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Elastin-Derived Peptides Are New Regulators of Thrombosis

Charlotte Kawecki, Nathalie Hézard, Olivier Bocquet, Gaël Poitevin, Fanja Rabenoelina, Alexandre Kauskot, Laurent Duca, Sébastien Blaise, Béatrice Romier, Laurent Martiny, Philippe Nguyen, Laurent Debelle, Pascal Maurice

Objective—Elastin is the major structural extracellular matrix component of the arterial wall that provides the elastic recoil properties and resilience essential for proper vascular function. Elastin-derived peptides (EDP) originating from elastin fragmentation during vascular remodeling have been shown to play an important role in cell physiology and development of cardiovascular diseases. However, their involvement in thrombosis has been unexplored to date. In this study, we investigated the effects of EDP on (1) platelet aggregation and related signaling and (2) thrombus formation. We also characterized the mechanism by which EDP regulate thrombosis.

Approach and Results—We show that EDP, derived from organo-alkaline hydrolysate of bovine insoluble elastin (kappa-elastin), decrease human platelet aggregation in whole blood induced by weak and strong agonists, such as ADP, epinephrine, arachidonic acid, collagen, TRAP, and U46619. In a mouse whole blood perfusion assay over a collagen matrix, kappa-elastin and VGVAPG, the canonical peptide recognizing the elastin receptor complex, significantly decrease thrombus formation under arterial shear conditions. We confirmed these results *in vivo* by demonstrating that both kappa-elastin and VGVAPG significantly prolonged the time for complete arteriole occlusion in a mouse model of thrombosis and increased tail bleeding times. Finally, we demonstrate that the regulatory role of EDP on thrombosis relies on platelets that express a functional elastin receptor complex and on the ability of EDP to disrupt plasma von Willebrand factor interaction with collagen.

Conclusions—These results highlight the complex nature of the mechanisms governing thrombus formation and reveal an unsuspected regulatory role for circulating EDP in thrombosis. (*Arterioscler Thromb Vasc Biol.* 2014;34:2570-2578.)

Key Words: extracellular matrix ■ platelet ■ thrombosis ■ vascular remodeling ■ von Willebrand Factor

Vascular remodeling underlies the pathogenesis of major cardiovascular diseases and involves degradation and reorganization of the extracellular matrix of the vessel wall. Elastin is the major component of elastic fibers and represents one of the main structural matrix components of the vascular wall together with collagens. This highly stable molecule with longevity comparable to human lifespan¹⁻³ is essential for maintaining the strength, resilience, and integrity of the vessel wall.⁴⁻⁶ Local inflammation concurrently with increased elastase activity, calcium, and lipid deposits are typical features of physiological and pathological vascular aging that contribute to elastin degradation.⁷⁻¹¹

Elastin degradation releases bioactive elastin-derived peptides (EDP) known as elastokines. Among them, peptides harboring the GxxPG motif, such as the canonical VGVAPG peptide, have been shown to act on several cells, such as endothelial cells, monocytes, and smooth muscle cells.⁸ Most of their effects have been assigned to their interaction with the elastin receptor complex (ERC). The ERC is a singular membrane receptor composed

of 3 subunits: a peripheral 67-kDa subunit called elastin-binding protein (EBP), a 55-kDa protective protein/cathepsin A (PPCA), and a 61-kDa neuraminidase (Neuraminidase-1, Neu-1).¹²

Considered for a long time as a mechanical support for cells, the extracellular matrix and its degradation products are now regarded as important regulators of cell physiology and development of vascular diseases. Accumulating evidence in the literature suggest that EDP may play important roles in the progression of age-related vascular diseases. For instance, a proatherogenic role of EDP has been recently described in ApoE^{-/-} and LDLR^{-/-} mice.¹³ In addition, chronic accumulation of EDP has been shown to promote hyperglycemia and insulin resistance in mice.¹⁴ Vascular aging is also associated with hemostasis dysregulation and increased thrombosis tendency.¹⁵ However, the effect of EDP on thrombosis has never been studied to date.

In this study, we investigated the role of EDP in platelet aggregation and thrombus formation *in vitro* and *in vivo* using both human and mouse whole blood. We show for the first time that EDP regulate thrombosis by an original mechanism

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Nonstandard Abbreviations and Acronyms	
EBP	elastin-binding protein
ECM	extracellular matrix
EDP	elastin-derived peptides
ERC	elastin receptor complex
kE	kappa-elastin
Neu-1	neuraminidase-1
PPCA	protective protein/cathepsin A
vWF	von Willebrand factor

involving effects both at the platelet level and at the matrix level by competing for von Willebrand factor (vWF) immobilization on collagen. At the platelet level, we further demonstrate the existence of a functional ERC that is able to decrease washed platelet aggregation and to regulate thrombus formation *in vitro* and *in vivo*.

Materials and Methods

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

Results

EDP Decrease Human Platelet Aggregation and α IIB β 3 Activation in Whole Blood

The effects of EDP on platelet aggregation were first investigated in whole blood by impedance aggregometry with the Multiplate[®] analyzer. As shown in Figure 1, kappa-elastin (kE) significantly decreased platelet aggregation induced by weak (ADP, epinephrine, arachidonic acid) and strong (collagen, TRAP, U46619) platelet agonists. At 100 μ g/mL kE, whole blood platelet aggregations were inhibited by \approx 20%, 48%, and 36% for ADP, epinephrine, and arachidonic acid, respectively, and by 35%, 25%, and 45% for collagen, TRAP, and U46619, respectively (Figure 1A and 1B).

We next evaluated the effects of kE on α IIB β 3 activation and CD62P surface exposure by flow cytometry. At resting state, kE had no effect on the activation level of α IIB β 3, P-selectin (CD62P), and phosphatidylserine surface expression (Figure I in the online-only Data Supplement) and did not trigger tyrosine phosphorylation of platelet proteins (Figure II in the online-only Data Supplement). In collagen-, TRAP-, and ADP-activated platelets, 100 μ g/mL kE significantly decreased α IIB β 3 activation by 41.3% \pm 8.8%, 46.3% \pm 5.7%, and 37.1% \pm 6.9%, respectively (Figure 1C). In contrast, no effect was observed on platelet granule secretion induced by collagen, TRAP, and ADP, as evidenced by anti-CD62P binding levels (Figure 1D).

Taken together, these data indicate that EDP affect platelet function in whole blood by decreasing both platelet aggregation and activation of α IIB β 3 without modifying platelet secretion. Importantly, when used alone, kE has no effect on platelet viability and activation.

EDP and VGVAPG Reduce Thrombus Formation *In vitro* Under Flow Conditions

The effects of EDP on platelet interaction with collagen under flow conditions were assessed using a whole blood perfusion

assay over a fibrillar collagen matrix at 1500/s. As shown in Figure 2B, preincubation of mouse whole blood with 100 μ g/mL kE significantly decreased the area covered by platelets from 60 seconds of perfusion. At 150 seconds, the inhibition reached 43% in the presence of kE (12.9% \pm 2.2%) compared with control (22.8% \pm 2.1%). To examine whether the ERC can reduce thrombus formation *in vitro*, we used the canonical VGVAPG peptide. This peptide was used at a 4-fold higher concentration than kE (400 μ g/mL) because it has been demonstrated that a 4-fold higher concentration is necessary to achieve the equivalent physiological stimulation levels of bioactive elastin peptides released after elastin degradation.^{13,14,16} As negative control, a scrambled VVGPGA peptide was used.^{17,18} As shown in Figure 2A and 2C, VGVAPG significantly decreased the area covered by platelets from 30 seconds of perfusion. At 150 seconds of perfusion, VGVAPG decreased by 30% the area covered by platelets compared with scrambled peptide (27.0% \pm 3.7% versus 38.7% \pm 4.5%).

All these data demonstrate that EDP and VGVAPG reduce thrombus formation *in vitro* and suggest the likely contribution of the ERC in this process.

