (Figure 5D, line 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 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 diminishes both
Discussion

PT Is Loaded on VSMC Exosomes and Protects Against Calcification in a Gla-Dependent Manner

Vitamin K antagonist treatment aimed at the prevention of thromboembolic complication causes accelerated vascular calcification in both animal models and patients,\textsuperscript{32,33} and this effect is thought to be associated with the accumulation of uncarboxylated endogenously expressed Gla proteins, such as MGP and GRP.\textsuperscript{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 that acts by directly binding to nascent growing calcium phosphate crystals to limit growth,\textsuperscript{5,37} PT inhibits exosome-mediated calcification via a PS/Gla interaction, thus disrupting nucleation site formation. Pulse-chase experiments indicated that there are 2 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\textsuperscript{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 interaction,\textsuperscript{40} and elevated calcium conditions have been observed in atherosclerotic plaques\textsuperscript{41} and in patients with chronic kidney disease, 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.\textsuperscript{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 signaling, but this hypothesis is yet to be tested.

Importantly, extensive calcification of elastic fibers is commonly observed in patients with pseudoxanthoma elasticum...
h harboring mutations in γ-glutamyl carboxylase, which causes multiple coagulation factor deficiency, 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 stones. 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 imbalance. Interestingly, as opposed to MGP clinical data, the appearance of minute levels of undercarboxylated PT in the circulation (>2 ng/mL, which corresponds to ≈0.00003 μmol/L) is not associated with vascular calcification as long as the total circulating PT levels remain high (>1.4 μmol/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 bisphosphonates. 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 blood, and these complexes have been recently associated with coronary artery calcification scores and aortic pulse wave velocity in patients with chronic kidney disease. 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. Although VSMC EV thrombogenic activity exceeds the potency of platelet-derived EVs, most likely because of the presence of TF and PS, the mechanisms of thrombogenic EV secretion by VSMCs remained unknown. 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. 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 anticoagulant pathways. 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-thrombin. This specificity may be defined by specific membrane proteins, such as integrins, as reported earlier for platelets. 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 2 possible explanations for this pool of PT that cannot be activated. First, PT may be loaded inside the exosomal lumen as discussed above. Second, activation of PT by factor Xa, by cleavage of the R320 bond in particular, requires membrane binding of factor Xa, suggesting steric hindrance may prevent activation of PT and Pre2.

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, whereas gradual loading of exosomes with PT and PT activation products reduces both procalcification and procoagulant 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, for example, GlA peptide derivative from protein S. Modulation of exosome biogenesis pathways, such as SMPD3 that is activated in vascular pathologies, may also provide alternative therapeutic approaches to reduce plaque thrombogenicity causing late stage thrombotic events in patients after myocardial infarction or coronary artery stenting.

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**Disclosures**

None.

**References**


37. Danziger J, Young RL, Shaan KM, Duprez DA, Jacobs DR, Tracy RP, Ix JH, Jenny NS, Maksalal KJ. Circulating Des-gamma-carboxy prothrombin is not associated with cardiovascular calcification or stiffness.


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**Highlights**

* Circulating Gla proteins are abundant in vascular smooth muscle cell exosomes.
* PT (prothrombin) is a novel circulating vascular calcification inhibitor.
* Gla domain but not proteolytic activity is required for vascular smooth muscle cell calcification inhibition by PT.
* PT accumulates at sites of calcification, and PT circulation level is reduced in patients with vascular calcification.
* Vascular smooth muscle cell exosomes display tissue factor–and phosphatidylinerine-dependent thrombogenic activity.
Prothrombin Loading of Vascular Smooth Muscle Cell–Derived Exosomes Regulates Coagulation and Calcification

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Supplementary Figure I. A. Alignment of MGP, coagulation factors IX, X and PT and anticoagulant proteins C and S. Sequences were aligned using CLUSTAL O (1.2.1) multiple sequence alignment.

