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Matrix ageing and vascular impacts: focus on elastin fragmentation

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Abstract

Cardiovascular diseases (CVDs) are the leading cause of death worldwide and represent a major problem of public health. Over the years, life expectancy has considerably increased throughout the world, and the prevalence of CVD is inevitably rising with the growing ageing of the population. The normal process of ageing is associated with progressive deterioration in structure and function of the vasculature, commonly called vascular ageing. At the vascular level, extracellular matrix (ECM) ageing leads to molecular alterations in long half-life proteins, such as elastin and collagen, and have critical effects on vascular diseases. This review highlights ECM alterations occurring during vascular ageing with a specific focus on elastin fragmentation and also the contribution of elastin-derived peptides (EDP) in age-related vascular complications. Moreover, current and new pharmacological strategies aiming at minimizing elastin degradation, EDP generation, and associated biological effects are discussed. These strategies may be of major relevance for preventing and/or delaying vascular ageing and its complications.

Keywords

Ageing • Extracellular matrix remodelling • Elastin fragmentation • Vascular diseases

1. Elastin in the vessel wall

The vessel wall extracellular matrix (ECM) is an active and dynamic structure playing a fundamental role in regulating vascular function in normal and pathophysiological conditions. The vascular ECM is composed of collagens, elastin, and others ECM components such as glycoproteins, amorphous or soluble proteoglycans, growth factors, and proteases sequestered in the matrix. ECM composition varies according to the vessel wall tunicae (intima, media, and adventitia) and all along the vascular tree.¹ Collagens and elastin remains by far the most abundant ECM proteins in the arterial wall.

Mature elastin is an insoluble and hydrophobic protein formed by cross-linking of its soluble precursor, tropoelastin which is mainly produced by smooth muscle cells in the media and fibroblasts in the adventitia. An elastic fibre consists of fibrillin-rich microfibrils surrounding an amorphous core of elastin ($\approx 90\%$ of elastic fibre content). Elastin is the dominant ECM protein in the arterial wall, comprising 50% of its dry weight.² It allows vascular elasticity and recoil. In the tunica media, elastic fibres are arranged in fenestrae sheets, or lamella, and circumferentially aligned with smooth muscle cells. The number of lamellar units, defined as an elastic lamella and adjacent smooth muscle cells, formed the functional resilient unit of the arterial wall.³ Elastogenesis is

restricted to foetal and infancy, and mature elastin fibres remain for lifespan. Indeed, its strong reticulation makes elastin a highly stable molecule with longevity comparable with human lifespan and any proteolytic damage that does occur with age and disease is essentially irreparable.⁴ Under pathological conditions, vascular and inflammatory cells can, however, produce tropoelastin, but these tropoelastin molecules fail to cross-link into mature elastic fibres.⁵

2. Vascular alterations occurring with ageing

Ageing is a multifactorial process defined as the consequence of time-dependent loss of function, resistance, and adaptability to stress in all organs.⁶ Vascular ageing depends on both intrinsic and extrinsic mechanisms and is considered to be the result of time and gradual modifications of the vascular tree.⁷ The cellular and molecular mechanisms involved in vascular ageing are complex and multifactorial.

During ageing and in the absence of observable vascular disease, several changes in morphology and composition of conduit arteries occur and contribute to decrease compliance, resilience, and increase stiffness seen in aged conduit arteries⁸ (Figure 1). The migration and

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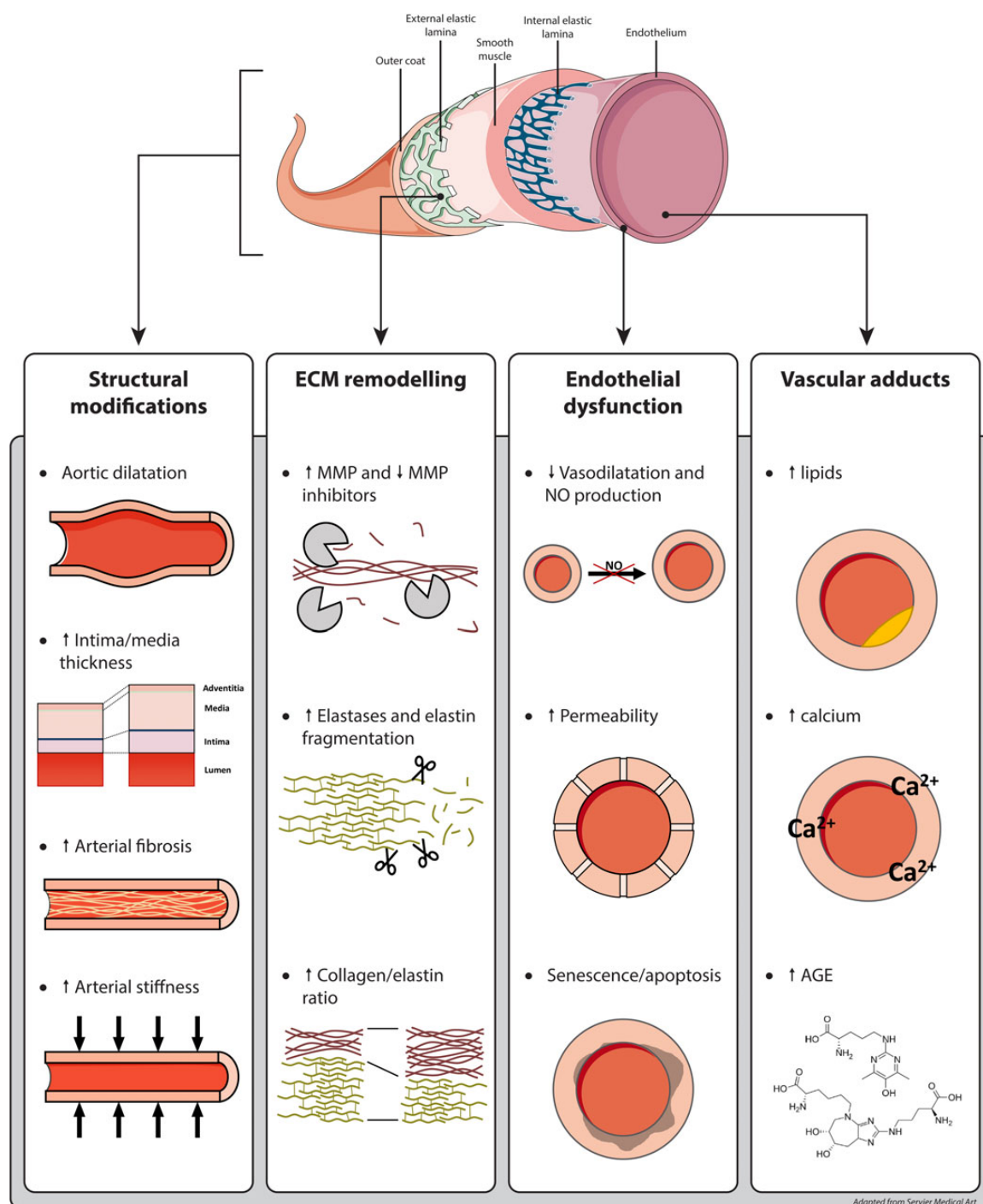