EDP Disrupt Plasma vWF Immobilization Onto Collagen

Platelet interaction with collagen under flow conditions requires the initial rolling and slowing down of platelets through vWF immobilized onto collagen. We therefore investigated if part of the inhibitory effects observed in Figure 2 could be explained by disruption of vWF immobilization onto collagen. Increasing concentrations of kE (25–200 μ g/mL) significantly decreased mouse and human plasma vWF binding to immobilized collagen (Figure 3A and 3B). The maximal effect was observed at 100 μ g/mL kE with 77.9% \pm 7.1% and 87.0% \pm 5.0% inhibition for mouse and human, respectively. Although statistically significant, VGVAPG decreased slightly mouse plasma vWF binding to immobilized collagen compared with kE. At 400 μ g/mL, the inhibition was of 12.7% \pm 2.3% (Figure 3C). No effect was observed for human plasma (Figure 3D). We next evaluated whether EDP could directly interact with vWF or collagen. Although both human and mouse plasma vWF did not interact with immobilized kE (Figure 3E), soluble kE dose-dependently interacted with immobilized collagen (Figure 3F).

Taken together, these data indicate that EDP, but not VGVAPG, are able to disrupt plasma vWF immobilization onto collagen by interacting with collagen and thereby competing for vWF binding.

VGVAPG Directly Affects Human Platelet Aggregation and Related Signaling

As the inhibitory effects of the VGVAPG peptide suggested the contribution of the ERC in platelets, we evaluated whether this peptide could inhibit collagen-induced washed platelet aggregation and related signaling. As shown in Figure 4A, washed platelet aggregation induced by 1 μ g/mL collagen was significantly decreased by VGVAPG (63.9% \pm 12.0%) compared with scrambled peptide (100.9% \pm 5.4%). Comparable inhibition was reached at 100 μ g/mL kE (Figure 4A). Platelet

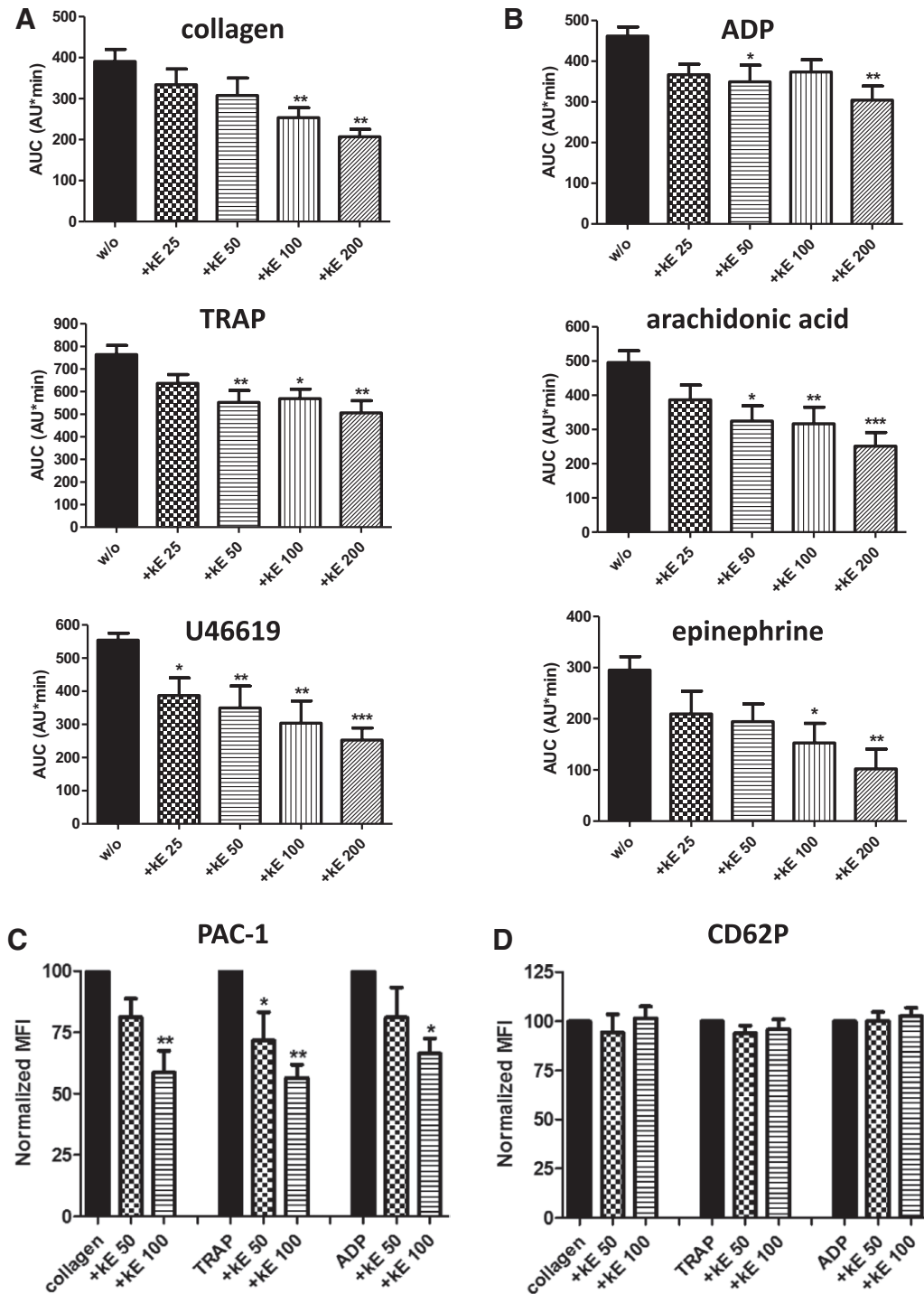


Figure 1. Elastin-derived peptides decrease human platelet aggregation and activation of α IIb β 3 in whole blood but not CD62P surface exposure. **A** and **B**, Platelet aggregation was measured in whole blood using the Multiplate[®] analyzer. Whole blood was incubated with saline buffer containing different concentrations of KE (25–200 μ g/mL). Platelet aggregation was induced by **(A)** 0.32 μ g/mL Horm collagen, 32 μ M TRAP, or 0.5 μ M U46619, or by **(B)** 6.5 μ M ADP, 0.25 mmol/L arachidonic acid, or 10 μ M epinephrine, and monitored for 6 minutes. Results were expressed as AUC of arbitrary units (AU min) and compared with control (w/o, without KE). Data represent the mean \pm SEM of 3 to 11 independent experiments. * P <0.05; ** P <0.01; *** P <0.001. **C** and **D**, Activation of α IIb β 3 and surface exposure of CD62P were determined by flow cytometry with FITC-conjugated PAC-1 **(C)** and anti-CD62P **(D)** antibody, respectively. Whole blood was incubated with 50 or 100 μ g/mL KE and stimulated, or not, by 20 μ g/mL Horm collagen, 10 μ M TRAP, or 20 μ M ADP for 5 minutes at 37°C without stirring to prevent platelet aggregation. Data represent the mean fluorescent intensity (MFI) \pm SEM of 3 to 5 independent experiments normalized to control without KE. * P <0.05; ** P <0.01.

aggregation was stopped 180 seconds after the addition of collagen by adding sodium dodecyl sulfate. Probing for tyrosine-phosphorylated proteins showed that collagen activation

increased the phosphorylation pattern of platelet proteins (Figure 4B and 4C). KE and VGVPAG seem to affect these phosphorylations as evidenced by densitometry analysis of a

