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Liberation of Desmosine and Isodesmosine as Amino Acids from Insoluble Elastin by Elastolytic Proteases

Hideyuki Umeda, Ph.D., Masanori Aikawa, M.D., Ph.D., and Peter Libby, M.D.

Division of Cardiovascular Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, USA

Abstract

The development of atherosclerotic lesions and abdominal aortic aneurysms involves degradation and loss of extracellular matrix components, such as collagen and elastin. Releases of the elastin cross-links desmosine (DES) and isodesmosine (IDE) may reflect elastin degradation in cardiovascular diseases. This study investigated the production of soluble elastin cross-linking structures by proteinases implicated in arterial diseases. Recombinant MMP-12 and neutrophil elastase liberated DES and IDE as amino acids from insoluble elastin. DES and IDE were also released from insoluble elastin exposed to monocyte/macrophage cell lines or human primary macrophages derived from peripheral blood monocytes. Elastin oxidized by reactive oxygen species (ROS) liberated more unconjugated DES and IDE than did non-oxidized elastin when incubated with MMP-12 or neutrophil elastase. These results support the exploration of free DES and IDE as biomarkers of elastin degradation.

Keywords

atherosclerosis; biomarker; cross-link; desmosine; elastin degradation; isodesmosine

1. Introduction

The cross-links characteristic of mature elastin render it highly stable and contribute to its essential structural and functional contributions to the mammalian extracellular matrix in general and to the pathophysiology of arteries in particular [1]. All cross-links in elastin form spontaneously after oxidative deamination of specific lysine residues of tropoelastin by lysyl oxidase in the extracellular space. Allysine, a formed reactive aldehyde, reacts with lysine and/or another allysine to form polyfunctional cross-links such as desmosine (DES) or isodesmosine (IDE) [2]. Such cross-links in elastin may resist elastolysis, and contribute to the biomechanical properties of this macromolecule that are essential for normal arterial function and are often deranged in diseases such as hypertension, arteriosclerosis, atherosclerosis, certain arteritides, and aneurysm formation. In particular, elastin degradation contributes to the formation and complication of atherosclerotic lesions. Several classes of extracellular proteases participate in the breakdown of elastin and can thus influence various aspects of vascular remodeling in atherosclerosis [3–5]. Disorders of elastic fibers increase

*Correspondence should be addressed to: Peter Libby, M.D., Division of Cardiovascular Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA. Phone: +1 617 525 4383; Fax: +1 617 525 4999. plibby@rics.bwh.harvard.edu.

Conflicts of interest

The authors have no conflicts of interest.

with age and contribute importantly to several non-vascular diseases, including pulmonary emphysema [3,6,7].

Common histological and biochemical findings with aging and in inflammatory diseases include decreased elastin content and fewer elastin cross-links in affected tissues [8–10]. The reduction of cross-links in tissues may have particular biological importance, because such cross-links maintain the resilience elasticity and strength of elastin. Insoluble elastin with partial degradation of cross-link structures has greater susceptibility to elastolysis than does native insoluble elastin [9,11]. Elastases also degrade tropoelastin, a precursor to insoluble elastin, more readily than insoluble elastin [12]. The mechanisms of elastolysis by elastases and the utility of elastin cross-links as biomarkers of elastin degradation, however, remain incompletely explored in atherosclerosis.

This study investigated the liberation of two major elastin cross-links, DES and IDE, from insoluble elastin by elastolytic enzymes implicated in human arterial diseases associated with vascular inflammation. Our results indicate that MMP-12 and neutrophil elastase promote the release of DES and IDE as amino acids from insoluble elastin, and that free DES and IDE may serve as useful biomarkers of elastin degradation in inflammatory arterial diseases, including atherosclerosis, leukocytoclastic vasculitis and abdominal aortic aneurysm (AAA).