Figure 1 Molecular and cellular events occurring during vascular ageing. As depicted in the figure, ageing is associated with critical modifications of the vascular wall with structural alterations, extracellular matrix remodelling, endothelial dysfunction, and deposits of lipids, calcium, and advanced glycation end-products. All these events contribute to decrease compliance, resilience, and increase stiffness in aged conduit arteries. ECM, extracellular matrix; MMP, matrix-metalloproteinases; NO, nitric oxide; AGE, advanced glycation end-products.

proliferation of medial smooth muscle cells, switching from a contractile to a synthetic, proliferative, mitotic and migratory phenotype, causes intima and media remodelling. This is accompanied by a progressive increase in lumen diameter and lengthening of loosely tethered vessels, such as the abdominal aorta, leading to tortuosity.⁹ In addition, changes occur on the two most abundant matrix proteins present in the vessel wall, collagen, and elastin, also contributing to increase vascular

stiffness. The collagen content increases with age, giving rise to a corresponding reduction in elastin amount and transferring the mechanical load to collagen fibres, which are 100–1000 times stiffer than elastic fibres. In addition to the age-related decrease in elastin content, the structure of the elastic lamellae changes with age due notably to repeated mechanical loading and oxidative stress, showing clear signs of fragmentation and rupture.⁸ At the same time, the remaining elastin

become calcified^{10,11} while the collagen molecules progressively acquire cross-links¹² altering their structural and functional properties. In addition, as arteries age or undergo pathological changes, the balance between proteases and their inhibitors is lost and protease release increases by the induction of matrix-metalloproteinases (MMP) gene expression, the activation of zymogens or the secretion of enzymes by inflammatory cells. This chronic increase in MMP activation plays a central role in age-associated arterial structural remodelling. Ageing has been shown to enhance MMP-2/-7/-9/-14 activity in the aortic walls of rodents, non-human primates, and humans.¹³

Finally, another determinant processes in vascular ageing are the increased oxidative stress and inflammation. Indeed, ageing is associated with a chronic, low-grade proinflammatory state and referred to 'inflammaging'.¹⁴ Considerable data have demonstrated an increase in systemic inflammatory markers during ageing in addition to proinflammatory transcription factors, integrins and proteases.¹⁵ Ageing also generates excess of reactive oxygen species that compromise the vasodilatory activity of nitric oxide, facilitate the formation of deleterious radicals,¹⁶ and play critical roles in vascular dysfunction.

3. Elastin fragmentation and generation of elastin-derived peptides

The progressive deterioration in vascular structure and function with medial degeneration, aortic stiffening and dilation, are the basis of the

so-called vascular aging continuum introduced by O'Rourke *et al.* in 2010.¹⁷ This continuum, which explains cardiovascular events occurring with ageing in patients without classic cardiovascular risk factors, highlights the fatigue then fracture of elastic lamellae in the proximal aorta as the main trigger. As mentioned above, the very low turnover rate of elastin *in vivo* leads to accumulation of age-related changes caused by mechanical fracture, proteolysis, and calcification/glycation. Mechanical fracture is due to fatigue failure caused by the pulsatile wall strain (repetitive stretches and relaxations) experienced by the aorta over the number of cardiac cycles throughout the lifetime. Elastic fibres undergo more than 3 billion cycles of extension and recoil over human lifespan. This purely mechanical issue is enhanced by pathophysiological processes related to ageing such as calcification, glycation, and lipid peroxidation. A positive correlation has been shown between the extent of calcification and the degree of elastin fragmentation. Elastin has several nucleation sites for apatite minerals favouring calcium accumulation.^{18,19} Moreover, aldehydes generated from peroxidation of polyunsaturated fatty acids, particularly 4-hydroxynonenal, malondialdehyde, and acrolein, were described to link elastin and to induce a loss of flexibility, and altered conformation leading to increased enzymatic digestion susceptibility.^{20–22} Finally, non-enzymatic cross-links of elastin with advanced glycation end-products (AGE) and, more recently, carbamylation products have been also reported.^{23,24} All these adducts will contribute to elastin fragility and fragmentation that is further amplified by inflammation and subsequent secretion of MMP^{13,25} (Figure 2).