B. Percent identity between Gla-containing regions;

C. Prothrombin activation by factor Xa (R271 and R320) or thrombin (R155 and R284). *, γ-carboxyglutamic acid residues (Gla)
Supplementary Figure II. A. Sequence of matrix Gla protein (MGP) residues 1-53. \( \gamma \) indicate \( \gamma \)-carboxyglutamic acid residues; * indicate potential (Ser) phosphorylation sites. B. Sequence of protein S residues 1-46 (Gla domain). \( \gamma \) indicates \( \gamma \)-carboxyglutamic acid; * indicate cysteines involved in an intramodule disulfide bond. C. Electrospray ionization quadrupole time-of-flight mass spectrum (ESI-Q-TOF-MS) of protein S residues 1-46 (Gla domain) revealed three m/z ratio peaks at m/z 1024.6 (6H\(^+\)), 1229.3 (5H\(^+\)) and 1536.4 (4H\(^+\)) resulting in a final calculated average mass of 6141.6 ± 0.1. The obtained peptide mass fitted well between the calculated masses of 6138.5 (mono-isotopic mass) and 6141.9 (average mass) for the protein S Gla domain with a disulfide bond between Cys-18 and Cys-23. Asterisks indicate decarboxylation of intact peptide due to mass spectrometry ionization effects (\( \Delta \) Mr -44; -88; -132). ESI-Q-MS of MGP1-53 revealed four m/z ratio peaks at m/z 1040.8 (7H\(^+\)), 1214.6 (6H\(^+\)), 1457.1 (5H\(^+\)), and 1821.9 (4H\(^+\)) resulting in a final calculated mass of 7281.1 ± 2.1. The obtained peptide mass fitted well between the calculated masses of 7278.4 (mono-isotopic mass) and 3230.7 (average mass) for the TSR with a disulfide bond between Cys-18 and Cys-23.
Supplementary Figure III. A. PT is not expressed by human VSMCs as detected by PCR.
B. FITC-labeled Annexin A5 binds to PS externalized on VSMC-derived exosomes. Exosomes were isolated from VSMCs, immobilized to beads, probed with Annexin A5-FITC and analysed by flow cytometry. Note that PT competed with annexin A5 binding to exosomes as shown by decreased fluorescence. Right. Quantification of AnxA5 binding (N=3, mean±SD. **p<0.01.)
**Supplementary Figure IV.** A. Alizarin red S staining of VSMCs shows reduction of calcification in the presence of PT. B, C. VSMCs were incubated with different concentrations of Ca (B) or P (C), for 24h in serum-free (SF) medium to induce calcification in the presence of $^{45}$Ca and PT (1.25 µM). Note that PT was able to inhibit calcification induced by either condition but was more effective at reducing Ca induced calcification. Student’s T-test for p values. Ca, calcium; P, phosphate; PT, prothrombin, VSMC, vascular smooth muscle cell. D, E. PT reduces VSMC apoptosis triggered by Ca/P. TUNEL staining; VSMCs were incubated in M199 supplemented with 0.5% BSA for 24h and then treated with Ca/P in the absence or presence of PT (1.25µM). Fluorescence transferase-mediated dUTP nick-end labeling (TUNEL) assays were performed at time points between 6 and 24h as described previously. Coverslips were mounted in DAPI-containing medium, and random images were captured digitally (Olympus, Tokyo, Japan) from each coverslip (D). Apoptosis was measured by direct counting of morphologically apoptotic cells by a time-lapse microscope at 37°C over 24h (E).
Detailed Methods

Cell culture
VSMCs were obtained from explants of human aortic tissues from male and female donors of various ages as previously described\(^1\). Cells were maintained in M199 medium (Sigma-Aldrich) supplemented with 20% fetal calf serum (FCS) and used between passages 3 and 12.

Proteins and antibodies
Carboxylated and decarboxylated osteocalcin were gift from Dr Henri Spronk (Maastricht University, The Netherlands), osteopontin from R&D systems, recombinant annexin A5 from BioVision, bovine fetuin-A from Sigma-Aldrich and corn trypsin inhibitor (CTI) from Haematologic Technologies Inc, human prothrombin, prothrombin fragment 1 (F1), human factor Xa, bovine factor Va were from Enzyme Research. Sheep anti-human Thrombin (#SAHT-HRP; 1:2000) and sheep anti-human F1 prothrombin fragment 1 antibodies (#SAFII-F1AP 1:2000) were from Affinity Biologicals, mouse anti-human CD63 (#556019; 1:2000) from BD Pharmingen, mouse anti-TF (TF9-10H10; #sc80952, 1:1000) from SantaCruz Biotechnology, mouse anti-human vinculin (V9264; 1:1000) from Sigma.