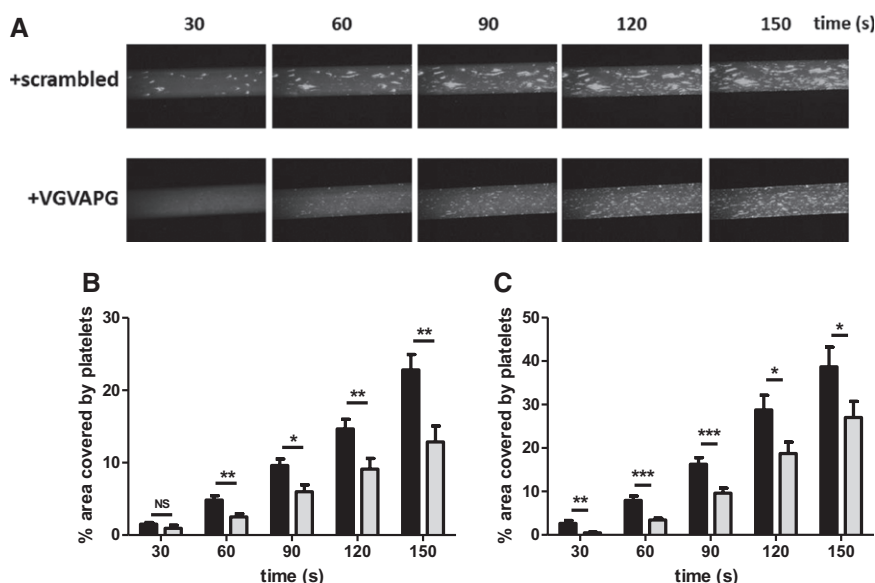


Figure 2. Elastin-derived peptides and VGVAPG impair platelet interaction with collagen under flow conditions. The effects of elastin-derived peptides on platelet interaction with collagen were investigated in a whole blood perfusion assay over a Horm collagen matrix at 1500/s. Mouse whole blood was collected in 40 μ M PPACK and 5 U/mL low-molecular-weight heparin, incubated with saline buffer, kE (100 μ g/mL), VGVAPG (400 μ g/mL), or VVGPGA (400 μ g/mL) together with rhodamine 6G (10 μ g/mL) for 5 minutes at 37°C and perfused for 180 seconds. **A**, The progression of platelet adhesion and thrombi formation was observed under an epifluorescent microscope (representative of 4 experiments). **B** and **C**, Results were expressed as the mean percentage of area covered by platelets \pm SEM from 4 independent experiments. **B**, kE (gray bars) vs saline buffer (black bars); **C**, VGVAPG (gray bars) vs scrambled VVGPGA (black bars). * P <0.05; ** P <0.01; *** P <0.001.

protein migrated at \approx 65 kDa (Figure 4B and 4C). Its phosphorylation level was significantly decreased by kE and VGVAPG compared with their respective control (Figure 4C).

Taken together, these results indicate that a functional ERC is present on platelets and is able to directly modulate platelet aggregation and related signaling.

EBP, Neu-1, and PPCA Are Expressed in Platelets and Form a Functional ERC

The Neu-1 subunit has been shown to play a critical role in ERC-mediated effects.¹⁹ On binding of EDP to the EBP subunit, Neu-1 catalyzes the local conversion of GM₃ ganglioside into lactosylceramide as a result of its sialidase activity.¹⁹ We checked for the presence of Neu-1 and PPCA in both human and mouse platelets by western blotting. Both ERC subunits were detected in human and mouse platelet lysates (Figure 5A and 5B). Overall, the migration patterns were similar between the 2 species with the exception of PPCA, suggesting differences in posttranslational modifications. Because of the unavailability of selective antibodies to detect EBP, we were unable to demonstrate the presence of EBP at the protein level. We therefore checked at the mRNA level in human megakaryocytes and normalized the expression to GAPDH. As shown in Figure 5D, EBP, Neu-1, and PPCA transcripts were unambiguously expressed in 2 different human megakaryoblastic cell lines (CHRF-288-11 and MEG-01) and in human CD34⁺ hematopoietic stem cells at 6 and 11 days after in vitro differentiation.²⁰ Similar results were observed when normalizing to another housekeeping gene, RPL32 (Figure 5E). To confirm the functionality of the receptor in human platelets, nonpermeabilized washed platelets were incubated with 100 μ g/mL kE, and sialidase

activity was assessed using the 2-*O*-(*p*-nitrophenyl)- α -*D*-N-acetylneuraminic acid as substrate. After 2 hours of incubation, kE significantly increased by 50.4% \pm 3.0% sialidase activity in platelets (Figure 5C).

Together, these results demonstrate that EBP, Neu-1, and PPCA are well expressed in platelets and form a functional receptor able to trigger activation of its Neu-1 subunit.

EDP and VGVAPG Delay Thrombosis In Vivo

The effects of kE were finally investigated in in vivo thrombosis using the FeCl₃-induced mesenteric arteriole thrombosis model. Ten minutes before the induction of thrombosis by 10% FeCl₃, mice were intravenously injected with 10 mg/kg kE or vehicle.¹⁴ The time for the formation of a stable occlusive thrombus was significantly prolonged in kE-treated mice compared with vehicle-injected control mice (37.4 \pm 5.1 minutes versus 22.8 \pm 2.8 minutes, respectively; P =0.022; Figure 6A and 6B). To examine whether binding of VGVAPG to the ERC also delays thrombosis in vivo, mice were injected with VGVAPG (40 mg/kg) and compared with scrambled VVGPGA. As shown in Figure 6C, injection of VVGPGA increased by \approx 2-fold the time to complete occlusion compared with saline-injected mice (Figure 6B), suggesting that this scrambled peptide had some unexpected effects in this model as in the in vitro model of thrombus formation wherein the scrambled peptide increased the area covered by platelets compared with saline (Figure 2B and 2C). However, when compared with VGVAPG-treated mice, occlusion times were significantly longer in these mice (75.3 \pm 2.7 minutes versus 50.5 \pm 4.7 minutes, respectively; P =0.001; Figure 6C), suggesting that binding of VGVAPG to the ERC can regulate in vivo thrombosis.