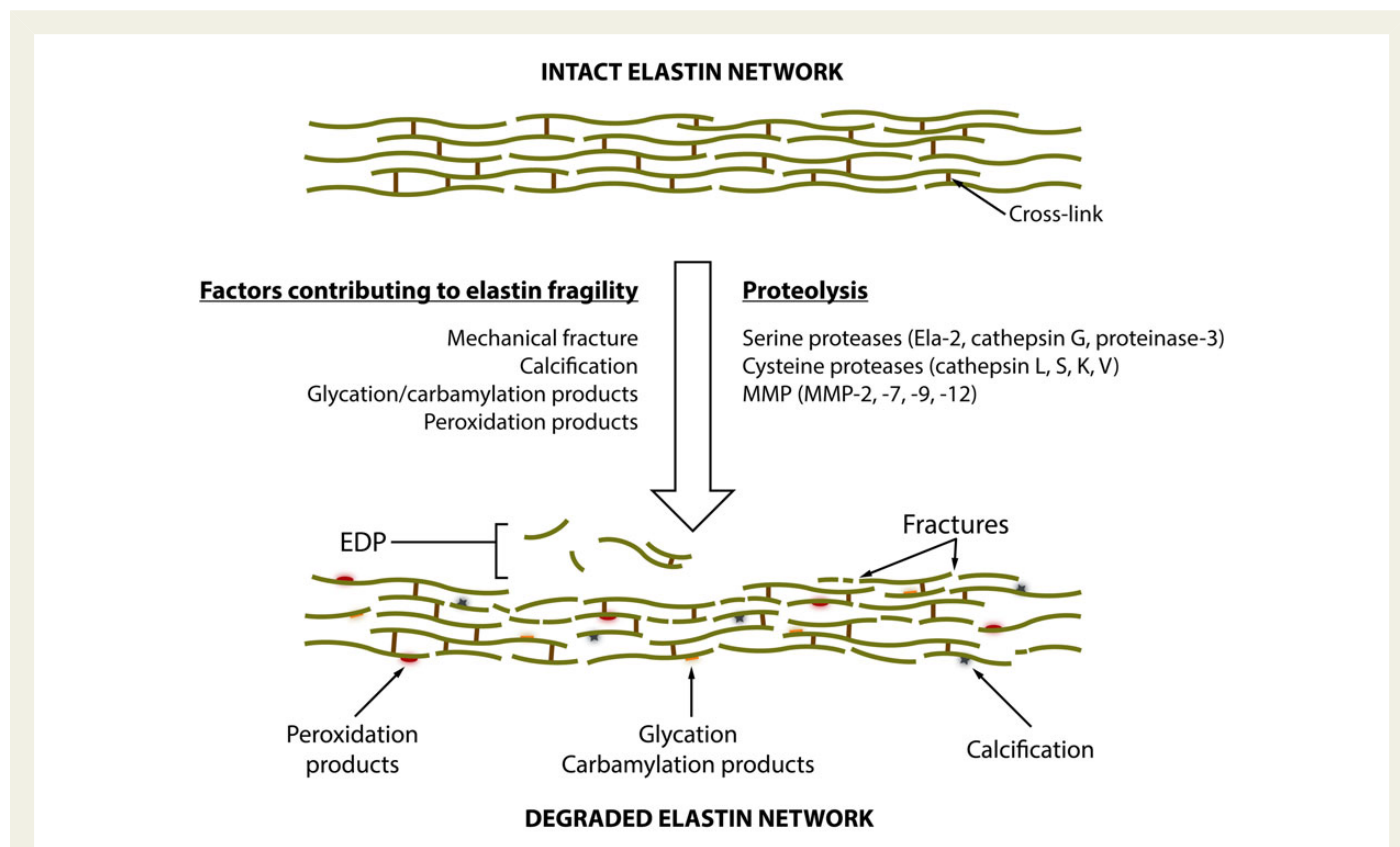


Figure 2 Elastin remodelling during vascular ageing. During ageing, elastin is subject to remodelling and fragmentation. Different factors can contribute to elastin fragility and rupture such as mechanical fracture, calcification, glycation, carbamylation, and peroxydation. In addition, these factors enhance elastin susceptibility to proteolysis by specific elastases such as serine, cysteine, and matrix-metalloproteinases. Ela-2, neutrophil elastase; MMP, matrix-metalloproteinases; EDP, elastin-derived peptides.

3.1 Elastases

Elastases are endopeptidases that cleave insoluble and soluble elastin. Elastases include serine-, cysteine- and metallo-proteinases²⁶ (Figure 2). The serine proteinases neutrophil elastase (Ela-2), cathepsin G, and proteinase-3 (myeloblastin) are mainly expressed by polymorphonuclear neutrophils and smooth muscle cells. Four members of the cysteine cathepsin family (cathepsin L, S, K, and V) display elastinolytic activity and are also mainly produced by macrophages and smooth muscle cells. Finally, four MMP are known as elastases, gelatinase A (MMP-2), matrilysin (MMP-7), gelatinase B (MMP-9), and macrophage elastase (MMP-12). While gelatinases are expressed ubiquitously, MMP-7 and MMP-12 are mainly produced by macrophages.²⁶

Elastin degradation by elastases generates elastin-derived peptides (EDPs). Among them, peptides harbouring a GxxPG consensus motif (where x represents any amino acid) are bioactive because this sequence motif stabilizes a type VIII β -turn conformation.²⁷ These bioactive EDP are referred to as elastokines. The typical elastokine is the VGVAPG peptide, a three-fold repetition sequence encoded by exon 24 of human tropoelastin. Other bioactive GxxPG motifs, GVYPG, GFPG, and GVLPG,²⁸ and longer elastokines have been reported.²⁹ For instance, MMP-7, -9, and -12 have been shown to generate the bioactive YTTGKLPGYGP, YGARPGVGVGGIP, and PGFGAVPGA peptides.²⁹

3.2 The elastin receptor complex

Tropoelastin and EDP have been shown to bind $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins,^{30,31} a lactose insensitive receptor,³² and galectin-3.³³ However, the elastin receptor complex (ERC) is the most documented elastin receptor and possibly the most important EDP signalling transducer reported so far. Indeed, elastin-binding protein (EBP) elicits most of their biological effects following their interaction with the ERC. This receptor has a singular composition: a peripheral 67 kDa subunit, named EBP or spliced-galactosidase, and two membrane-associated proteins, a 55 kDa protective protein/cathepsin A (PPCA) and a 61 kDa neuraminidase (neuraminidase-1, Neu-1).³⁴ EBP is a spliced variant of the lysosomal β -galactosidase resulting through the deletion of exons 3 and 4 and the introduction of a frameshift. A second frameshift occurs after the deletion of exon 6 restoring the initial frame. This spliced form of β -galactosidase is devoid of enzymatic activity but retains the ability to bind galactosugars (galactose, lactose, or chondroitin sulfate) that are efficient antagonists of EDP binding. This singular splicing mechanism generates a 32-residue sequence-specific of EBP containing the elastin-binding site.³⁵ The role of the PPCA subunit within the ERC is still unknown but, by analogy to its function in the lysosome, it could protect the two other subunits and thereby contribute to the structural integrity of the whole receptor complex. In contrast to PPCA, the catalytic activity of Neu-1 is essential for signal transduction by the ERC.³⁶ Indeed, Neu-1 catalyses the local conversion of the GM₃ ganglioside into lactosylceramide, a critical second messenger of ERC signalling pathways.³⁷

