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Commentary

# Role of the endogenous elastase inhibitor, elafin, in cardiovascular injury From epithelium to endothelium

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ABSTRACT

Neutrophils and neutrophil-derived elastases play a major role in the regulation of vascular injury and inflammation, such as ischemia–reperfusion injury. Elafin is an endogenous inhibitor of neutrophil-derived elastases with numerous anti-inflammatory functions that include modulation of inflammatory cytokine release as well as innate and adaptive immunity. It is produced by epithelial tissues including the skin and respiratory system that have adapted to respond to the microbial and chemical insults that lead to inflammation. The production of peptides like elafin with multi-faceted anti-inflammatory activity is an important part of this adaptation. Although not directly expressed within the cardiovascular system itself, pre-clinical studies have suggested therapeutic benefit of elafin in cardiovascular disease.

The aim of this review is to highlight the role of neutrophil-derived elastases in vascular inflammation and injury. We will discuss the beneficial effects of elafin inhibition of neutrophil elastase and its extended anti-inflammatory activity in pre-clinical models of inflammatory vascular injury.



## 1. Introduction

### 1.1. Neutrophil-derived elastases and vascular inflammation

#### 1.1.1. Extracellular matrix turnover

Neutrophils lead the early phase of the inflammatory response gathering at sites of inflammation, and transmigrating through the endothelium to release granule contents and generate oxygen-derived free radicals that serve the host defence by providing microbicidal activity. Human neutrophil elastase (HNE) and proteinase-3 are amongst the contents of neutrophil azurophilc granules [1]. These enzymes degrade components of the extracellular matrix and the list of substrates for HNE is extensive [2] including fibrin, fibronectin, collagen, the glycoprotein IIb/IIIa receptor [3], elastin and cadherins [4]. Fig. 1 illustrates the effects of HNE and proteinase 3 within the vasculature.

HNE's action on extracellular matrix components exposes recognition sites that bind cellular integrin and tyrosine kinase receptors. These signals direct the cellular response to injury. Recognition of elastin-derived fragments by the elastin receptor results in migration and chemotaxis of monocytes and vascular smooth muscle cells [5,6].

Endothelial cells are susceptible to detachment when cultured with activated neutrophils or HNE [7]. Anchorage of cells to the extracellular matrix is necessary for survival and cleavage of matrix components and cadherins responsible for adhesion results in apoptosis [8,9]. HNE is joined by the matrix metalloproteinase (MMP) and the cathepsin family of proteases in modulating endothelial extracellular matrix degradation during acute inflammation [10]. There is considerable overlap of substrates between these protease families leading to apparent redundancy. HNE is distinct in having both a broad range of substrates and the ability to be released rapidly and in high concentration from neutrophil granules at sites of inflammation. By contrast, many of the extracellular MMP and cathepsin proteases are regulated by gene expression and MMPs are activated in a cascade of proteolytic steps [11]. HNE also modulates the activity of vascular extracellular proteases. It directly activates MMPs and inactivates their inhibitors (tissue inhibitors of metalloproteases: TIMPs) [12,13]. A complex interplay between extracellular proteases that share common substrates occurs during inflammatory endothelial

*Abbreviations:* HNE, human neutrophil elastase; PAR, protease activated receptor; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteases; PAI-1, plasminogen activator inhibitor type-1; TFPI, tissue factor pathway inhibitor; α1-PI, α1-antitrypsin; SLPI, secretory leucocyte protease inhibitor; WAP, whey acidic protein; LPS, lipopolysaccharide; EMPIRE, elafin myocardial protection from ischemia reperfusion.