2. Materials and Methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA), phenylmethanesulfonyl fluoride (PMSF), *p*-aminophenylmercuric acetate, E-64, aprotinin, trichloroacetic acid, plasminogen, plasmin, human neutrophil elastase, recombinant human MMP-12 (catalytic domain), recombinant human MMP-9, and human cathepsins S and L were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human cathepsin K and MMP-2 (catalytic domain) were obtained from Enzo Life Sciences (Plymouth Meeting, PA). RPMI 1640 medium without phenol red was purchased from GIBCO Products Invitrogen (Grand Island, NY). Acetonitrile (HPLC-grade), sodium dihydrogen phosphate, and hydrochloric acid were purchased from Nacalai Tesque (Kyoto, Japan). RAW264.7, THP-1, and U937 cells were obtained from American Type Culture Collection (Manassas, VA). DES and IDE standards were purchased from Elastin Products Company (Owensville, MO).

2.2. Cell cultures

RAW264.7, THP-1, and U937 cells were maintained at a density of $0.2\text{--}1 \times 10^6/\text{ml}$ in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Human peripheral blood monocytes were isolated from buffy coat by density gradient centrifugation with Cappel LSM Lymphocyte Separation Media (ICN Biomedical, Santa Ana, CA), and adherence on 24-well plates for 2 hours in RPMI 1640, as described previously [4].

2.3. Preparation of insoluble elastin and oxidized insoluble elastin

Preparation of insoluble elastin from bovine aortic tissues used extraction with 1M NaCl treatment, as described previously [8]. Oxidation of insoluble elastin by Fe^{2+} (Cu^{2+})/ H_2O_2 generated oxidized insoluble elastin, as described previously [9]. A suspension of 25 mg of dried insoluble elastin was exposed to 50mM H_2O_2 in the presence of 0.5 mM FeSO_4 (or 0.1 mM CuSO_4), in the total volume of 5 ml of 50 mM sodium phosphate buffer for 48 hours at 37°C .

2.4. Digestion of insoluble elastin with elastolytic enzymes

Dried insoluble elastin powder (10 mg) or oxidized insoluble elastin (10 mg) were dispersed in 1 ml of 50 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl₂, pH 7.4, and incubated with recombinant human MMP-12 catalytic domain (1 µg), recombinant human MMP-9, recombinant human MMP-2 catalytic domain (1 µg) or neutrophil elastase (1 µg), for 72 hours at 37°C. Before digestion with recombinant human MMP-9 (1 µg), this recombinant protein was activated with *p*-aminophenylmercuric acetate (1 mM) for 3 hours. Incubation of insoluble elastin powder (10 mg) or oxidized insoluble elastin (10 mg) with human cathepsin S (1 µg), human cathepsin L (1 µg), or recombinant human cathepsin K (1 µg) were carried out in suspension at pH 5.5 (100 mM sodium acetate, 5 mM dithiothreitol, 5 mM EDTA) for 72 hours at 37°C. The enzyme–substrate ratio was 1:10000 (m/m). After incubation, the remaining insoluble elastin was sedimented (13,000 × *g*, 20 minutes), and the supernatant analyzed by HPLC.

2.5. Assay of elastolytic activity

Human monocyte–derived macrophages or monocyte/macrophage cell lines (RAW264.7, THP-1, U937) were incubated with insoluble elastin (10 mg), then placed in 24-well plates in serum-free RPMI 1640 without phenol red. After 72 hours in a humidified incubator (37°C, 5% CO₂), the reaction mixtures were centrifuged (5 minutes at 1,000 × *g*). Part of the supernatant was used for zymography and Western blotting analysis. The remaining supernatant was centrifuged (20 minutes at 13,000 × *g*) and used for analysis of liberated DES and IDE from insoluble elastin by HPLC.

2.6. siRNA knockdown of MMP-9, MMP-12, and neutrophil elastase

Silencing RNA oligonucleotides (siRNA) used included MMP-9 (SI02692718, SI02715328, QIAGEN), MMP-12 (SI0017758, SI02709644, QIAGEN, Valencia, CA), neutrophil elastase (SI03126172, SI04158644, QIAGEN). Transfection of siRNA was performed with a HiPerFect Transfection Reagent (QIAGEN) following the manufacturer's instructions (QIAGEN).