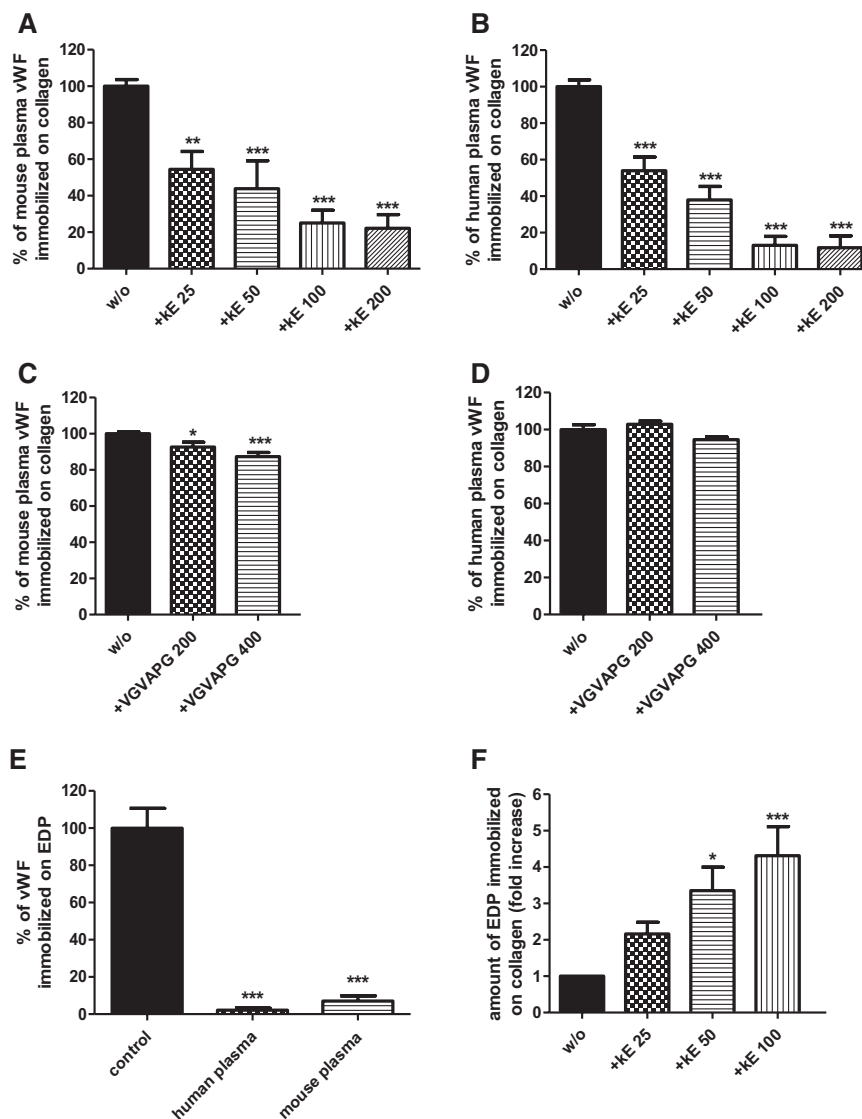


Figure 3. Elastin-derived peptides disrupt plasma vWF/collagen interaction by specifically interacting with collagen. Mouse (**A** and **C**) or human (**B** and **D**) plasma (1/200) were added for 2 hours at 37°C in collagen-coated wells (25 $\mu\text{g}/\text{mL}$) previously incubated with different concentrations of (**A** and **B**) kE (25–200 $\mu\text{g}/\text{mL}$) or (**C** and **D**) VGVAPG (200 or 400 $\mu\text{g}/\text{mL}$). Bound vWF was revealed by a horseradish peroxidase-coupled rabbit anti-human vWF antibody. Results are expressed as mean \pm SEM of 4 to 6 independent experiments, each run in triplicate, and normalized to the control (w/o, without kE). **E**, Mouse or human plasma (1/200) were added for 2 hours at 37°C in kE-coated wells (100 $\mu\text{g}/\text{mL}$) and bound vWF was revealed as above. Results are expressed as mean \pm SEM of 9 experiments, each run in triplicate, and normalized to their corresponding control (mean binding of human or mouse plasma vWF to collagen). **F**, Increasing concentrations of kE (25–100 $\mu\text{g}/\text{mL}$) were added for 2 hours at 37°C in collagen-coated wells (25 $\mu\text{g}/\text{mL}$). Bound elastin-derived peptides were revealed by the BA4 antibody followed by a horseradish peroxidase-coupled anti-mouse secondary antibody. Results are expressed as mean \pm SEM of 3 independent experiments, each run in triplicate, and normalized to the control (w/o, without kE). * P <0.05; ** P <0.01; *** P <0.001; NS, nonsignificant.

Together, these observations strongly indicate that circulating EDP affects thrombus formation in vivo and that the ERC is an important regulator in this process. The effects of EDP and VGVAPG were then evaluated in a tail-bleeding assay and bleeding times for kE- (707 \pm 17 seconds) and VGVAPG- (593 \pm 52 seconds) treated mice were significantly longer than those for saline- (360 \pm 40 s) and VVGPGA- (413 \pm 51 s) treated mice (Figure 6D). However, 33% of VVGPGA-treated mice showed no cessation of bleeding in the observed time period in contrast to 11% for VGVAPG-treated mice. Therefore, additional experiments are required to definitively prove that the ERC is involved in the regulation of tail bleeding time.

Discussion

EDP originating from elastin fragmentation are one of the major products of extracellular matrix degradation released during vascular aging. Generation of EDP is a typical feature of inflamm-aging⁸ and correlates with occurrence of vascular stiffness, calcification, and lipid deposits.^{9–11,21,22} A growing body of evidences now suggests that these extracellular matrix degradation products can also affect adjacent/circulating cells and contribute to the progression of age-associated vascular diseases.^{21,23} In this context, we recently demonstrated that chronic injection of EDP in mice potentiates atherosclerosis development and promotes insulin resistance,^{14,24} 2 events that mainly occur during aging.

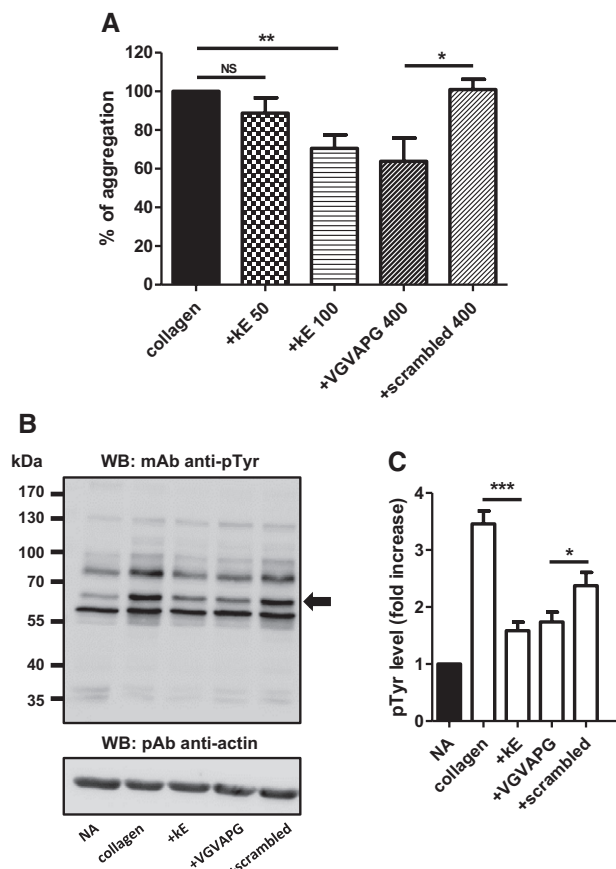


Figure 4. Elastin-derived peptides and VGVAPG decrease collagen-induced aggregation of washed platelets and related protein tyrosine phosphorylations. Washed human platelets (3.10^9 /mL) were incubated with saline buffer, kE (50 or 100 μ g/mL), VGVAPG (400 μ g/mL), or VVGPGA (400 μ g/mL) peptide for 3 minutes at 37°C. **A**, Washed platelet aggregation was induced by 1 μ g/mL Horm collagen and monitored by measuring light transmission using a SODEREL aggregometer. Results were expressed as percent of aggregation and normalized to control. Data represent the mean \pm SEM of 7 to 8 independent experiments. **B**, At 180 seconds after the addition of collagen, platelet aggregates were solubilized and proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Tyrosine-phosphorylated proteins were detected using the monoclonal anti-phosphotyrosine antibody PY20 and the amount of proteins loaded was evaluated by a polyclonal anti-actin antibody. A representative tyrosine phosphorylation pattern is shown. **C**, Quantification of the tyrosine phosphorylation level of a platelet protein of \approx 65 kDa (indicated by an arrow on panel B) by densitometry analysis (pTyr/actin ratio). Results are expressed as mean \pm SEM of 3 independent experiments, each run in duplicate, and normalized to nonactivated platelets (NA). * P <0.05; ** P <0.01; *** P <0.001; NS, nonsignificant.