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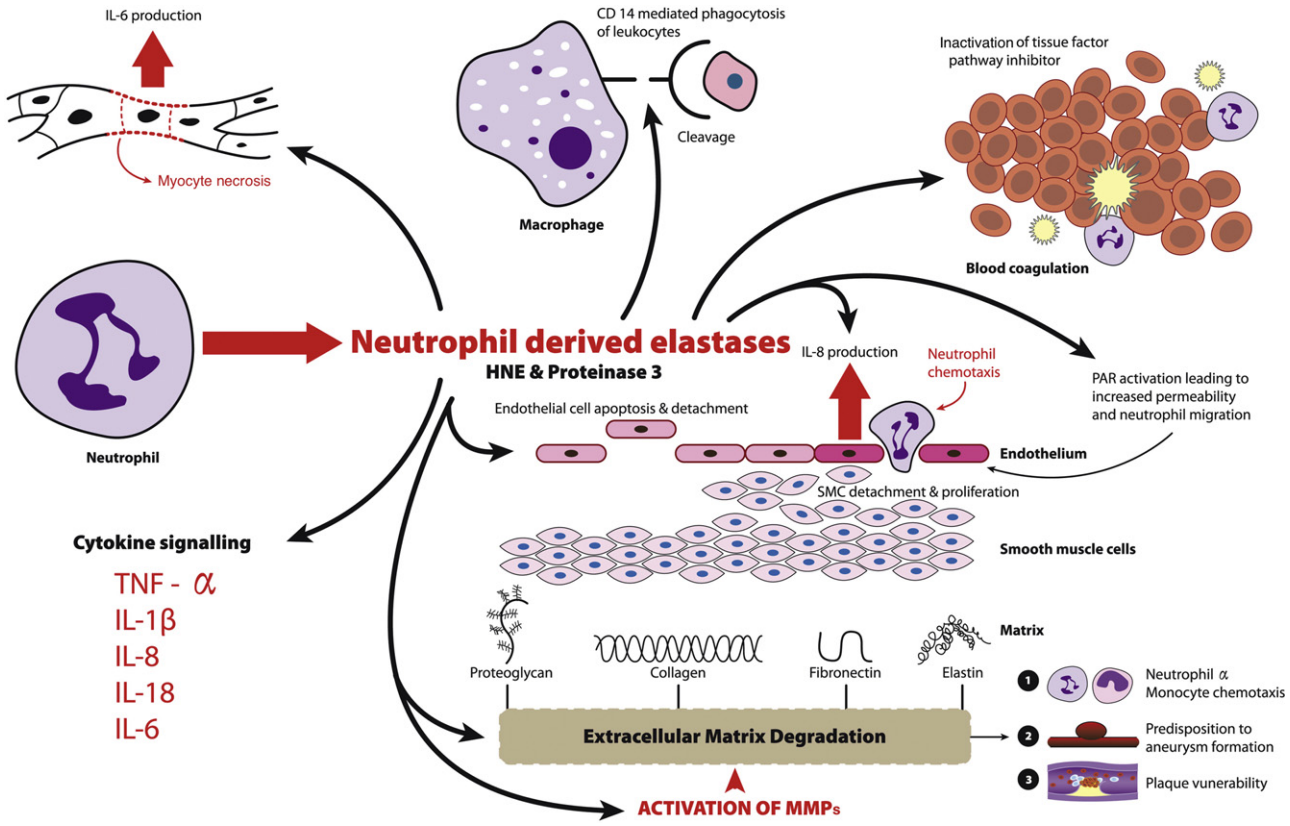


**Fig. 1.** Effects of neutrophil derived elastases. Actions of neutrophil-derived elastases: human neutrophil elastase and proteinase 3.
Activated neutrophils release HNE and proteinase 3 leading to endothelial disruption and extracellular matrix degradation. These processes contribute to aneurysm development and disruption of vulnerable atherosclerotic plaques. HNE and proteinase-3 modulate the activity of TNF-α, IL-1β, IL-8 and IL-18 by proteolytic cleavage. HNE activation of protease activated receptors (PAR) activation may contribute to changes in endothelial permeability and migration of neutrophils. HNE stimulates production of IL-8 production from endothelial cells and induces IL-6 release from necrotic myocardium. HNE cleavage of the CD14 receptor on macrophages impedes resolution of inflammation by preventing recognition and clearance of apoptotic cells. HNE also promotes thrombosis by inactivating tissue factor pathway inhibitor.