Binding of EDP to the ERC triggers a host of signalling events that depends on the cell type (Figure 3). In fibroblasts, two signalling modules are activated and converge to mitogen-activated protein kinase kinases 1/2 (MEK1/2) activation: the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/B-Raf and G_i protein/phosphoinositide 3-kinase γ (PI3K γ)/Raf-1 modules.³⁸ In smooth muscle cells, EBP-mediated cell proliferation involves activation of G_i proteins concomitantly with opening of calcium L-type channels.³⁹ Finally, a PI3K γ /Akt/endothelial

nitric oxide synthase (eNOS)/nitric oxide (NO)/protein kinase G1 (PKG1) module involved in extracellular-regulated protein kinases 1/2 (ERK1/2) activation has been reported in endothelial cells.⁴⁰ Importantly, a progressive age-dependent uncoupling of the ERC has been shown to occur, impairing its transduction pathways.⁴¹ For many years, the mechanisms involved in this uncoupling remained elusive. Recently, our group has demonstrated that ERC uncoupling observed during ageing involves a rise in basal Neu-1 activity to a level too high to be modulated by EDP stimulation.⁴² Consequently, EDP-mediated lactosylceramide production and Neu-1 sialidase activity are reduced in ageing cells. These results, proposing that age-related ERC uncoupling could originate from its inability to regulate Neu-1 activity and lactosylceramide production, are of crucial importance considering the fact that EDP biological effects could not only vary during ageing, but also that pharmacological approaches targeting this peculiar receptor have to be adapted.

3.3 Challenges in the determination of EDP levels in biologic fluids

Several attempts have been made to determine elastin fragmentation products levels in biologic fluids of normal individuals and patients suffering from vascular diseases. Elastin degradation was assessed either by detecting circulating levels of elastin cross-links (i.e. desmosine/isodesmosine) or by elastin degradation products, whatever their nature. These strategies were applied on serum, plasma, or urine samples and mostly used immunological or colorimetric methods.^{43–47} The use of HPLC coupled to mass spectrometry has been developed more recently and seems to be suitable for monitoring desmosine/isodesmosine levels.^{48–51}

The reported studies agreed that circulating EDP levels increase with ageing and correlate with the progression of vascular diseases. Nevertheless, discrepancies exist in the reported EDP concentrations (ng/mL to μ g/mL range). This variation can be explained by the early observation made by Wei *et al.* on the selectivity of monoclonal antibodies raised against soluble forms of elastin that depends on both the purification protocol used for extracting fibrous elastin and the method used for its solubilization.⁴⁷ Consequently, there is currently no recognized and standardized method at hand to quantify EDP levels in biologic fluids. The current view is that EDP concentration in healthy subjects should be in the nanograms per millilitre order.^{45,52} and that higher concentrations (in the μ g/mL order) could be reached in pathological situations.⁵³ Even if this concentration seems extremely high, it has to be emphasized that where elastin is degraded, the local concentration of released EDP might be in that range.

Most EDP assay protocols aim at determining the global concentration of EDP. We now know that elastokines define a specific class of EDP, i.e. biologically active matrix fragments able to modify cell behaviour. Undoubtedly, the availability of a robust method to quantify their presence in biological fluids would be of great clinical value in the detection and monitoring of vascular ageing and related complications.

4. Pathophysiological consequences of elastin remodelling during vascular ageing

As mentioned above, the wall of large conduit arteries, especially aorta, thickens, loses elasticity, and stiffens with ageing. Arterial stiffness, as measured by pulse wave velocity, is correlated with high blood