Human blood concentrations of EDP reported in the literature fluctuate tremendously according to the method used for EDP assay.^{25–28} However, blood EDP production has been shown to increase with the progression of various vascular diseases, such as in atherosclerosis, aneurysms, and chronic kidney disease.^{22,26,28} For instance, serum EDP concentration in patients with chronic kidney disease at stages 3 and 4 has been measured at 57 μ g/mL ranging from 43 to 78 μ g/mL.²² In wild-type C57Bl/6J male mice, blood EDP concentration was estimated at 7.7 ± 1.1 mg/kg culminating at 14.2 ± 1.1 mg/kg in high fat diet mice and 15.2 ± 1.2 mg/kg in diabetic (*db/db*) mice.¹⁴ Importantly, local concentrations

of EDP at the site of injury are predicted to be even higher. Indeed, a specific feature of locally produced EDP is to increase further the elastolytic activity by acting on infiltrating cells at the site of vascular injury. This higher elastolytic activity will further increase elastin degradation and release of large amounts of EDP. This phenomenon is referred to as a self-propelled vicious circle, which could contribute to the progression and chronicity of vascular disease.²¹

In this study, we investigated the effects of EDP in thrombosis, another age-related vascular disease. We took advantage of kE, widely used for a long time for research and industrial purposes. kE is a complex mixture of EDP that contains bioactive peptides harboring the GxxPG motif recognized by the ERC. By combining both in vitro and in vivo approaches, we demonstrated that circulating EDP regulate thrombosis and hemostasis by an original mechanism involving effects both at the platelet and at the matrix levels. At the platelet level, EDP were shown to significantly decrease human platelet aggregation in whole blood induced by several agonists, such as collagen, TRAP, U46619, ADP, epinephrine, and arachidonic acid, with a maximal inhibition culminating at 20% to 48% according to the agonist used. This inhibitory effect was associated with decreased α IIB β 3 activation, suggesting that binding of EDP to platelets may affect the inside-out signaling pathways leading to α IIB β 3 activation. No effect was, however, observed on α -granule secretion as evidenced by measuring CD62P binding to platelets in whole blood on agonist challenge. Because of the complex nature of kE, we systematically used the VGVAPG peptide versus a scrambled peptide (VVGPGA) in the following experiments to specifically focus on the ERC in these processes because this peptide is known to bind specifically to the EBP subunit of this receptor.^{29,30} Indeed, \approx 29 different peptides were identified in kE mixtures by mass spectrometry analysis with one third containing the bioactive GxxPG motif (not shown).¹⁴ In contrast, the scrambled VVGPGA is unable to adopt the type VIII β -turn conformation because of the lack of GxxPG motif.³⁰ However, previous studies have reported that simple proline-containing peptides, such as PG, WP, and PGP (putative fragments of collagen and elastin), possess significant antithrombotic and anticoagulant effects in vitro and in vivo.³¹ This probably explains that the scrambled peptide used in the current study had some effects on platelets (Figure 4C) and on in vitro (Figure 2C) and in vivo (Figure 6) experiments. Other scrambled peptides (GGVVAP, VGVGAP, GAGVVP, AVPVGG) were tested and showed similar effects (data not shown).

Using washed platelets, we next demonstrated that kE and the ERC directly affect platelet function by decreasing collagen-induced platelet aggregation and related signaling. We show here that the level of collagen-induced tyrosine phosphorylation is significantly reduced by kE and VGVAPG as evidenced by decrease of the tyrosine phosphorylation of a 65-kDa platelet protein that remains to be identified in further studies. Importantly, when tested alone, kE did not induce neither platelet activation nor platelet secretion or exposure of phosphatidylserine, suggesting that kE did not cause desensitization of platelets or affect platelet viability over the time course of the in vitro experiments. The

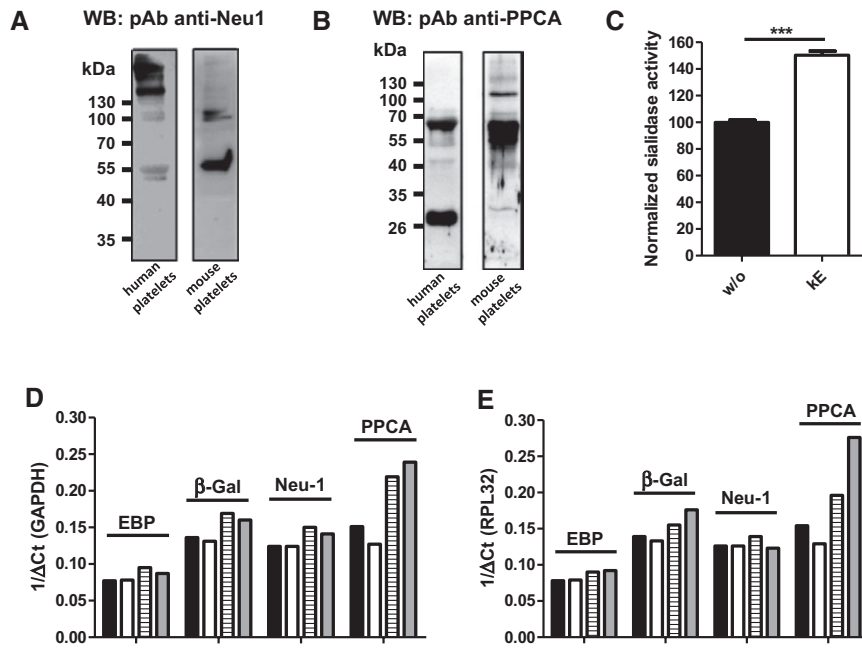


Figure 5. EBP, Neu-1, and PPCA are expressed in platelets and form a functional receptor. Western blot of human and mouse washed platelet lysates (25 µg) probed with a rabbit polyclonal (A) anti-Neu-1 or (B) anti-PPCA antibody. Data are representative of 4 independent experiments. C, Sialidase activity was measured in nonpermeabilized washed platelets (6.10^7 /well) after 2 hours of incubation at 37°C in the presence of 200 µM 2-O-(*p*-nitrophenyl)- α -D-N-acetylneuraminic acid substrate together with vehicle or kE (100 µg/mL, final concentration). Results are expressed as mean \pm SEM of 6 independent experiments, each run in triplicate, and normalized to the control (w/o, without kE); *** P <0.001. D and E, RT-qPCR analysis of mRNA levels of EBP, β -Gal, Neu-1, and PPCA in the CHRF-288-11 (black bars) and MEG-01 (white bars) human megakaryoblastic cell lines and human CD34⁺ hematopoietic stem cells at 6 (hatched bars) and 11 (gray bars) days after in vitro differentiation. mRNA levels were normalized to (D) GAPDH or (E) RPL32 mRNA levels. Data are expressed as relative expression (1/ Δ Ct). EBP indicates elastin-binding protein and PPCA, protective protein/cathepsin A.

existence of a functional ERC in platelets was further demonstrated by the presence of both Neu-1 and PPCA in human and mouse platelets at the protein level and of EBP, Neu-1, and PPCA in human megakaryocytes at the mRNA level. These 3 subunits form a functional receptor in human platelets that is able to trigger an increase in sialidase activity on binding to EDP. In contrast to PPCA, the catalytic activity of Neu-1 has been shown to be essential for ERC-mediated effects both in vitro^{19,32} and in vivo^{13,14} by catalyzing the local conversion of GM₃ ganglioside into lactosylceramide.^{19,32} Expression of Neu-1 and PPCA has been already reported in platelets. Neu-1 has been shown to be up-regulated at the cell surface after refrigeration and to participate in vWF receptor (GPIb α) desialylation.³³ PPCA has been shown to be secreted on platelet aggregation,³⁴ but its role in platelet activation remains unknown to date. Unfortunately, because of the unavailability of anti-EBP antibodies, we were unable to demonstrate the presence of EBP at the protein level in platelets. However, the increase in platelet sialidase activity triggered by EDP confirm that EBP is well present at the protein level in platelets.

Deciphering the influence of EDP and the VGVAPG peptide on platelet interaction with collagen under flow conditions led to the unexpected finding that EDP also disrupt immobilization of human and mouse plasma vWF onto collagen fibers as a result of a high affinity of EDP for collagen. Indeed, at 25 µg/mL that represents 0.33 µmol/L (average molecular weight of kE=75 kDa), a strong inhibition of 45% was observed with a maximal effect culminating at 80% to 85% inhibition. In our

conditions, both human and mouse plasma vWF were unable to interact with immobilized kE as already reported by Matsui et al³⁵ and VGVAPG was without effect, suggesting that longer EDP seem to be involved in disruption of plasma vWF immobilization onto collagen.