injury. HNE's broad ranging activity and modulating activity over other proteolytic pathways suggest a central role at the onset of the proteolytic cascade in pathologies where neutrophil degranulation is present. HNE has a central role alongside MMPs and cysteine proteases in atherosclerotic aneurysm development. Thrombus is present over the surface of larger aneurysms providing an active interface for neutrophil recruitment and activation. The thrombus from abdominal aortic aneurysms is rich in HNE particularly within the luminal compartment. This contributes to aneurysm growth by preventing in-growth of smooth muscle cells and re-colonisation by circulating progenitors [14]. Thrombus is continuously turning over and together with neutrophil recruitment provides a supply of HNE, plasmin and activated MMPs. These proteases permeate to the abluminal side of the thrombus to contribute to expansive arterial wall modelling

### 1.2. Modulation of thrombosis and fibrinolysis

The capacity of HNE to degrade components of the coagulation and fibrinolytic pathways has been demonstrated *in vitro*. Cleavage of plasminogen activator inhibitor type-1 (PAI-1) shortens clot lysis time *in vitro* [15]. Plasminogen is degraded to miniplasminogen by HNE. This plasminogen fragment is more readily activated and the resulting miniplasmin retains fibrinolytic activity but may be relatively resistant to inhibition by α-2 antiplasmin [16]. More recently, the role of neutrophil-derived elastases in thrombus formation has been demonstrated in knockout mice [17]. Compared to wild type mice the animals deficient in neutrophil elastase had markedly reduced fibrin formation in response to chemical injury on intravital videomicroscopy. The mechanism involved proteolytic inactivation of an endogenous anticoagulant, tissue factor pathway inhibitor (TFPI). TFPI and neutophil elastase were observed to co-localise on the external surface of neutrophils in nucleosomes facilitating TFPI degradation. The formation and externalisation of nucleosomes is increased by neutrophil interaction with activated platelets. The combination of human neutrophils and platelets generates pro-coagulant activity measured by the production of active factor X and this was markedly reduced in the presence of HNE inhibitors [17]. These observations point to a hitherto unappreciated role for neutrophils and HNE in triggering coagulation and stabilising thrombus formation. Massberg et al. [17] suggested an innate immunity role for neutrophil elastase generated thrombosis promoting retention of invading pathogens within liver microvessels. This work demonstrated the capability of neutrophils to promote thrombus formation in larger vessels in the absence of infection. The circulating neutrophil count is associated with clinical events including myocardial infarction [18]. Neutrophils from patients with acute coronary syndromes exhibit evidence of activation and degranulation [19]. Together these finding suggest a more direct pro-thrombotic role for neutrophils in coronary disease.

### 1.3. Regulation of inflammatory signalling

HNE and proteinase-3 modulate cytokine signalling. HNE and Proteinase-3 can proteolytically activate or process the inflammatory cytokines TNF-α, IL-1β, IL-8 and IL-18 [20–23]. HNE is capable of degrading IL-1β and TNF-α possibly acting as a negative

regulator of inflammation. Chemerin is a chemoattractant protein that promotes recruitment of antigen presenting cells such as macrophages and dendritic cells [24]. HNE activates chemerin from prochemerin by proteolytically cleaving its C-terminal peptide. This provides one mechanism whereby initial infiltration of neutrophils may orchestrate subsequent antigen presenting cell recruitment at inflammatory sites [25].

HNE mediated cleavage of the CD14 receptor on monocytes and fibroblasts reduces responsiveness and TNF-α production in response to LPS [26] as well as impairing recognition and clearance of apoptotic cells by phagocytosis [27]. HNE mediated cleavage of the complement receptor 1 from the surface of erythrocytes generates a fragment that acts as an inhibitor of complement [28]. These findings indicate divergent effects on inflammatory signalling and may reflect changing roles for neutrophil derived elastases depending on variables such as local concentration, stage of inflammation and signalling context.