2.7. Western blot analysis

Samples were concentrated with 10% trichloroacetic acid, and diluted in sample buffer and heated at 70°C for 10 minutes. Electrophoresis was performed under reducing conditions through a Novex 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes according to the manufacturer's instructions. Western blotting detection of MMP-12 and neutrophil elastase was performed using with specific antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), according to the manufacturer's instructions. MMP-12 and neutrophil elastase in the blots were visualized with an enhanced chemiluminescence method.

2.8. High-performance liquid chromatography (HPLC)

Analysis of liberated DES and IDE as amino acids from insoluble elastin was performed as previously described [8].

2.9. Statistics

Data are presented as mean ± SD. Data analysis employed GraphPad prism software (version 5, GraphPad Software, San Diego, CA), using one-way ANOVA with Bonferroni correction to determine significant differences between group means. In all cases, *P* values less than 0.05 were considered significant.

3. Results

3.1. Liberation of DES and IDE as amino acids by recombinant elastolytic proteases

Little is known about the liberation of elastin cross-links by various elastolytic proteases. We hypothesized that such enzymes release DES and IDE as amino acids from insoluble elastin. Several previous studies have proposed that MMP-12 and neutrophil elastase may cleave alanine-rich cross-link regions [13–15]. We used recombinant matrix metalloproteinases (MMP-2, MMP-9, MMP-12), serine proteases (neutrophil elastase), and cysteine proteases (cathepsins S, K, and L) to evaluate this hypothesis. To determine the liberation of DES and IDE from insoluble elastin, we examined the supernatant after centrifugation of the reaction mixtures of recombinant proteinases and insoluble elastin *in vitro* by HPLC (Fig. 1A). Liberated DES and IDE each yield a single sharp peak. MMP-12 and neutrophil elastase released IDE, and to a lesser extent DES as amino acids from insoluble elastin, but two gelatinases (MMP-2, MMP-9) and three cysteinyl proteinases with endoelastolytic activity (cathepsins S, K, and L) did not liberate DES and IDE (Fig. 1B).

While native insoluble elastin generally resists enzymatic breakdown, due in part to its highly cross-linked structure [1], insoluble elastin with partial degradation of cross-link structures may have greater susceptibility to elastolysis by disease-related proteinases [9, 11]. Testing this hypothesis used insoluble elastin oxidized by Fe^{2+} (Cu^{2+})/ H_2O_2 . We previously described non-enzymatic degradation of cross-links in oxidized elastin [9]. When exposed to MMP-12, oxidized insoluble elastin liberated more DES and IDE as amino acids into the soluble fraction than did untreated insoluble elastin (Fig. 1C and 1D). These results affirm the hypothesis that oxidative modification can promote enzymatically-mediated elastin breakdown, and further suggest that release of free DES and IDE into biological fluids (blood plasma, urine, and sputum) could serve as a novel biomarker in inflammatory conditions associated with oxidative stress such as atherosclerosis, AAA, and pulmonary emphysema.

3.2. Cell-derived elastases liberate DES and IDE as amino acids from insoluble elastin

Tissue macrophage accumulation characterizes many important chronic inflammatory diseases, and plentiful previous work points to proteinases produced by these phagocytes in extracellular matrix remodeling. Our previous work showed that mononuclear phagocytes can express “neutrophil” elastase, showing that this enzyme may participate in chronic inflammatory conditions as well as more acute diseases characterized by a predominantly polymorphonuclear cell infiltrate [4]. We therefore examined liberation of DES and IDE from elastin by macrophage-derived enzymes. Evidence suggests that monocyte/macrophages degrade elastin *in vitro* when cultured in contact with this substrate [16]. In general, during differentiation of monocytes into macrophages in the arterial wall, human mononuclear phagocytes express a broad palette of proteases (all of the proteases indicated in Fig. 1) that can degrade insoluble elastin and other extracellular matrix components [4, 17]. After 3, 5, and 10 days in culture, differentiating human peripheral blood monocyte-derived macrophages (PBMC) were co-cultured with insoluble elastin under serum-free conditions for 72 hours. DES and IDE were released as amino acids from insoluble elastin with no stimulation, and the levels were increased over time until day 10, when macrophages were fully differentiated (Fig. 2).