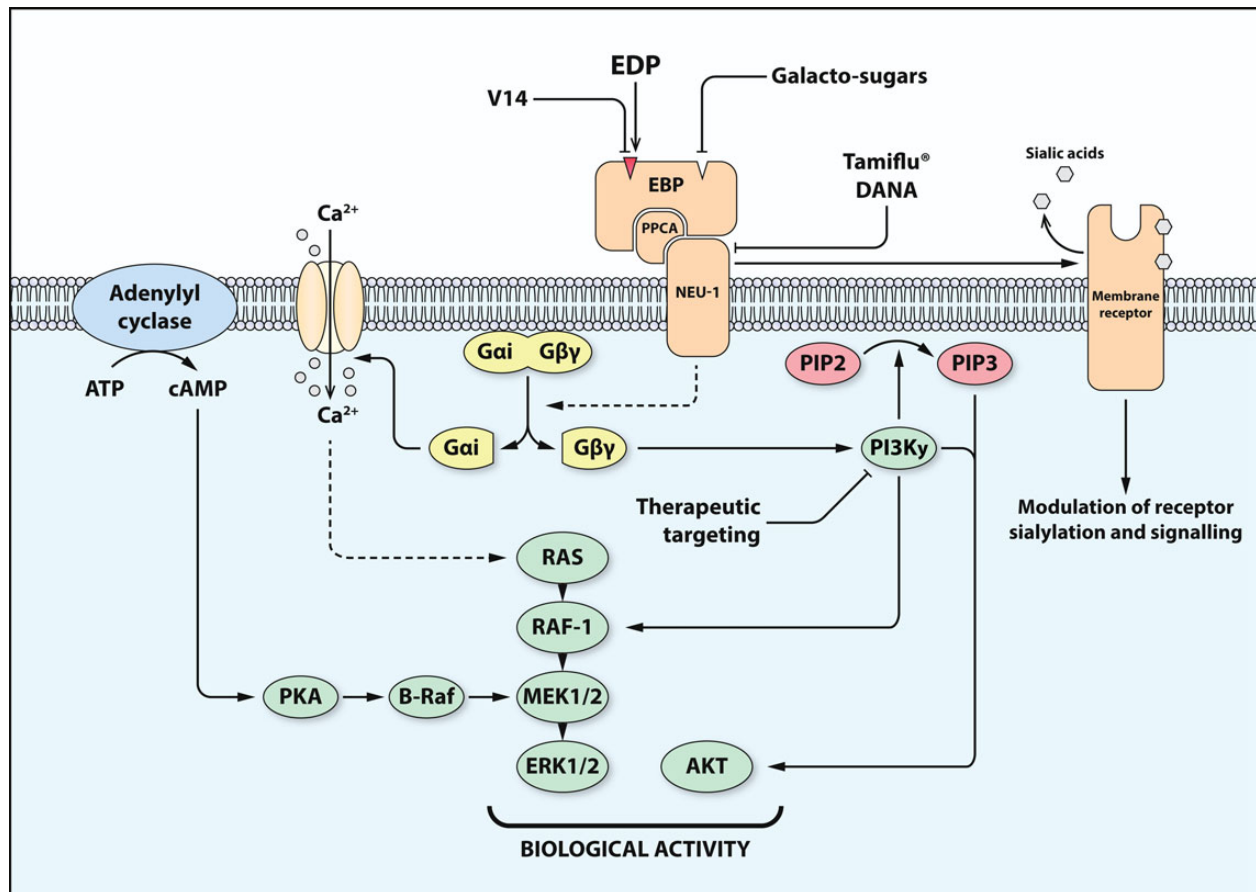


Figure 3 Signalling pathways mediated by the elastin receptor complex and related pharmacological inhibition strategies. EDP binding to the elastin receptor complex triggers signalling pathways. Moreover, the Neu-1 subunit can modulate the sialylation level of other membrane receptors and their related signalling. Different pharmacological strategies to block the elastin receptor complex signalling are depicted. EDP, elastin-derived peptides; EBP, elastin-binding protein; PPCA, protective protein/cathepsin A; NEU-1, neuraminidase-1; DANA, 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid; MEK1/2 mitogen-activated protein kinase kinases 1/2; ERK1/2 extracellular-regulated protein kinases 1/2; PKA, protein kinase A; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PI3K, phosphoinositide 3-kinase.

pressure and is a causative factor in arterial hypertension.⁵⁴ Elastin and collagen determine the passive mechanical properties of large arteries and any proteolytic degradation or chemical alterations that affects elastin will modify its mechanical properties, thereby influencing arterial stiffness and blood pressure. The contribution of elastin to arterial stiffness has been extensively studied by the group of Mecham using genetically modified mice.⁵⁵ *Eln*^{-/-} and *Eln*^{+/-} mice have increased left ventricular pressure, high blood pressure, and increased arterial stiffness compared with wild-type mice.^{56,57} It was found that stiffness is significantly increased in the *Eln*^{+/-} aorta by postnatal day (P) 7, while systolic blood pressure is not significantly increased until P14, indicating that increased large artery stiffness preceded the increase in blood pressure in this animal model.⁵⁸ Conversely, expression of the human elastin gene in *Eln*^{+/-} mice which increases elastin amounts from ~60 to 80% of normal levels reverses the changes in both arterial stiffness and hypertension.⁵⁹ However, a paradoxical increase in arterial elasticity has been observed in patients suffering from Williams–Beuren syndrome in which abnormalities of elastic fibres are observed.^{60,61} Williams–Beuren syndrome is a genetic disorder caused by the deletion of one allele of the elastin gene and is associated with typical

cardiovascular abnormalities including arterial wall hypertrophy, supra-valvular aortic stenosis, and systemic hypertension. Together with the above-cited animal models, this human genetic disease demonstrates that elastin has functions other than providing elasticity. It has been shown that the increased proliferation of arterial smooth muscle cells, in a quiescent contractile state, from arteries of patients suffering from Williams–Beuren syndrome is due to decreased deposition of elastin.⁶² Therefore, beyond elasticity, elastin also acts as an autocrine factor for smooth muscle cells.

Two other features of elastin that may contribute to increase arterial stiffness are its ability to bind calcium and to cross-link adducts such as AGE. Calcification of media of large arteries increases significantly with ageing and there is a strong correlation between calcium content and arterial stiffness in humans. Cumulative data from the literature showed a gradual deposition of calcium phosphate minerals on elastic lamellae, also known as medial elastocalcinosis. This was recently confirmed by Khavandgar et al.⁶³ in a model of Matrix Gla protein knockout (*MGP*^{-/-}) mice cross-bred with *Eln*^{+/-} mice. In these mice, elastin haploinsufficiency impedes the progression of arterial calcification. Finally, elastin stiffness is also increased through cross-linking. Owing

to its long biological life, elastin as well as collagen are exposed to various non-enzymatic post-translational modifications that can contribute to functional and structural alterations of their properties. For instance, AGE-mediated cross-links of human aortic elastin increase with ageing²³ and elevated concentrations of glycated EDP in sera of diabetic patients with arterial hypertension have been reported.⁶⁴ Recently, it has been shown that elastin is also subject to carbamylation,²⁴ another non-enzymatic reaction involving binding of isocyanic acid to free amino groups. Isocyanic acid is mainly formed by the spontaneous dissociation of urea but can also be derived from thiocyanate through myeloperoxidase. Whether carbamylation of elastin affects its rigidity and susceptibility due to proteolysis remains to be determined.