Specific involvement of EDP in regulating thrombosis and hemostasis was finally confirmed in vivo because kE prolonged mesenteric arteriole occlusion and tail-bleeding time in mice. kE was used at 10 mg/kg, a concentration slightly above the blood EDP concentration measured in wild-type mice (7.7 ± 1.1 mg/kg) and well below the concentrations estimated in high fat diet (14.2 ± 1.1 mg/kg) and diabetic (15.2 ± 1.2 mg/kg) mice.¹⁴ Importantly, injection of the VGVAPG peptide prolonged mesenteric arteriole occlusion and may also increase tail-bleeding time in mice, confirming the specific involvement of the ERC in vivo and most probably of the ERC-mediated effects described here on platelets. This is further strengthened because the VGVAPG peptide has no major effect on vWF binding to collagen.

In conclusion, the current study demonstrates for the first time that EDP originating from elastin fragmentation during vascular aging may be involved in the regulation of thrombosis and hemostasis in vivo. It now remains to clarify the contribution of endogenous EDP in murine models harboring the pathophysiological features of vascular aging because healthy vessels are devoid of atherosclerotic plaques and do not recapitulate all the steps and players involved in the complex process of thrombus formation at sites of atherosclerotic lesions. In addition, these antithrombotic

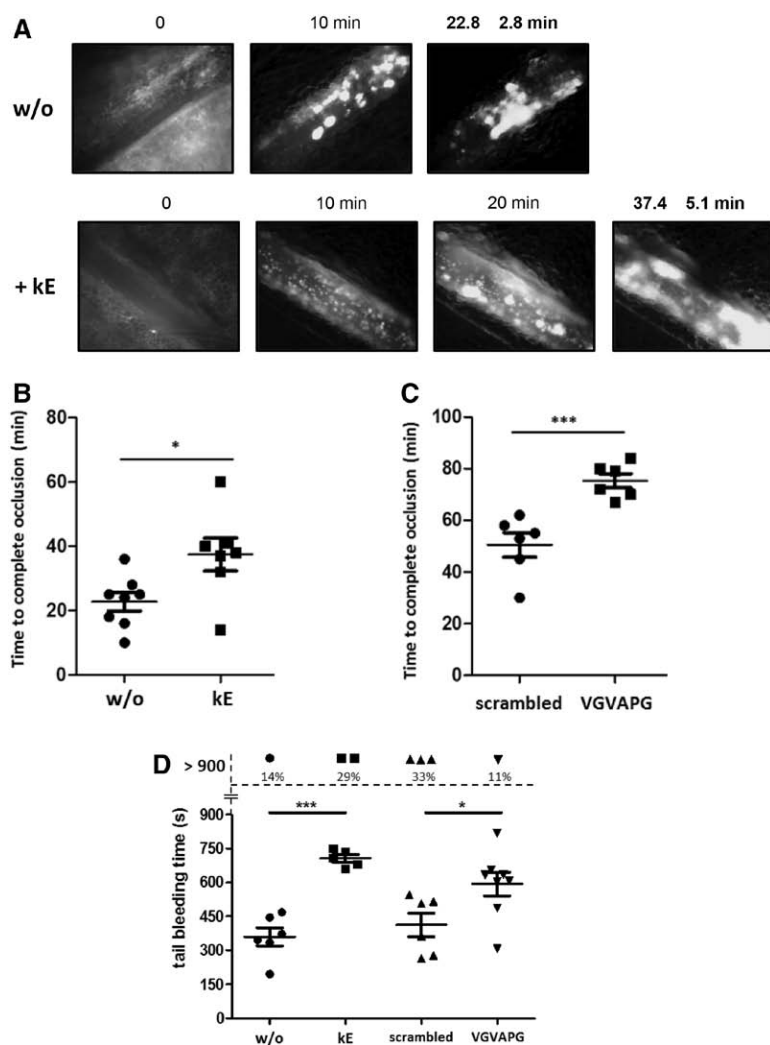


Figure 6. Elastin-derived peptides and VGVAPG delay thrombus formation in vivo. The effects of elastin-derived peptides were investigated in a mouse model of mesenteric arteriole thrombosis induced by 10% FeCl₃. **A**, Photographs show a representative progression of thrombus formation for kE- versus vehicle (w/o)-injected mice. **B** and **C**, The dot plots show times to complete occlusion on vascular injury in mice injected with (B) saline or kE (10 mg/kg) and (C) VGVAPG (40 mg/kg) or scrambled VVGPGA (40 mg/kg). Means are indicated by horizontal bars. **D**, Bleeding times of mice treated with vehicle (●), 100 μg/mL kE (■), and 400 μg/mL scrambled VVGPGA (▲) or VGVAPG (▼) peptide. **P*<0.05; ****P*<0.001.

effects of EDP may have deleterious consequences on atherosclerosis progression because intraplaque hemorrhage has been associated with increased risk of plaque rupture. In this study, we further demonstrated that the regulatory role of EDP on thrombosis rely on an original mechanism that involves both EDP-mediated disruption of vWF immobilization onto collagen fibers at the matrix level (receptor-independent) and EDP-mediated effects at the platelet level, leading to decreased aggregation and increase in sialidase activity. Importantly, platelets express a functional ERC that is able to recapitulate the effects observed with kE. However, whether EDP and the VGVAPG peptide are acting by the same mechanism on platelets remains to be elucidated. Indeed, the hypothesis that EDP may also regulate platelet function by a receptor-independent mechanism cannot be totally discarded. Anyway, this study demonstrates for the first time that a functional ERC is present in platelets. On binding to an elastin-derived bioactive GxxPG peptide, such as VGVAPG, the ERC is able to decrease washed platelet aggregation and related signaling and to prolong arteriole occlusion and tail-bleeding time. Because the signaling pathways mediated by the ERC have been shown to transduce signals through the catalytic activity of its Neu-1 subunit,^{13,14,36} this study opens new avenues in the complexity of

the platelet signaling pathways involved during thrombosis and hemostasis and offers the exciting possibility of a potential fine-tuning of platelet receptor sialylation and activation by the ERC.

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We thank Pr Robert P. Mecham for kindly providing the BA4 antibody, Pr Alessandra d' Azzo for the anti-PPCA antibody, Dr Roselyne Garnotel for the analysis of elastin purity by chromatography, and Jean-Luc Breda for animal maintenance.

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Disclosures

None.

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Significance

This is the first study establishing the importance of elastin-derived peptides, proteolysis products of the vascular extracellular matrix (ECM), in the regulation of hemostasis and thrombosis. Vascular aging is associated with extensive ECM remodeling leading to the production of ECM-derived peptides, known as matrikines, which present new biological activities. These ECM-derived peptides therefore represent promising pharmaceutical targets. Elastin is a good example of such vascular ECM macromolecules subject to proteolysis. However, the impact of elastin-derived peptides on thrombosis and hemostasis is yet underestimated to date. In this study, we show that elastin-derived peptides regulate thrombosis by an original mechanism involving effects both (1) on platelets, that express a functional elastin receptor complex, by reducing platelet aggregation, and impairing related signaling and (2) at the matrix level by disrupting plasma von Willebrand factor immobilization onto collagen.