To characterize further the elastases responsible for the release of DES and IDE, we used mouse RAW264.7 cells and human THP-1 cells commonly used monocytoid cell lines that produce more consistent results than primary human monocyte-derived macrophage preparations that may display considerable donor variation. MMP-12 and neutrophil elastase led to increased release of DES and IDE. RAW264.7 cells have features similar to

macrophages and express elastolytic proteases, such as MMP-12 [18]. THP-1 monocyte-like cells mainly express serine elastases such as neutrophil elastase [19]. Although after treatment with phorbol 12-myristate 13-acetate (PMA), THP-1 cells become adherent and exhibit morphological similarities to macrophages, and their expression of neutrophil elastase decreases [20]. We found that RAW264.7 cells express MMP-12 but not neutrophil elastase, and that THP-1 cells produce neutrophil elastase but not MMP-12 (not shown). The present study thus used THP-1 cells in their unstimulated form to ascertain whether cell-derived neutrophil elastase can release DES and IDE from insoluble elastin, while RAW264.7 cells served to examine the role of MMP-12. Co-incubation of these cells with insoluble elastin yielded release of free DES and IDE into the medium, as determined by HPLC (Fig. 3A and 3B). Heat treatment of the conditioned medium abrogated the liberation of DES and IDE, suggesting that proteases mediated this process (not shown). The other monocytic cell lines, U937 and HL-60, also liberated DES and IDE as amino acids, in a manner similar to that of THP-1 (not shown).

Many extracellular proteinases undergo activation by other proteolytic enzymes, highlighting the interactions between these enzymes. MMPs are typically secreted in zymogen form (pro-MMP) and require extracellular activation. In particular, activation of pro-MMP by plasmin likely occurs in vivo. Tissue plasminogen activator and urokinase plasminogen activator generate plasmin from plasminogen. In vitro, plasmin activates pro-MMP-1, pro-MMP-3, and pro-MMP-9 [21]. Urokinase plasminogen activator-generated plasmin can activate MMP-12 [22]. The addition of plasmin or plasminogen did not increase DES/IDE liberation by either cell line (not shown), in agreement with a previous report [23]. Thus, the elastolytic proteases elaborated by mononuclear phagocytes such as MMP-12 and neutrophil elastase appear not to require exogenous plasmin to degrade insoluble elastin.

To identify proteases responsible for the release of DES and IDE, we investigated the effect of various protease inhibitors in RAW264.7 or THP-1 cells (Fig. 3C and 3D). The serine protease inhibitors (PMSF and aprotinin) inhibited DES and IDE liberation by THP-1. In U937 cells, PMSF produced similar effects as in THP-1 cells (data not shown), suggesting that neutrophil elastase secreted from THP-1 and U937 can liberate IDE from insoluble elastin. These data indicate that the generation of free DES and IDE by these monocytic cells depend on a secreted serine protease. None of the inhibitors tested reduced release of free DES and IDE by RAW264.7. Nor did neutralizing antibodies to MMP-9, MMP-12, or neutrophil elastase inhibit the liberation of DES and IDE by RAW264.7 (not shown).

Purified recombinant neutrophil elastase or MMP-12 released DES and IDE from insoluble elastin (Fig. 1B). Thus, we tested the hypothesis that silencing the genes encoding these proteases would limit liberation of DES and IDE by mononuclear phagocytes. Efficiency of silencing by siRNA oligos for MMP-12 and neutrophil elastase was determined by Western blotting (Fig. 3E and 3F). Silencing of MMP-12 in RAW264.7 cells and neutrophil elastase in THP-1 substantially decreased the release of DES and IDE from insoluble elastin.