Proteolysis of elastin is another feature of elastin remodelling during vascular ageing. In most of the studies reported so far, elastin degradation and/or presence of EDP have shown detrimental effects on vascular complications associated with ageing. By cross-breeding MMP (MMP-2, -7, -9, -12) or cathepsin (K, L, S)-deficient mice with LDLR^{-/-} or ApoE^{-/-} mice, two established models of atherogenesis,^{65,66} elastin degradation was shown to contribute to progression of atherosclerosis. Contradictory data have been reported on plaque stability, but elastin fragmentation may well play important role in plaque destabilization and rupture, as recently reported by two recent studies from a same group.^{67,68} By cross-breeding ApoE^{-/-} mice with mice containing a heterozygous mutation (C1039G^{+/-}) in the fibrillin-1 gene that exhibit impaired elastin structure and elastin fragmentation in the vascular wall, increased arterial stiffness, development of highly unstable plaques with intraplaque neovascularization and plaque rupture have been reported.^{67,68} Direct evidence for the involvement of EDP in the progression of atherosclerosis has been recently reported by Gayral et al.⁶⁹ Chronic injections of EDP or VGVAPG into LDLR^{-/-} or ApoE^{-/-} mice were shown to increase atherosclerotic plaque size formation in these mice. The absence of PI3K γ in bone marrow-derived cells prevented EDP effects of atherosclerosis *in vivo* as well as EDP-mediated monocyte migration and reactive oxygen species (ROS) production *in vitro*. In addition, degradation of elastic lamellae in LDLR^{-/-} mice fed with an atherogenic diet correlates with atherosclerotic plaque formation. Using a model presenting a drastic reduction of Neu-1 activity,^{70,71} the ERC was shown to be essential to atherogenesis in condition of endogenous elastin fragmentation. The defect in Neu-1 activity in this model was correlated with a decrease in monocyte and lymphocyte infiltration, clearly demonstrating the role of this receptor complex in inflammatory processes of atherogenesis and the involvement of endogenous EDP. Another critical player that contributes to atherosclerotic plaque development and progression are vascular smooth muscle cells.⁷² In this context, EDP have been shown to stimulate *in vitro* smooth muscle cell migration and proliferation^{39,73} and to differentiate rat aortic smooth muscle cells⁷⁴ and rat dermal fibroblasts⁷⁵ into cells exhibiting an osteoblast-like phenotype. These phenotypic transitions mediated by EDP are potentiated by transforming growth factor- β 1 (TGF- β 1) and could influence vascular calcification occurring during ageing.

Destruction of elastic fibres also play a predominant role in abdominal aortic aneurysm (AAA) development, a chronic degenerative vascular condition that is associated with ageing.⁷⁶ The pathophysiology of aortic aneurysms is complex and characterized by an infiltration of the vessel wall by lymphocytes and macrophages, destruction of elastin and collagen in the media and adventitia, loss of smooth muscle cells with thinning of the media, and neovascularization. The infusion of porcine pancreatic elastase into rat aortas, in addition to the well-known

angiotensin II and calcium chloride animal models, have been extensively used as a model of AAA and revealed that destruction of elastic fibres play a critical role in AAA development. These detrimental effects in AAA development were also demonstrated using MMP-deficient mice such as MMP-2 and MMP-9, and more recently using mice deficient in cathepsin S,⁷⁷ K,⁷⁸ and L.⁷⁹ In these cathepsin-deficient mice, the significant reduction in AAA development was associated with decreased in elastin fragmentation, cell proliferation and apoptosis, inflammatory cell infiltration, protease expression, and angiogenesis to different extent according to the cathepsin deficiency. Several studies based on human cohorts have proposed that circulating EDP could be used as a biomarker predicting AAA progression.⁸⁰ These EDP may be important signals to a variety of cells involved in the development of AAA, stimulating chemotaxis and MMP production. Direct evidence for the involvement of EDP has been provided by using the VGVAPG peptide and BA4, a blocking antibody directed against the EBP recognition sequence GxxPG. Hance et al. were among the first to demonstrate *in vitro* that EDP released from human AAA tissues can attract monocytes through the ERC. They showed that AAA-derived monocyte/macrophage chemotactic activity was eliminated by competition with the VGVAPG peptide and decreased by about 40% in the presence of BA4.⁸¹ Comparable results were obtained using aortic extracts from mgR/mgR mice, a mouse model of Marfan syndrome.⁸² Marfan syndrome is an autosomal-dominant inherited disorder of connective tissue caused by mutations in the gene for fibrillin-1, with prominent clinical manifestations in the cardiovascular, skeletal, and ocular systems. Aneurysm formation and aortic dissection remain the most life-threatening complications of Marfan syndrome. Using this fibrillin-1 hypomorphic mouse model (mgR/mgR), the same group recently showed direct involvement of EDP and the ERC in the development of AAA.⁸³ Intraperitoneal injections of BA4 were shown to reduce MMP-2 and MMP-9 expression, as well as elastin fragmentation and macrophage infiltration in the aorta of treated animals.

New emerging effects of EDP have recently been reported in diabetes mellitus. Diabetes mellitus is a chronic metabolic disease associated with hyperglycaemia, macro- and micro-vascular complications, and considered as a major risk factor for CVD.⁸⁴ Type 2 diabetes is the most common form of diabetes mellitus and typically considered as adult-onset diabetes. Type 2 diabetes is characterized by hyperglycaemia and peripheral insulin resistance mostly caused by reduced insulin sensitivity in skeletal muscle, adipose tissue, and liver. Diabetes mellitus accelerates vascular ageing and is associated with extensive vascular ECM remodelling. Increased MMP plasma concentrations, such as MMP-2 and MMP-9,⁸⁵ and serum levels of EDP and glycated EDP have been reported in diabetic patients.^{64,86,87} Indeed, the presence of EDP in the blood leads to the formation of anti-elastin antibodies, and both are associated with the development of diabetic vascular complications.^{64,86–89} How elastin fragmentation and EDP contribute to the development of diabetes mellitus and/or vascular complications associated with diabetes mellitus remains unknown so far. Recently, insulin resistance has been linked to the expression of cathepsin S,⁹⁰ neutrophil elastase,⁹¹ and imbalance between serum levels of neutrophil elastase and the neutrophil elastase inhibitor α 1-antitrypsin,⁹² raising the possibility of EDP involvement in the development of insulin resistance. This hypothesis was confirmed in wild-type mice in which administration of EDP and the VGVAPG peptide was shown to increase hyperglycaemia and to trigger insulin resistance.⁹³ Importantly, these effects were blocked by the neuraminidase inhibitor 2,3-didehydro-2-deoxy-N-acetylneuraminic acid (DANA) or

chondroitin sulfate. In addition, Blaise *et al.*⁹³ showed that EDP trigger interaction between Neu-1 and the insulin receptor. This EDP-mediated interaction results in lower sialylation and phosphorylation levels of the insulin receptor and decreased insulin receptor signalling. Taken together, these data suggest that EDP may be involved in the insidious development of insulin resistance occurring during ageing through the regulation of insulin receptor signalling by the Neu-1 subunit of the ERC.