Gla peptides synthesis

**MG1-53**
MG1 peptide (primary sequence provided at the Supplementary Figure X) was synthesized by manual solid-phase peptide synthesis (SPPS) using the in situ neutralization/HBTU activation procedure for Boc chemistry as previously described\(^2\). In brief, each coupling cycle consisted of N\(_{a}\)-Boc removal by a 1–2-min treatment with neat TFA, a 1-min DMF-flow wash, and a 10-min coupling (Arg, Asn: 20 min) with 1.0 mmol preactivated Bocamino acid in the presence of excess DIEA, followed by a second DMF-flow wash. The following amino acids were side-chain protected: Boc-Arg (p-toluenesulfonyl)-OH, Boc-Asn(xanthyl)-OH, Boc-Asp(O-cyclohexyl)-OH, Boc-Cys(4-methylbenzyl)-OH, Boc-Gla(di-O-cyclohexyl)-OH, Boc-Glu(O-cyclohexyl)-OH, Boc-His(dinitrophenyl)-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Ser(benzyl)-OH, Boc-Thr(benzyl)-OH, and Boc-Tyr(2-Br-Z)-OH. Synthesized peptides were cleaved from the resin by treatment with anhydrous HF for 1 h at 0°C using 4% anisole, precipitated and washed with ice-cold diethylether, dissolved in aqueous acetonitrile and lyophilized.

**Protein S Gla peptide**

Gla peptide (1–46: A NSLL g g TKQGNL g R g C I g g LCNK g g A R g V F g NDP g T D Y F Y P K Y L G) was synthesized using a thioester generating linker on MBHA resin (TAMPAL) to yield a C-terminal mercaptopropionic acid-leucine (MPAL)- activated thioester moiety (COSR) as previously described\(^3\). Val46 residue was substituted to Gly to accelerate
chemical ligation rate. After deprotection and cleavage from the resin, the peptide was folded, HPLC-purified and lyophilized.

**PT Alexa488 labeling and uptake**

Human PT was labeled using the Alexa488 labeling kit according to the manufacturer’s protocol (Invitrogen). To study PT uptake by immunocytochemistry VSMCs were incubated in M199 supplemented with 0.5% bovine serum albumin (BSA) for 16h and then cells were incubated with 20µg/ml (0.28µmol/L) Alexa488-labeled PT in the absence or presence of elevated Ca (3.6mmol/L) or unlabelled PT (2.8µmol/L), washed three times in PBS and fixed in 3.7% paraformaldehyde (PFA). VSMCs were visualized using 63x/1.4-0.6 Oil Plan Apo objective on a Leica TCS SP5 confocal microscope (Leica Microsystems) at room temperature. Images were processed and analyzed using Volocity 5.5 software (Perkin Elmer).

To examine PT uptake by western blotting, VSMCs were incubated with PT (0.73µmol/L) for 6h. Then cells were washed with 0.1mol/L glycine (pH2.5) to remove surface-bound PT and analyzed by western blotting in reducing conditions using F1 antibody.

To quantify PT uptake by flow cytometry, VSMC were serum-starved for 16h and then trypsinised and incubated with 50µg/ml (0.7µmol/L) Alexa488-labeled PT in M199 supplemented with 0.5%BSA and 1.8mmol Ca or 3.6mmol/L Ca for 30min on ice. Then aliquot of sample was incubated either on ice (to determine surface PT binding levels) or at +37°C (to measure the total PT binding/uptake level) for 30 min. The competitor (3.5µmol/L unlabeled PT) was added to block specific uptake of Alexa488-labeled PT in some samples. Cells were rinsed with PBS, fixed in 3.7% paraformaldehyde and analyzed by flow cytometry. Data were analyzed using FlowJo software (FlowJo X 10.0.6) and PT uptake was calculated by subtraction of the mean fluorescent units (MFU) values of surface-bound PT from the MFU values of total PT binding/uptake level.