4. Discussion

In chronic inflammatory states, the degradation of elastin-rich extracellular matrix in vascular and other tissues associates with the local accumulation of macrophages that express a diverse panel of proteolytic enzymes. Many elastolytic proteases participate in extracellular matrix homeostasis in humans, and abundant data support their dysregulation in many diseases, including those that affect arteries [3–6]. This study examined whether macrophage-derived elastases lead to increased release of DES and IDE.

MMP-12 cleaves bonds preferentially N-terminal to Leu, as do other enzymes of the MMP family [12, 13], but it is unique among MMPs in that it scissions bonds N-terminal to charged residues [24]. Elastin has hydrophobic residues at the P₁' site, a feature that may contribute to the general lack of efficient cleavage of linkages N-terminal to charged species by most MMPs. Tropoelastin has seven sites cleaved by MMP-12 with Lys at P₁' positions, all at Ala-Lys linkages [13]. Taddese et al. reported that such cleavages were not detected in the case of insoluble elastin [25], which may be partly because the majority of lysine residues in elastin participate in the formation of cross-links [14]. This study, however, detected DES and IDE as amino acids liberated from insoluble elastin by recombinant MMP-12 active domain — this MMP can cleave sites with Ala-Lys, because this cleavage may result in the liberation of DES and IDE as amino acids into the soluble fraction [14].

Neutrophil elastase is the most widely studied among several elastases belonging to the serine proteinase family of enzymes. Neutrophil elastase can cleave many extracellular matrix proteins, including several types of collagen, fibronectin, proteoglycans, heparin, cross-linked fibrin, and elastin. A previous study [15] supports our present data, showing the release of free DES and IDE into the soluble fraction; these studies showed that neutrophil elastase cleaves elastin selectively near cross-linking residues.

This study used oxidized insoluble elastin to explore the mechanisms of elastolysis that may pertain to diseased tissues where oxidative conditions prevail. Compared to normal insoluble elastin, oxidized elastin contains more oxidative modified cross-links, oxodesmosine (OXD) and isoxodesmosine (IOXD) - generated from DES and IDE, respectively, by reactive oxygen species (ROS) [9]. Oxidized elastin may have more cleavage regions, as the formation of OXD and IOXD during the oxidation of insoluble elastin by ROS can cause solubilization of insoluble elastin [9]. This study showed that oxidized insoluble elastin liberated more DES and IDE into the soluble fraction than could normal insoluble elastin. This higher susceptibility to elastolysis may have implications for understanding elastin turnover in the arterial wall, particularly in diseases such as atherosclerosis and AAA. Iron derived from extravasated erythrocytes can accumulate in atheromata, where it could catalyze Fenton reaction-related oxidative chemistry. Also, a variety of oxidants that generate ROS including peroxynitrite, superoxide, and hypochlorous acid show elevated activity in atheromata [26, 27].

Cultured monocyte/macrophage cell lines or human peripheral blood monocyte-derived macrophages in contact with insoluble elastin can generate the degradation products DES and IDE (Fig. 2, 3A and 3B). In the present study, silencing of MMP-12 or neutrophil elastase using siRNA oligonucleotides reduced the generation of free DES and IDE (Fig. 3E and 3F). We also observed increased DES and IDE liberation by silencing of MMP-9 (data not shown). Inhibition of one protease can influence the activity of other proteases [28]. Thus, the reduction of MMP-9 activity might increase accessibility of active MMP-12 or neutrophil elastase to DES and IDE regions in the insoluble elastin.