HNE stimulates production of the neutrophil chemokine IL-8 and augments endothelial cell production of IL-8 in response to other stimuli such as lipopolysaccharide [29]. The observation that HNE stimulates cytokine production from a variety of cell types has raised the possibility of receptor interaction. Devaney et al. [30] demonstrated that HNE up-regulation of IL-8 mRNA and protein was dependent on expression of the toll-like receptor 4 in a kidney cell line. HNE and proteinase-3 also activate the protease activated receptors (PAR) [31,32] that influence a wide range of physiological responses including platelet activation, intimal hyperplasia and the maintenance of vascular tone and barrier function [33]. Activation of PAR-1, PAR-2 and PAR-4 stimulates IL-6, IL-8 and prostaglandin E2 release [34]. Selective activation of PAR-1 and PAR-2 by HNE results in increased epithelial permeability and transepithelial migration of neutrophils. This effect is blocked by PAR antagonists and is not related to cleavage of gap junctions [35]. Recently, HNE has been shown to activate PAR-2 through cleavage of the N-terminus [36]. This results in selective activation of downstream MAP kinase signalling pathways, and PAR-2 dependent calcium signalling is silenced. This recent observation of interaction with specific cell surface receptors indicates the potential for discriminate signalling by HNE.

### 1.4. Antimicrobial actions of HNE

Neutrophil elastase deficient mice are susceptible to bacterial and fungal infection. [37,38]. HNE has direct antimicrobial action against Gram-negative bacteria such as *Escherichia coli* by degrading the bacterium's outer membrane protein A [37]. Flagella are bacterial components with strong pro-inflammatory activity on epithelial and inflammatory cells. Degradation of flagellin the structural component of flagella in *Pseudomonas aeruginosa* by HNE results in loss of this virulence factor's stimulatory activity [39–41]. HNE cleaves other virulence factors of enterobacteria and has direct antifungal activity against *Candida albicans* and *Aspergillus fumigatus* [38,42]. Antimicrobial activity of HNE and proteinase-3 against Gram-positive bacteria was recently demonstrated with direct *in vitro* killing activity on *Streptococcus pneumoniae* [43].

Cathelicidins are antimicrobial peptides that are stored and released from the lysosomes of neutrophils. HNE and proteinase 3 regulate the activity of cathelicidins by proteolytic release of the antimicrobial C-terminal fragment [44]. Neutrophil derived elastases can therefore affect microbial killing by direct and indirect mechanisms.

### 1.5. Neutrophil elastase activity is regulated by endogenous inhibitors

Neutrophil derived elastases have wide ranging inflammatory effects on a broad range of substrates. Endogenous serine protease inhibitors (serpins) block activity by complexing with the elastase molecule. α1-antitrypsin (α1-PI) is the major circulating serpin produced in the liver with inhibitory activity against HNE. α1-PI is present at saturating levels within the circulation providing systemic inhibitory activity against HNE. Elafin and secretory leucocyte protease inhibitor (SLPI) are serpins produced locally at sites of inflammation by epithelial cells in response to inflammatory stimuli such as TNF-α and HNE. The ability to raise this local defence of 'alarm' antiproteases illustrates the extent to which epithelial tissues have evolved mechanisms to respond to, and contain, neutrophil-mediated inflammation [45]. Cardiovascular tissues do not express these alarm antiproteases and are more vulnerable to HNE mediated injury as a result.

HNE can evade high local concentrations of inhibitors through a series of mechanisms. Large quantities of oxidants and proteases released by leukocytes recruited to the site of inflammation can overwhelm and inactivate protease inhibitors. Adhesion of neutrophils to the extracellular matrix leads to the compartmentalisation of the released proteases between the neutrophil and matrix, and this microenvironment excludes the large circulating protease inhibitors such as α1-PI [46]. A large proportion of the serine proteases released from azurophil granules bind to the plasma membrane with catalytic activity preserved. Owen et al. [47] suggested that this tight binding of extracellular neutrophil serine proteases to the cell membrane makes them inaccessible, and therefore resistant, to circulating, high-molecular-weight, endogenous inhibitors such as α1-PI. Surface bound HNE is inhibited by small molecule inhibitors including SLPI suggesting a specific locale for alarm antiproteases to control HNE [47]. This local antiprotease shield is not present within the cardiovascular system and strategies to introduce or mimic it will reduce HNE mediated tissue injury and inflammation. Sections 2–4 will examine the therapeutic potential of elafin in diseases characterised by neutrophil mediated vascular injury.