**Isolation of exosomes and apoptotic bodies (AB)**

Exosomes and apoptotic bodies (AB) were isolated as previously reported
\(^1\). Briefly, VSMCs were rinsed with EBSS and incubated in serum-free conditions in DMEM supplemented with 0.1% BSA for 16h. The medium was collected and centrifuged 2,500xrpm (Sorvall RF7) to remove apoptotic bodies (AB). Exosomes were isolated from the supernatant by centrifugation at 100,000xg for 40min at 4°C and resuspended in PBS for further analysis.

To study PT recycling in exosomes (“pulse/chase”) VSMCs were incubated in the presence or absence of PT (1.25µmol/L) (PT “pulse”) in DMEM supplemented with 0.1% BSA for 16h.
Then cell conditioned media was collected for exosome isolation and cells were extensively washed in 0.1mol/L Glycine (pH 2.5) and PBS. Next, VSMCs were incubated further 24hrs in DMEM supplemented with 0.1% BSA in the absence of PT (PT “chase”). Exosomes were isolated from PT “pulse” and “chase” media and analyzed by western blotting.

**Western blotting**

Samples (5µg/lane, reducing conditions) were separated by 10% polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore) by using semi-dry blotting system (BioRad). Membranes were incubated in blocking buffer (PBS supplemented with 5% milk and 0.05% tween-20) 1h and incubated with primary antibody overnight at +4°C. Then membranes were washed with blocking buffer, incubated with the corresponding secondary horseradish peroxidase-conjugated antibodies and visualized by using enhanced chemiluminescence (ECL, Pierce). Samples for CD63 and TF analysis were prepared in non-reducing buffer.

**Immobilization of exosomes to beads and flow cytometry**

Ten µg of exosomes were immobilized to 4µm surfactant-free aldehyde/sulfate latex beads (Invitrogen) as previously described. An aliquot of immobilized exosomes or control beads (with immobilized BSA) were incubated in 10mmol/L HEPES, pH 7.4 supplemented with 0.14mol/L NaCl; 2.5mmol/L CaCl₂ in the presence or absence of FITC-labeled annexin A5 (0.6µmol/L; BD Biosciences, Oxford, UK) or 0.28µmol/L Alexa488-labeled PT and a blocking unlabeled protein (6.9µmol/L): PT, PTF1, annexin A5 or fetuin-A for 30min at room temperature. Then samples were diluted with binding buffer and analyzed by flow cytometry with a BD FACScalibur (BD Biosciences).

**Thrombin generation assay and PT activation with prothrombinase in vitro**

Thrombin generation by exosomes was measured as previously described. In brief, exosomes (1µg in 10µL of PBS) were incubated in the absence or presence of 1µmol/L annexin A5 and then mixed with 70µl of platelet poor plasma which was prepared by ultracentrifugation at 100,000xg for 4h (to remove all EVs) and supplemented with 20µg/µl CTI. 20µl of PRP reagent or thrombin calibrator (Thrombinoscope BV) were added to each well and the plate was incubated at 37°C for 10min. The reaction was started by addition of 20µl of FluCa starting kit (Thrombinoscope BV) and fluorescent intensity was measured continuously for 120min (Fluoroskan Ascent FL, Thermo Electron Corporation).
To study PT activation by prothrombinase \textit{in vitro}, exosomes were isolated from VSMC incubated in the absence or presence of PT (0.4\,\mu\text{mol/L}). Next, exosomes (3\,\mu\text{g}) isolated from VSMC incubated in the absence of PT were treated with 0.5\,\mu\text{mol/L} Factor Va, 0.5\,\mu\text{mol/L} Factor Xa and 0.5\,\mu\text{mol/L} PT in 10\,\text{mmol/L} Hepes buffer (pH 7.4) supplemented with 140\,\text{mmol/L} NaCl and 2.5\,\text{mmol/L} CaCl\textsubscript{2} at 37°C for 30\,\text{min}. Exosomes isolated from VSMC incubated in the presence of PT were also treated with 0.5\,\mu\text{mol/L} Factor Va and 0.5\,\mu\text{mol/L} Factor Xa in 10\,\text{mmol/L} Hepes buffer (pH 7.4) supplemented with 140\,\text{mmol/L} NaCl and 2.5\,\text{mmol/L} CaCl\textsubscript{2} at 37°C for 30\,\text{min}. Then exosomes were separated by ultracentrifugation and pellet (Exosomes) and supernatant were analyzed by western blotting using antibody for catalytic domain or F1.