In inflammatory conditions such as atherosclerosis and AAA, elastolysis may cause the release of DES/IDE into biological fluids such as blood, plasma, or urine. Figure 4 shows postulated pathways for progressive elastolysis through the liberation of DES and IDE from insoluble elastin. One research group found free DES and IDE in biological fluids such as blood and urine [29, 30]. While these studies suggested that changing DES and IDE levels in biological fluids reflect tissue elastase activity and elastin degradation, the specific elastases responsible for DES/IDE release remained unidentified. The current data suggest that elevated MMP-12 and neutrophil elastase in inflamed arteries participate in elastin degradation through the liberation of DES and IDE during the progression of vascular diseases. Thus, this study sheds new mechanistic light on the mechanisms of elastolysis in

inflammatory conditions, and provides a justification for further study of elastin breakdown products like, DES and IDE as biomarkers of extracellular matrix turnover in disease.

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Abbreviations

AAA	abdominal aortic aneurysm
DES	desmosine
IDE	isodesmosine
IOXD	isooxodesmosine
MMPs	matrix metalloproteinases
OXD	oxodesmosine
PBMC	peripheral blood monocyte-derived macrophages
PMA	phorbol 12-myristate 13-acetate

PMSF phenylmethanesulfonyl fluoride
ROS reactive oxygen species

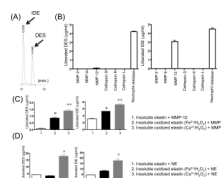


Fig. 1. Liberation of DES and IDE as amino acids from insoluble elastin by elastolytic enzymes (A) Representative HPLC profiles of DES and IDE standards. (B) Insoluble elastin (10 mg) was incubated with elastolytic enzymes (1 μg) indicated on the abscissa for 72 hours at 37 °C. (C) MMP-12 catalytic domain (1 μg), (D) neutrophil elastase (1 μg) were incubated with insoluble elastin (10 mg, lane 1), oxidized insoluble elastin (10 mg, lane 2), from oxidation of insoluble elastin by Fe²⁺/H₂O₂, or oxidized insoluble elastin (10 mg, lane 3) from oxidation of insoluble elastin by Cu²⁺/H₂O₂, for 72 hours at 37°C. Data are presented as mean ± SD (n=5). *, ** *P*<0.05 when compared with lane 1.

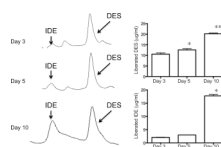


Fig. 2. Liberation of DES and IDE as amino acids from insoluble elastin by human PBMC
Human PBMC (after 3, 5, and 10 days in culture) were co-cultured with insoluble elastin (10 mg) under serum-free conditions for 72 hours at 37 °C. Data are presented as mean $\pm$ SD (n=5). *, ** $P < 0.05$ when compared with 3 days. HPLC profiles in the figure are representative of three independent experiments.



Fig. 3. Liberation of DES and IDE as amino acids from insoluble elastin by proteases secreted from macrophage-like and monocyte-like cell lines

RAW 264.7 macrophage-like cells (A) or THP-1 monocyte-like cells (B) were co-cultured with insoluble elastin under serum-free conditions for the times indicated (top) at 37°C. RAW 264.7 macrophage-like cells (C) or THP-1 monocyte-like cells (D) were co-cultured with insoluble elastin under serum-free conditions in the absence (top) or presence of E-64 (100 μ M), aprotinin (100 μ g/ml), or PMSF (1 mM) for 72 hours at 37°C. (E) After 24 hours of transfection of siRNA for MMP-12, RAW 264.7 macrophage-like cells were co-cultured with insoluble elastin under serum-free conditions for 72 hours at 37°C. Efficiency of silencing of MMP-12 was determined by western blotting (bottom section of panel E). (F) After 24 hours of transfection of siRNA for neutrophil elastase, THP-1 monocyte-like cells were co-cultured with insoluble elastin under serum-free conditions for 72 hours at 37°C. Efficiency of silencing of neutrophil elastase was determined by western blotting (bottom section of panel F). HPLC profiles in the figure are representative of three independent experiments.