Finally, another emerging effect of EDP came from the work of Kawecki *et al.*⁹⁴ on thrombosis. Vascular ageing is associated with haemostasis dysregulation and increased thrombosis tendency.⁹⁵ In their study, Kawecki *et al.* have reported, for the first time, that EDP decrease human platelet aggregation in whole blood induced by different weak and strong platelet agonists and that both EDP and the VGVAPG peptide strongly reduce thrombus formation in a mouse model of thrombosis induced by chemical injury. Moreover, EDP and VGVAPG also prolonged tail-bleeding times.⁹⁴ This unexpected regulatory role of EDP on thrombosis seems to rely on a dual mechanism involving effects on platelets that express a functional ERC able to trigger an increase of platelet sialidase activity, and on the ability of EDP to disrupt plasma von Willebrand factor interaction with collagen by specifically binding to collagen fibres.⁹⁴ This latter correlated with reduced platelet interaction with collagen under flow conditions and therefore may have critical impact on platelet adhesion with the vessel wall *in vivo*. Whether EDP also affect clotting and fibrin formation remains to be determined. These effects of EDP on thrombosis, which remain to be further investigated, in addition to their reported proangiogenic roles, may have important consequences on the AAA outcome and atherosclerosis progression because neovascularization and intraplaque haemorrhage have been associated with increased risk of plaque rupture. This hypothesis seems to be supported by the phenotype of the ApoE^{-/-}/fibrillin-1^{C1039G+/-} mice. As previously mentioned, these mice that exhibit impaired elastin structure and severe elastin fragmentation show highly unstable plaques with intraplaque neovascularization and plaque rupture.^{67,68}

5. Pharmacological strategies

Limiting or blocking the deleterious effects of EDP during age-related vascular complications can be achieved using several strategies: (i) targeting elastases to limit elastin fragmentation and EDP generation; (ii) blocking the interaction between EDP and the ERC; (iii) inhibiting Neu-1-associated sialidase activity; and (iv) blocking downstream signalling molecules involved in ERC signal transduction.

5.1 Inhibiting elastases

Elastases are targets of high therapeutic potential in a large number of diseases. These elastin-degrading enzymes are organized as interconnected proteolytic cascades regulating each other. Consequently, targeting these enzymes is challenging. Moreover, due to their involvement in the regulation of several tissue functions, their inhibition often results in adverse side effects. Current examples of elastase inhibition strategies are briefly described below.

Human neutrophil elastase plays important role in ECM remodelling in response to injury. This protease can be rapidly released with proteinase-3 by neutrophil degranulation and is at the crossroad of numerous regulatory pathways, notably in the vascular context.⁹⁶ Synthetic small molecule inhibitors have been developed to inhibit its activity and clinical studies have focused on inflammatory lung diseases.

Unfortunately, those inhibitors have shown limited effects. New strategies based on endogenous inhibitors of human neutrophil elastases are now considered. Data obtained from two serpins, secretory leucocyte protease inhibitor (SLPI) and elafin, suggest that these natural inhibitors may have therapeutic potentials.⁹⁶ For instance, elafin-overexpressing mice had reduced neutrophil infiltration of cardiac tissue, decreased left ventricular fibrosis and infarct size in a myocardial ischaemia model.⁹⁷ Likewise, smooth muscle cell proliferation and inflammatory cells invasion were suppressed in a model of arterial injury using mice overexpressing elafin.⁹⁸ These preclinical observations suggested that elafin could be a good candidate to prevent elastase contribution to deleterious inflammation after major surgery. Consequently, elafin administration was evaluated in clinical trials aiming at limiting elastase involvement. Unfortunately, a recent report shows that this strategy gives limited results when elafin is administrated as a single dose.⁹⁹ Consequently, continuous delivery of the elastase inhibitor or higher dose could be required to reach an efficient protective effect.

Inhibition of MMP elastases has been mostly considered in the context of cancer research as these enzymes are critically involved in tumour invasion. Considerable efforts have been made to develop potent inhibitors, but results have been disappointing due to important undesired effects. However, the development of specific MMP inhibitors is an absolute priority.¹⁰⁰ The current inhibitor design considers the targeting of secondary binding sites to improve inhibition specificity. For instance, small molecules have been developed to bind the collagen-binding domain of MMP-2 and MMP-9. In addition, current data show that a strategy based on exosite binding is promising as selectivity is greatly enhanced.¹⁰⁰

As mentioned previously, cathepsins are also potent elastases. Therapeutic targeting of this enzyme family has mainly focused on cathepsin K, the main bone-degrading protease in the osteoclasts and thereby the major target for osteoporosis. Potent peptide-derived inhibitors displaying a reversible binding nitrile or ketone warhead were synthesized.¹⁰¹ For further details on cathepsin inhibitors, the reader is referred to the excellent review of Fonovic and Turk¹⁰² describing the current existing inhibitors. In the context of vascular wall remodelling, cathepsin S targeting seems to be the most promising target.¹⁰³

5.2 Blocking EDP/ERC interaction

Blocking EDP binding to the EBP subunit of the ERC can be achieved either using the V14 peptide or galactosides (Figure 3). The V14 peptide (VVGSPSAQDEASPL) mimics the EBP sequence on which EDP bind. Consequently, the V14 peptide has the potential to trap circulating EDP, thereby blocking their biological effects.^{104,105} Another possibility is to use galactosugars, lactose, or chondroitin sulfate, because their interaction with EBP leads to shedding of EBP from the ERC and blocking of the corresponding signalling pathways. These compounds were used to demonstrate the beneficial effects of EDP in heart protection against ischaemia/reperfusion injury¹⁰⁴ and in tissue repair.¹⁰⁶ Although the V14 peptide and galactosugars allowed better understanding in the field of EDP biology, their selective delivery at the site of vascular injury remains an issue.