### 1.6. Clinical application of biological and synthetic inhibitors of HNE

Endogenous and synthetic small molecule inhibitors have been developed to combat the pro-inflammatory activity of HNE. Clinical studies with elastase inhibitors have focussed on inflammatory lung disease.

SLPI belongs to the same family of four disulphide core proteins as elafin. It shares many properties including inhibition of HNE and interference with lipopolysaccharide signalling, transcription factor NF-κB activation and TNF-α production. Delivery of recombinant SLPI was protective in rat and murine models of ischemia reperfusion injury [48]. A clinical study examining the effect of aerosolised SLPI in cystic fibrosis patients demonstrated reduced elastase activity, IL-8 and neutrophil levels in treated patients [49]. α1-PI is regarded as the major inhibitor of HNE in the lung and intravenous formulations derived from human plasma (Prolastin; Talecris Corporation, Aralast; Alpha Therapeutic Corporation and Zemaira; CSL Behring) have been trialled in patients with α1-antitrypsin deficiency with minimal impact on disease progression [50]. DX-890 (Depelstat; Dyax Corporation/Debiopharm) is a potent HNE inhibitor derived from human inter-α-inhibitor. It may have application as an aerosol elastase inhibitor in the treatment of cystic fibrosis.

Several synthetic neutrophil elastase inhibitors have been developed. Preclinical studies have shown promise in demonstrating reduced neutrophil elastase injury and inflammation but clinical translation has been frustrated by lack of efficacy and concerns over toxicity. Sivelestat (Ono Pharmaceutical) is a low molecular weight reversible competitive inhibitor of HNE. In observational studies, administration was associated with reduced mortality in critically ill patients and attenuated pulmonary

dysfunction in patients with acute respiratory distress syndrome [51,52]. In prospective double-blinded controlled trials, it has been shown to reduce IL-8 production and reduce acute lung injury after cardiopulmonary bypass, and reduce duration of ventilation in intensive care [53,54]. The only multi-centre double-blind placebo-controlled trial of sivelestat failed to show a decrease in mortality or reduced ventilator requirement in critically ill patients [55]. ONO-6818 (Ono Pharmaceutical) is a non-peptide selective neutrophil elastase inhibitor that reduced IL-8 production and complement activation in a simulated cardiac bypass circuit [56]. Clinical studies in patients with lung disease were halted because of liver injury associated with the drug. Mr889 is a less potent, reversible and slow-binding competitive inhibitor of HNE developed by Medea Research. Clinical evaluation demonstrated the drug to be safe but ineffective in modifying biochemical markers of lung destruction [57].

## 2. Elafin

### 2.1. Elafin is a potent endogenous inhibitor of neutrophil elastase

Elafin is an endogenous inhibitor of human neutrophil elastase and proteinase-3 that was first isolated from psoriatic skin and human bronchial secretions [58,59]. Cloning of elafin cDNA indicates that initial transcription produces a protein of 117 amino acid residues, which undergoes intracellular cleavage of an N-terminal hydrophobic signal sequence to produce pro-elafin [60–62]. The pro-elafin protein is composed of 2 domains: a C-terminus consisting of 57 amino acids and an N-terminus consisting of 60 amino acids also known as the cementoin domain [63,64]. The N-terminus contains VKGQ sequences that provide the substrate for transglutaminase, with glutamine and lysine acting as acyl donors and acceptors in formation of isopeptide inter-protein cross-links [64]. Transglutaminisation allows elafin to be cross-linked to the extracellular matrix where it may persist as a tissue bound inhibitor of HNE. Sumi et al. [65] demonstrated elafin immunoreactivity within the intima of human coronary arteries in association with transglutaminase. The C-terminus is responsible for the elastase inhibition. It has a four-disulphide core and shows structural similarity with the whey acidic protein (WAP) family [64,66]. This combination of a transglutaminase substrate area and a WAP/four-disulphide core has similarities with other proteins that have been named "trappins" [62]. Elafin has 40% sequence homology with SLPI and is more specific in its spectrum of activity exhibiting potent inhibition of HNE and proteinase-3. It has equilibrium dissociation constants for these enzymes of $0.8 \times 10^{-10}$ M and $1.2 \times 10^{-10}$ M, respectively [67].