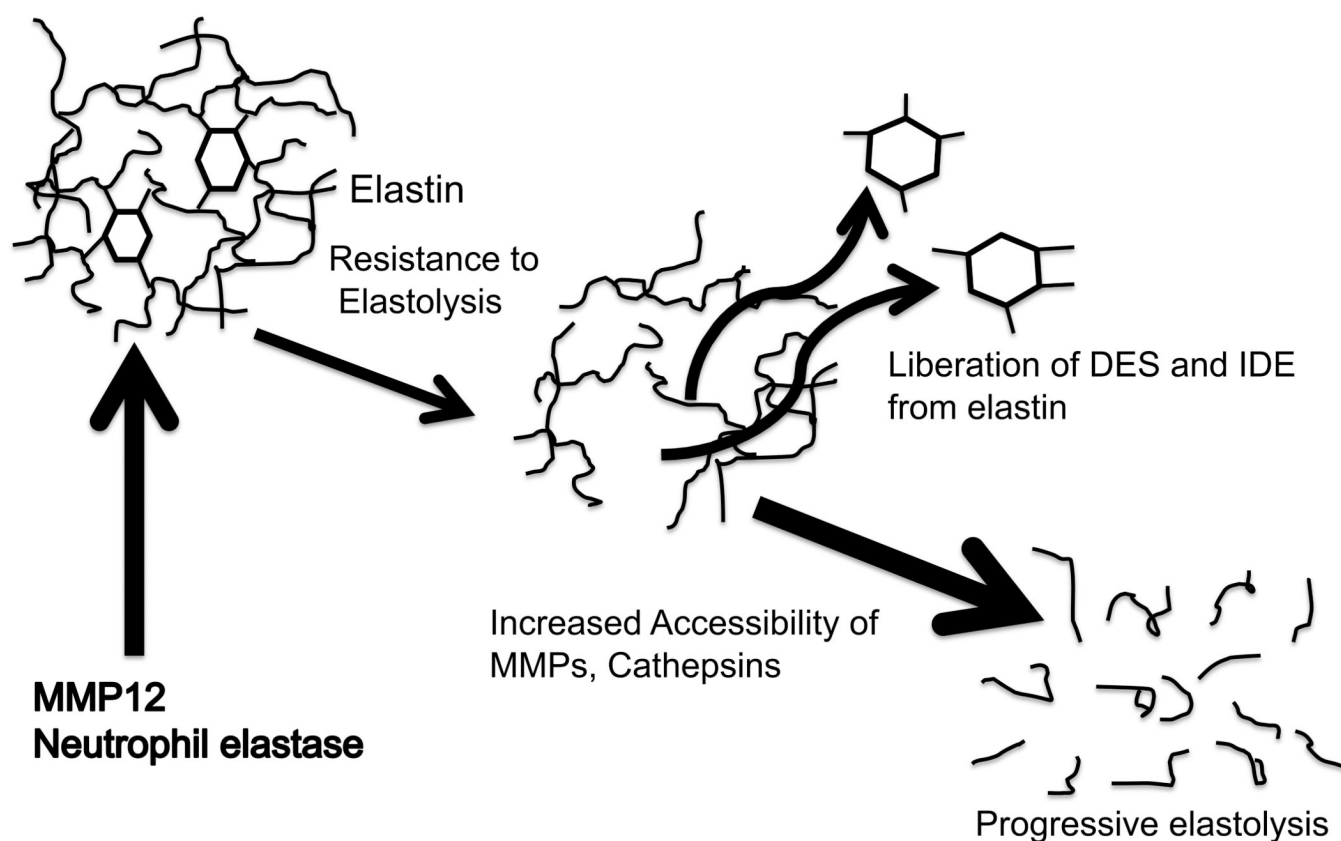


Fig. 4. Postulated pathways for progressive elastolysis through the liberation of DES and IDE from insoluble elastin

DES and IDE greatly contribute to the stability of insoluble elastin. Therefore, the liberation of DES and IDE from insoluble elastin may result in progressive elastolysis.