5.3 Inhibiting Neu-1 sialidase activity

DANA is currently used as a sialidase inhibitor to selectively block ERC-mediated signalling.^{42,69,93} However, this compound is not specific to Neu-1 and inhibits all sialidases, precluding its therapeutic use. An attempt was made to design and synthesize inhibitors for human neuraminidases, but the results were not satisfactory.¹⁰⁷ Indeed, selectivity

was not achieved, most probably because the structures of human sialidases are not fully described. Recently, O'Shea et al.¹⁰⁸ used oseltamivir phosphate (Tamiflu®) to target Neu-1 and block cancer cell survival in human pancreatic cancer with acquired chemoresistance. This study suggests that Tamiflu®, used in the treatment of influenza infection, could possibly be used to selectively block Neu-1. Nevertheless, this possibility has still to be validated in a vascular injury context.

5.4 Blocking ERC-mediated signalling pathways

The biological effects mediated by the ERC could be the resulting of either direct and/or indirect actions (Figure 3). Direct effects are the results of lactosylceramide production in the cell following Neu-1 activation. Lactosylceramide is considered as a critical proximal second messenger in ERC signalling and blocking lactosylceramide production and related signalling pathways will block ERC effects. In this context, a downstream signalling protein involved in ERC signal transduction is PI3K γ .^{38,69} This class IB PI3K is largely expressed in inflammatory cells¹⁰⁹ but has also been identified in cells of the cardiovascular system, particularly in endothelial cells¹¹⁰ and smooth muscle cells.¹¹¹ This original expression pattern, different from other class of PI3K, places this protein as a good candidate for drug targeting in CVD.^{109,112} In this context, our team has played a large part in determining the *in vitro* and *in vivo* immuno-modulatory functions of PI3K γ in arterial pathologies of atherosclerosis¹¹³ and restenosis.¹¹⁴ By driving the monocyte response to EDP, we recently identified that PI3K γ is a key signalling protein involved in EDP-induced atherosclerosis *in vivo*.⁶⁹ Our work reveals one of the possible mechanisms by which PI3K γ drives atherosclerosis. The role of PI3K γ downstream of ERC in the modulation of the inflammatory functions of immune cells in atherogenesis raises the specific role of this pathway in the complications of atherosclerosis such as neointima formation in arterial damage after angioplasty or intra-stent neoatherosclerosis development. Indeed, the importance of EDP signalling remains to be elucidated in these arterial diseases, and such results will reinforce the use of specific PI3K γ inhibitors in this pathological context. Nevertheless, specific attention should be paid to the development of safer PI3K γ inhibitors to follow new therapeutic strategies in the prevention of CVD. Another important aspect to elucidate is the molecular mechanism explaining how PI3K γ is activated downstream of the ERC since this kinase was demonstrated to be specifically activated by downstream G protein-coupled receptors.

Indirect effects could originate from Neu-1 activity on adjacent membrane receptors (Figure 3). Indeed, during the last decade, Neu-1 has been shown to be involved in the modulation of insulin receptor signalling⁹³ and regulation of toll-like receptor 4 (TLR4),¹¹⁵ tropomyosin receptor kinase A (Trk A),¹¹⁶ PDGF subunits BB (PDGF-BB) and insulin-like growth factor (IGF) receptors,¹¹⁷ epidermal growth factor (EGF) and mucin 1 (MUC1) receptors,¹¹⁸ and more recently platelet endothelial cell adhesion molecule (PECAM-1), also known as cluster of differentiation 31 (CD31).¹¹⁹ It is not excluded that Neu-1 can also modify G protein-coupled receptors leading to PI3K γ recruitment to the plasma membrane. Consequently, Neu-1 now emerges not only as a catabolic enzyme but also as a key factor in cell signalling regulation.¹²⁰ Efforts are now made to understand how Neu-1 interacts with these membrane proteins in order to conceive new strategies aiming at selectively blocking these interactions.

6. Concluding remarks and future directions

Considered for a long time as an inert supporting network for cells, the vascular ECM is definitely an active and dynamic structure playing central role in vascular ageing and related complications. Over the last decade, elastin fragmentation and generation of EDP have emerged as major contributors of vascular ECM remodelling and related diseases occurring with ageing. Owing to their pathophysiological importance, limiting or blocking the deleterious effects of elastin fragmentation and EDP may represent promising therapeutic targets. As mentioned previously, different strategies can be considered: the design of specific and selective elastase inhibitors to limit elastin fragmentation, or compounds that can block the interaction between EDP and the ERC, Neu-1 activity or intracellular proteins involved in ERC signalling pathways (Figure 3). However, a large number of questions remain to be answered. For instance, our understanding on the various MMP networks and their mutual relationships is incomplete. Furthermore, how the expression of elastases changes during ageing and the progression of vascular complications needs to be addressed.

Another unresolved question is the organization and stoichiometry of the ERC at the plasma membrane. For instance, the presence of a plasma membrane-bound Neu-1 regulating a plethora of membrane receptors by desialylation has been consistently documented from the last 10 years. However, Neu-1 is, by definition, a lysosomal sialidase and current structural data cannot account for such membrane localization. How the different subunits of the ERC organize within the plasma membrane will undoubtedly help in the design of specific inhibitors of the receptor.

It is also unclear how the progressive age-dependent uncoupling of the ERC occurs.⁴¹ As previously mentioned, we recently demonstrated that this age-related ERC uncoupling could originate from its inability to regulate Neu-1 activity and lactosylceramide production in cultured human fibroblasts.⁴² If this phenomenon could also apply for endothelial cells, smooth muscle cells and circulating cells remains to be shown. Finally, the biological effects of elastin degradation products described in this review highlight the influence of matrix-directed proteolysis during ageing. Hence, it can be reasonably expected that remodelling and proteolytic fragments of another vascular ECM proteins could also play a significant role in vascular ageing and related complications.

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