SUPPLEMENTAL FIGURES

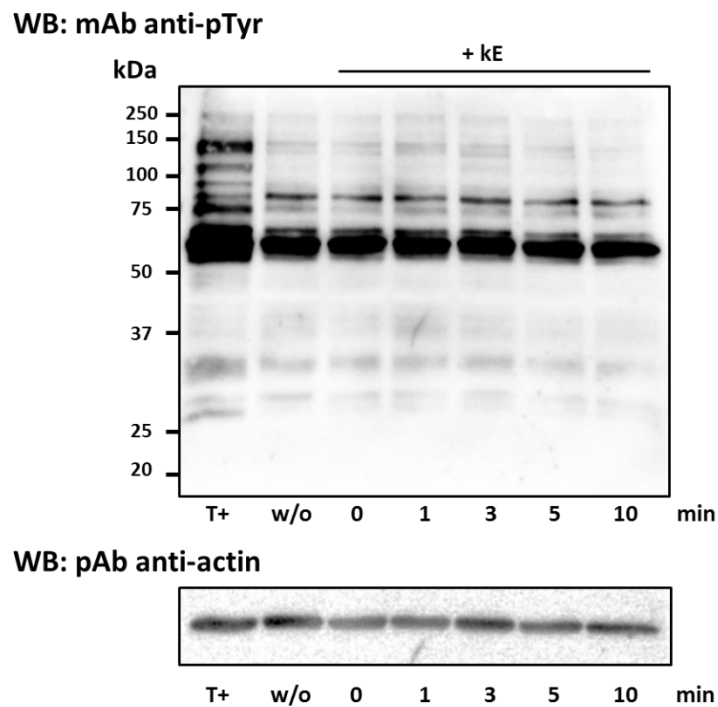
A	
PAC-1	
Normalized MFI	
w/o	1.00
kE 50	0.97±0.08
kE 100	1.19±0.19

B	
CD62P	
Normalized MFI	
w/o	1.00
kE 50	0.99±0.04
kE 100	1.02±0.08

C	
Annexin V	
%age of Annexin V-positive CD41⁺ cells	
w/o	0.80±0.20
A23187	96.27±1.84***
kE 50	0.43±0.15
kE 100	0.50±0.20
kE 200	0.60±0.15

Supplemental Figure I. Incubation of whole blood with EDP has not effect on platelet α IIb β 3 activation, CD62P and PS surface exposure.

Activation of platelet α IIb β 3 and surface exposure of P-selectin (CD62P) and phosphatidylserine (PS) were determined by flow cytometry with FITC-conjugated PAC-1 (A), anti-CD62P (B) antibodies, and FITC-conjugated Annexin V (C), respectively. Whole blood was incubated with 50, 100 or 200 μ g/mL kE for 5 minutes at 37°C before acquisition. (A,B) For positive controls, see Figure 1. (C) As positive control, whole blood was incubated for 5 minutes at 37°C with the calcium ionophore A23187 (10 μ M). (A,B) Data represent the mean fluorescent intensity (MFI) \pm SEM of 3 to 5 independent experiments normalized to saline buffer (without kE, w/o). (C) Data represent the percentage of CD41⁺ cells positive for Annexin V labeling and are mean \pm SEM of 3 independent experiments. *** $P < 0.001$.



Supplemental Figure II. Incubation of washed platelets with EDP has no effect on platelet protein tyrosine phosphorylations.

Washed human platelets ($3 \cdot 10^8$ /mL) were incubated with saline buffer or kE (100µg/mL) for 0, 1, 3, 5 and 10 minutes at 37°C with stirring in the cuvette of an aggregometer. As positive control (T+), washed platelets were activated by 10µM TRAP. Then platelets were solubilized and proteins separated by SDS-PAGE. Tyrosine phosphorylated proteins were detected using the monoclonal anti-phosphotyrosine antibody PY20 and the amount of proteins loaded was evaluated by a polyclonal anti-actin antibody. A representative tyrosine phosphorylation pattern, of four independent experiments, is shown.

MATERIALS AND METHODS

Reagents and antibodies

Horm collagen was purchased from Nycomed. ADP and TRAP-6 (thrombin receptor activating peptide-6) were obtained as Multiplate[®] reagents from Roche Diagnostics. The thromboxane analog U46619 was from Tocris. Rhodamine 6G, apyrase, prostaglandin E₁ (PGE₁), ferric chloride, epinephrine, MTT assay, mouse monoclonal anti-phosphotyrosine (PY20) and goat polyclonal anti-actin antibodies were from Sigma. Arachidonic acid was purchased from Bio/Data Corporation. D-Phe-Pro-Arg chloromethylketone dihydrochloride (PPACK) was from Calbiochem. Enoxaparin (2000 UI anti-Xa/0,2 mL) was used as low molecular weight heparin (LMWH). The peptides VGVAPG and VVGPGA were synthesized by GeneCust (purity >98%). HRP-coupled polyclonal rabbit anti-human von Willebrand factor (vWF) antibody was from Dako. The FITC-coupled mouse PAC-1 and anti-CD62P antibodies, and FITC-Annexin V were from BD Biosciences. Rabbit polyclonal anti-Neu-1 (H-300) antibody was from Santa Cruz Biotechnology and anti-PPCA kindly provided by Pr Alessandra d'Azzo. The mouse monoclonal BA4 antibody was a kind gift of Pr Robert P Mecham. This antibody has been shown to recognize EDP containing the GxxPG motif and to be well suitable for enzyme-linked immunosorbent assay.¹⁻³ HRP-coupled anti-rabbit and anti-mouse IgG were from Cell Signaling

Kappa-elastin preparation

EDP were prepared as described previously.⁴ Briefly, insoluble elastin was prepared from bovine *ligamentum nuchae* by hot alkali treatment. Purity was assessed by comparing its amino acid composition to the one predicted from the elastin gene product. Soluble EDP were then obtained from insoluble elastin as described.⁴ The obtained mixture of EDP, termed kappa-elastin (kE), has been shown to contain several peptides harboring the bioactive motifs PGAIPG, GAVPG, GVLPG, GGVPG, GVVPG, and VGVAPG⁵. The composition of EDP from kE was recently compared to EDP obtained after proteolysis of human elastin by neutrophil elastase by mass spectrometry analysis and shown to contain similar peptides harboring the bioactive GxxPG motif.⁵ In addition, kE has been shown to exhibit the same biological properties than elastin hydrolysates obtained by human neutrophil elastase as both EDP mixtures increase pro-MMP1 production in human skin fibroblasts with comparable extent.⁶

Animals and treatment

Mouse experiments were performed in agreement with the European legislation on care and use of laboratory animals and were approved by the ethical committee of Reims Champagne Ardenne. C57Bl/6J male mice of 4 to 6 week-old were purchased from Janvier and maintained under standard laboratory conditions for 2 weeks before experiments.

Platelet aggregation in whole blood

Whole blood was obtained by venipuncture from consenting healthy human donors and anticoagulated with sodium citrate (0.129M) using Vacutainer[®] tubes. Whole blood aggregation was monitored by impedance aggregometry at 37°C with stirring using the Multiplate[®] analyzer. Whole blood was diluted in physiological saline (1:1) and incubated with different concentrations of kE for 3 minutes at 37°C before adding Horm collagen (0.32µg/mL), TRAP (32µM), U46619 (0.5µM), ADP (6.5µM), arachidonic acid (0.25mM), or epinephrine (10µM). Curves were recorded for 6 minutes and platelet aggregation was determined as area under the curve (AUC) of arbitrary units (AU*min).

Washed platelet aggregation

Human whole blood was anticoagulated with acid-citrate-dextrose (trisodium citrate 22g/L, citric acid 8g/L, dextrose 24.5g/L). Platelet-rich plasma (PRP) was obtained by centrifuging

whole blood for 15 minutes at 170g at room temperature. Platelets were obtained from PRP by centrifugation for 15 minutes at 1200g and washed in the presence of apyrase (0.3U/mL) and PGE1 (100nM) to minimize platelet activation. Then platelets were adjusted to 3.10^8 /mL in pH 7.5 Tyrode's buffer (137mM NaCl, 2.7mM KCl, 1.2mM NaHCO₃, 0.36mM NaH₂PO₄, 2mM CaCl₂, 1mM MgCl₂, 5mM HEPES, 5.5mM glucose). Aggregation was monitored by measuring light transmission using an 8-channel SODEREL aggregometer.

Flow cytometry analysis

Ninety five μ L of whole blood were incubated for 5 minutes with 5 μ L of kE (final concentrations of 0, 50 and 100 μ g/mL). Then, the mixtures were incubated, or not, for 5 minutes with 100 μ L of agonists (20 μ g/mL collagen, 20 μ M ADP or 10 μ M TRAP, final concentrations) then stopped by 250 μ L of PBS. Twenty μ L of each mixture were incubated with FITC-PAC1 or FITC-anti-CD62P antibody for 20 minutes at room temperature in darkness. Each mixture was then diluted in 1mL PBS and immediately analyzed with a FC500 flow cytometer (Coulter). Platelet population was gated using their forward and side scatter characteristics. Acquisition and processing data from 5,000 platelets were performed and analyzed using CXP software (Coulter). Mean Fluorescent Intensity (MFI) of the whole platelet population was used. For FITC-Annexin V labelling, the same protocol was used in the presence of Annexin V binding buffer. Platelets were labelled with PE-CD41 and acquisition and processing data from 10,000 platelets (CD41⁺ population) were performed and analyzed as mentioned above.