### 2.2. Tissue distribution and regulation of elafin

Elafin is secreted constitutively by the squamous epithelium of the skin, with expression raised in inflammatory skin conditions such as psoriasis [58,64]. It has also been isolated in other epithelia such as sweat glands, hair follicles, tongue, tonsils, gingiva, epiglottis, esophageal lining, the vagina, the pharynx [68], submandibular glands [69], trachea, stomach, intestine [64], and mammaries [70]. More recently leucocyte expression has been identified within human endometrial neutrophils and alveolar macrophages of the respiratory system [71,72]. The detection of elafin within human coronary arteries in association with atherosclerosis raises the question over whether it has originated from infiltrating inflammatory cells or free protein entering from the circulation.

IL-1β and TNF-α together with HNE are major inducers of elafin expression in human airway epithelial cells and keratinocytes [68,73]. Regulation by these inflammatory cytokines highlights the role elafin may play in the early orchestration of the inflammatory response as an 'alarm' antiprotease secreted by local cells.

## 3. Extended anti-inflammatory roles of elafin

### 3.1. Inhibition of inflammatory cytokine production

Our group demonstrated attenuated inflammatory cytokine production by human endothelial cells and macrophages following elafin overexpression using adenoviral vectors [29]. Endothelial cell IL-8 production was reduced in response to bacterial lipopolysaccharide (LPS), TNF-α and oxidised low-density lipoprotein, a key inflammatory stimulus and driver of atherosclerotic plaque development. Overexpression in monocyte-differentiated human macrophages reduced TNF-α production in response to low concentrations of LPS [29]. SLPI has similar properties reducing TNF-α and matrix metalloprotease production in response to LPS [74,75]. An intracellular mechanism was suggested by the finding that transfection of a non-secreted form of SLPI but not addition of recombinant SLPI to cultured macrophages suppresses the response to LPS [75]. In search of an intracellular target, we and others have identified the transcription factor NF-κB. NF-κB upregulates many inflammatory genes associated with the inflammatory response including IL-8 and TNF-α. Overexpression or incubation of elafin and SLPI with monocytes reduces LPS responsiveness [29,76]. This effect is seen in association with reduced proteolytic degradation of NF-κB's inhibitory subunits IκBα and IκBα suggesting an action by elafin and SLPI on the unbiquitin–proteosome pathway.

### 3.2. Innate and adaptive immunity functions

Elafin forms part of the complex antimicrobial defence screen on mucosal and epithelial surfaces. Elafin has direct antimicrobial activity against *Staphlylococcus aureus* and *P. aeruginosa* [77]. It is possible the positive charge of elafin allows it to disrupt the anionic membrane of bacteria directly and this mechanism has been proposed for other cationic antimicrobial peptides. The interaction of elafin with LPS is complex. When preincubated together, the elafin-LPS complex increases cytokine production from inflammatory cells suggesting that elafin is priming the innate immune response [78]. This facilitates recognition of bacterial invasion on epithelial surfaces where concentrations of elafin are high allowing complexes to form prior to signalling. This contrasts with the inhibitory effects of elafin on LPS signalling described above and the reduced production of TNF-α in response to systemic LPS administration observed in elafin expressing mice [79]. Cleavage of the surface receptor CD14 by HNE impedes apoptotic cell recognition and tethering by macrophages. Our group demonstrated that adenoviral over-expression of elafin in human macrophages prevents CD14 cleavage and preserved apoptotic cell tethering [80]. This rescues these cells from an HNE induced pro-inflammatory to anti-inflammatory phenotype favouring apoptotic cell recognition and clearance [81].

Elafin favours the development of a Th1-type immune response. Overexpression in transgenic mice or delivery using adenoviral vectors was associated with increased numbers and activation of antigen presenting dendritic cells [82]. Cytokine and antibody analysis from pulmonary cells, serum and bronchial washings suggested that immunity was biased toward a type 1 response with production of IL-12, IFN-γ and IgG2a antibody. Clinically, this finding is supported by high concentrations of elafin in bronchoalveolar lavage from farmer's lung and psoriatic skin [83,84]. Both conditions are characterised by a vigorous type-1 immune response.