\textit{Calcification assay}

VSMCs were serum-starved in M199 supplemented with 0.5\% BSA for 24\,\text{h}. Then cells were treated with the different additives and/or calcium and phosphate concentrations diluted in M199 supplemented with 0.5\% BSA for another 24\,\text{h}. CaCl\textsubscript{2} and NaH\textsubscript{2}PO\textsubscript{4} were added to supplement calcium and phosphate. Calcification was quantified by \textsuperscript{45}Ca incorporation or cresolphthalein assay as previously described\textsuperscript{1}.

\textit{VSMC Exosomes \textsuperscript{45}Ca Uptake assay}

Exosomes (5\,\mu\text{g}) isolated from VSMC were incubated in calcifying buffer containing 50\,\text{mmol/L} TES buffer (pH 7.6) supplemented with 2.2\,\text{mmol/L} CaCl\textsubscript{2}, 1.6\,\text{mmol/L} KH\textsubscript{2}PO\textsubscript{4}, 1\,\text{mmol/L} MgCl\textsubscript{2}, 85\,\text{mmol/L} NaCl, 15\,\text{mmol/L} KCl, 10\,\text{mmol/L} NaHCO\textsubscript{3} and \textsuperscript{45}Ca (50,000\text{cpm/ml}) at 37°C for 24\,\text{h} as previously described\textsuperscript{1}. Precipitates were spun down by centrifugation at 13,000rpm for 10\,\text{min}, washed with HBSS and spun down again. Next, pellets were resuspended in 0.1\,\text{mol/L} HCl and neutralised with 0.1\%SDS/0.1\,\text{mol/L} NaOH. Incorporated \textsuperscript{45}Ca was measured by liquid scintillation counting.

\textit{Alizarin red S staining}

Cells were grown in 12-well plates, washed with PBS and fixed in 10\% formaldehyde in PBS for 45\,\text{min} at 4°C. The cells were washed with distilled water and then incubated with 2\% Alizarin red S (Sigma-Aldrich) for 5\,\text{min} and briefly rinsed with water.

\textit{Crystal precipitation assay}

To study crystal precipitation in solution, 5\,\text{mmol/L} CaCl\textsubscript{2}, 3\,\text{mmol/L} NaHPO\textsubscript{4}, 50\,\text{mmol/L} Tris pH7.4, and 140\,\text{mmol/L} NaCl were incubated for 90\,\text{min} at 37°C in the absence or presence of fetuin-A or PT.
**Immunohistochemistry**

Aortic and carotid tissue sections from healthy (n=4) and patients with calcified artery specimen (n=6) were stained with a polyclonal antibody against F1 (Affinity Biologicals), which recognizes both whole PT and F1. For detection, a secondary anti-sheep antibody was used. Controls were performed with the primary antibody substituted for phosphate-buffered saline (PBS).

**Immunoassay for detection of PT in plasma**

Calcification of the iliac/femoral arteries was determined on plain pelvic X-rays in 81 hemodialysis patients, which were analyzed by two experienced physicians. The local Ethics Committee approved the study protocol and each patient gave informed consent. Patients were grouped according to the presence or absence of visible calcification on pelvic X-ray. PT concentrations were determined in the one-stage assay using a coagulometer (KC-4), a commercial thromboplastin preparation (Thromborel S; Dade Behring), and clotting factor II-deficient plasma (Dade Behring). PT concentrations were calculated with the aid of a reference curve from pooled normal plasma.

**Statistical analysis**

Data statistical analysis was conducted using Prism 4.01 (Graphpad, La Jolla, CA). All experiments were repeated at least three times in triplicates and data presented are Mean ± Standard Deviation. The assumption that data follows the Gaussian distribution was evaluated using Kolmogorov-Smirnov test (Prism 4.01). Accordingly, two groups were compared using paired t-test with two tailed P value and 95% confidence interval. Multiple groups were compared using one-way analysis of variance (ANOVA) with Bonferroni’s Multiple Comparison Post Hoc test.
Supplemental References