In vitro thrombus formation under flow conditions

Thrombus formation was evaluated in a whole blood perfusion assay under arterial shear condition (1500s⁻¹) over collagen-coated microchannels. Vena8 FLUORO+ biochips (Cellix) were coated with Horm collagen (30 μ g/mL, overnight, 4°C). Whole blood was collected from the vena cava of anesthetized mice in 40 μ M PPACK and 5U/mL LMWH.⁷ Blood from 2 mice were pooled, labeled with rhodamine 6G (10 μ g/mL) together with vehicle, kE (100 μ g/mL), VGVAPG or scrambled VVGPGA (400 μ g/mL), and incubated at 37°C for 5 minutes before use to resensitize platelets. For all experiments, mouse blood was used within 1 hour. Recruitment of platelets and real time thrombus formation was recorded with an epifluorescent microscope (Zeiss AX10) connected to a Nikon digital DXM1200 camera. Thrombus formation was determined as the mean percentage of total area covered by platelets using the ImageJ software.

Collagen binding assay of vWF

Plasma was obtained from anticoagulated human and mouse blood after centrifugation at 2,000g for 10 minutes. Ninety six-well microtiter plates (FluoroNunc MaxiSorp, Nunc) were coated overnight at 4°C with 25 μ g/mL Horm collagen diluted in PBS containing 25mM acetic acid and then blocked with 1% BSA. Plates were incubated with different concentrations of kE (1h, 37°C) followed by mouse or human plasma (1/200) for additional 2 hours at 37°C. After washings, bound vWF was detected using HRP-coupled rabbit anti-human vWF antibodies (1/2,000). Visualization was obtained with TMB-ELISA (ThermoScientific) and the reaction was stopped with 2M H₂SO₄. Binding of vWF to collagen in the presence of various concentrations of kE was compared to the binding in the absence of kE (normalized at 100%).

Collagen binding assay of kE

The same protocol was used as described above, except that bound EDP were detected using the BA4 monoclonal antibody (1/10,000) followed by HRP-coupled anti-mouse IgG (1/1,000).

kE binding assay of vWF

Ninety six-well microtiter plates were coated overnight at 4°C with kE (100 μ g/mL) diluted in PBS and then blocked with 1% BSA. Plasma (1/200) was added for 2 hours at 37°C and

bound vWF was detected as described above. Binding of human and mouse vWF to kE was compared to the average binding of human and mouse vWF to collagen, respectively, normalized at 100%.

Immunoblotting

For the detection of Neu-1 and PPCA, human and mouse washed platelets were prepared by centrifugation of anticoagulated whole blood at 700g for 15 minutes. The supernatant was recovered and platelets were pelleted by centrifugation at 1200g for 15 minutes. Platelets were then washed 2 times with washing buffer (36mM citric acid, 5mM glucose, 5mM KCl, 1mM MgCl₂, 103mM NaCl). Washed platelets were lysed in Laemmli buffer, heated at 100°C for 5 minutes and subjected to SDS-PAGE. Proteins were then transferred on a nitrocellulose membrane (Whatman GmbH). After saturation, membranes were incubated with rabbit polyclonal anti-Neu-1 (1/500) or anti-PPCA (1/1,000) overnight at 4°C. Immunoreactive bands were revealed using a HRP-conjugated anti-rabbit IgG (1/10,000) followed by enhanced chemiluminescence detection reagents (Amersham) and visualized with the Odyssey Fc LI-COR scanner (ScienceTec).

For the analysis of protein tyrosine phosphorylations, washed platelets were lysed by SDS 2% directly in the cuvette of the aggregometer. Samples were then diluted in Laemmli buffer, heated at 100°C for 5 minutes and subjected to SDS-PAGE. Platelet proteins phosphorylated on tyrosine residues were revealed using the monoclonal PY20 antibody followed by HRP-conjugated anti-mouse IgG (1/10,000).

PCR and real-time qRT-PCR

Total mRNAs were extracted with the Qiagen kit (RNeasy mini kit) from differentiated CD34⁺ human stem cells⁸ and two human megakaryoblastic cell lines (CHRF-288-11 and MEG-01). The RT-qPCR primers for detection of EBP, β -Gal and PPCA were designed as previously described.⁹ The forward and reverse primers for Neu-1 were TCCAAGGCTGAGAACGACTT and AGGTGTCCACTGAGCCGAT, respectively. All genes were normalized to the human housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or ribosomal protein L32 (*RPL32*). The forward and reverse primers for GAPDH were CCATCACCATCTTCCAGGAG and CCTGCTTCACCACCTTC, respectively. The forward and reverse primers for RPL32 were CATTGGTTATGGAAGCAACAAA and TTCTTGGAGGAAACATTGTGAG, respectively. All primers were synthesized by Eurogentec.

One μ g of total RNA were reverse-transcribed using the M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real-time PCR was performed using an Absolute SYBR Green Rox mix (Thermo Fisher Scientific), on a CFX 96 real time PCR detection system (Bio-Rad). PCR conditions were 15 min at 95 °C, followed by 40 cycles each consisting of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing/extension). The specificity of PCR amplification was checked using a heat dissociation curve from 65°C to 95°C following the final cycle. Delta Ct values (Δ Ct) were calculated from the difference of the Ct of the gene of interest to the Ct of the housekeeping gene GAPDH or RPL32 and are shown as $1/\Delta$ Ct values.

Sialidase activity

Platelet sialidase activity was assessed using the 2-O-(p-Nitrophenyl)- α -D-N-acetylneuraminic acid (Sigma) as substrate. Assays were performed in triplicate with 200 μ L of human washed platelets in Tyrode's buffer (3.10⁸/mL). Washed platelets were incubated with vehicle or 100 μ g/mL kE for 2 hours at 37°C together with 200 μ M of substrate (final concentration). Reactions were stopped by adding 300 μ L of 1M Na₂CO₃ and the absorbance of each sample was immediately measured at 405nm using an Infinite F200 PRO microplate reader (TECAN).

FeCl₃-induced in vivo thrombosis

Mice were anesthetized with 25% urethane and intravenously injected with kE (10mg/kg), VGVAPG (40mg/kg), scrambled VVGPGA (40mg/kg) or vehicle, and rhodamine 6G (4mg/kg) before induction of thrombosis. Rhodamine 6G was used to label endogenous platelets and leukocytes. The mesenteric arteriole was isolated from surrounding tissue and spread on a microscopic glass. Vascular injury was performed by applying a filter paper disc (6mm diameter, 1mm thickness) saturated with 10% FeCl₃ onto the mesenteric arteriole for 5 minutes. After removing the filter paper, thrombus formation was monitored in real time with the epifluorescent microscope connected to the digital camera. Time to complete occlusion was defined as blood flow being stopped for at least 30 seconds.

Tail bleeding time

Bleeding time assays were performed by cutting off the tip of the tail (5mm from the tip) of mice anesthetized with 25% urethane and intravenously injected with kE (10 mg/kg), VGVAPG (40mg/kg), scrambled VVGPGA (40mg/kg) or vehicle. The tail was immediately immersed in saline buffer heating at 37°C. Bleeding was followed visually and time to stable cessation of bleeding (no rebleeding within 1 minute) was recorded. When necessary, bleeding was stopped manually at 15 minutes.

Statistical analysis

Results are expressed as mean +/- SEM. Two groups were compared using Student *t* test or 2-tailed Mann-Whitney test. Three and more groups were compared using one-way ANOVA followed by Dunnett's multiple comparison test. *P* values of less than 0.05 were considered as statistically significant.

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