## 4. Neutrophil mediated cardiovascular injury: the role of HNE and therapeutic potential of elafin

The earlier sections outlined the pro-inflammatory actions of HNE that serve to initiate and sustain inflammatory tissue injury. Elafin is a potent endogenous inhibitor of HNE with multifaceted anti-inflammatory roles. Production of elafin in response to inflammatory injury represents a mechanism whereby epithelial tissues have evolved to contain and repair neutrophil-mediated injury. The following sections will outline the prominent role of neutrophils and HNE in cardiovascular pathologies including myocardial infarction and arterial inflammation. We will discuss the substantial evidence from pre-clinical studies (summarised in Table 1) indicating a therapeutic role for elafin in models of vascular injury.

### 4.1. Neutrophil-mediated ischemia–reperfusion injury

The role of the neutrophil in myocardial-reperfusion injury following myocardial infarction has been reviewed by Hansen and Jordan et al. [85,86]. Cardiomyocyte injury is exacerbated following reperfusion and neutrophils are pivotal mediators determining post-ischemic inflammatory reperfusion injury. Neutrophils accumulate within the reperfused myocardium releasing HNE and reactive oxygen species that further effect microvascular and myocardial injury. Preclinical studies have demonstrated that neutrophil depletion or inhibition of neutrophil elastase attenuates post-ischemic inflammatory reperfusion injury within the myocardium [87,88].

Plasma HNE and myeloperoxidase concentrations increase following myocardial infarction providing evidence of neutrophil activation [89,90]. Neutrophils are activated by a vast array of mediators released from endothelial cells, mast cells and myocytes within the myocardium following ischemia. The complement fragment C5a, IL-8 and platelet activating factor act as chemoattractants and stimulate adherence to the endothelium. TNF-α released from mast cells and IL-6 from ischemic cardiomyocytes further stimulate neutrophil superoxide production, transendothelial migration and degranulation [91].

Coronary occlusion without reperfusion is associated with ischemia and restricted infiltration of neutrophils into the border area of the infarcted zone over 24 h [92]. The goal of therapy in acute myocardial infarction is timely restoration of perfusion and whereas this reduces infarct size, it is associated with accelerated accumulation of neutrophils within the reperfused myocardium [93,94]. Neutrophil adhesion to the endothelium occurs within minutes of reperfusion [95,96]. Release of proteases and reactive oxygen species cause cardiomyocyte necrosis. Neutrophils also occlude microvessels and cause changes in endothelial permeability that contribute to myocardial edema [97]. Capillary plugging and obstruction by activated neutrophils contributes to failure of microvascular perfusion and increased infarct size within the "no-reflow" zone. Neutrophil depletion reduced this phenomenon and infarct size in a pre-clinical model [98]. Neutrophil mediated inflammation generates production of further chemokines and adhesion molecule expression that amplify inflammatory cell recruitment. The goal of therapy is both to reduce neutrophil-mediated injury and to break the vicious cycle of further neutrophil recruitment.

Administration or over-expression of elafin was associated with reduced infarct size and neutrophil infiltration in several models of ischemia–reperfusion injury. Tiefenbacher et al. [88] investigated cardiac reperfusion injury in a rat model inducing repeated ischemia and reperfusion with ligation of the left coronary artery. The animal received a bolus of recombinant elafin by tail vein injection prior to ischemia. Myocardial function measured by systolic fractional thickening was better in animals receiving elafin or a synthetic elastase inhibitor. Systolic fractional thickening of the myocardium measured by pulsed Doppler was reduced by 50% in the controls compared to 22% in

**Table 1**
Summary of clinical effects of elafin in animal models.

| Target | Pathology | Elafin effect |
|---|---|---|
| Arterial | Balloon angioplasty [86] | 43% Reduction in intimal cross-sectional area |
| | Wire induced injury [88] | Suppression of vessel enlargement, intimal thickening, media area and cellularity.<br>Decrease in neo-intima formation.<br>Lower number of proliferating smooth muscle cells.<br>Reduction in the macrophages and neutrophils. |
| | Pulmonary hypertension [91] | Resistance to chronic hypoxia induced pulmonary hypertension and pulmonary artery enlargement.<br>Reduced pulmonary arterial muscularisation.<br>Suppression of metalloproteinase-9 activity. |
| Venous | Atherosclerotic vein graft degeneration [87] | Reduced atherosclerotic plaque formation.<br>Reduced neo-intimal formation and medial/adventitial thickness.<br>Reduced intimal thickness.<br>Reduced macrophage infiltration and lipid accumulation. |
| Ischemia–reperfusion injury | Limb reperfusion injury [80] | Preserved muscle viability.<br>Reduced neutrophil recruitment. |
| | Cardiac reperfusion injury [68] | Reduced myocardial infarct size.<br>Improved left ventricular function.<br>Reduced neutrophil infiltration. |
| Cardiac inflammation | Myocardial infarction [79] | Decreased infarct size.<br>Reduced scar thinning and infarct expansion.<br>Less diastolic dysfunction. Reduced post-infarct fibrosis.<br>Suppressed increase in matrix metalloproteinase.<br>Lower myeloperoxidase activity and neutrophil recruitment. |
| | Myocarditis [92] | Preserved cardiac function.<br>Reduction in inflammation and myocardial injury.<br>Enhanced survival in transgenic mice |
| | Cardiac transplantation [94] | Reduced wet weight of transplanted hearts.<br>Preservation of myocyte integrity, reduced myocardial necrosis |

elafin treated animals. Mice expressing human elafin under the control of the preproendothelin promoter exhibit better cardiac function compared to littermates following myocardial infarction. Left ventricular dimensions in diastole were significantly increased in the wild-type mice (mean 4.75 mm) compared to sham operated mice (mean 3.95 mm). Elafin expressing mice had some increase in cavity size (mean 4.30 mm), which was not significantly different from sham operated mice. Elafin expressing mice had reduced infarct expansion and less scar thinning. The increases in myocardial tissue elastase and matrix metalloprotease activity following infarction were effectively suppressed in elafin transgenic animals compared to wild type littermates [99]. Tissue myeloperoxidase content can be used as a measure of neutrophil infiltration. In the rat myocardial infarction model myeloperoxidase levels increased 14-fold in control animals compared to only a 3-fold rise in elafin treated animals [88]. Similar reductions in neutrophil infiltration were observed in the elafin transgenic mice and following elafin administration prior to arterial ligation in a rodent limb ischemia model [100]. In the latter study, elafin attenuated the acceleration in neutrophil infiltration (myeloperoxidase content) following reperfusion and was associated with reduced myocyte necrosis.

Elafin administration or overexpression was consistently associated with reduced myocyte death and preserved function following ischemia and reperfusion across different models. The reduction in neutrophil infiltration suggests that elafin interrupts the positive feedback loop signalling further neutrophil recruitment during reperfusion. This may be through combined direct inhibitory action on HNE and proteinase 3 mediated tissue injury and suppression of the potent neutrophil chemokine IL-8.

### 4.2. Neutrophil elastase-mediated inflammation of the vessel wall

Endothelial function is compromised by neutrophil-mediated injury. Loss of permeability barrier leads to edema and hemorrhage and loss of antithrombotic activity is conducive to platelet adhesion and fibrin deposition. These effects manifest in diverse diseases that share neutrophil-mediated injury including the adult respiratory distress syndrome, vasculitides and disseminated intravascular coagulation. Activated endothelial cells release platelet activating factor and IL-8 facilitating paracrine activation and degranulation of neutrophils. HNE stimulates IL-8 production directly and augments endothelial cell production in response to other stimuli such as lipopolysaccharide [29]. HNE activation of endothelial PAR receptors (described above) may signal thrombus formation and neutrophil adhesion through mobilisation and secretion of Weibel–Palade bodies, which harbor vWF multimers and the P-selectin adhesion molecule [101].

The neutrophil is not a prevalent cell type within atherosclerotic plaque. Nevertheless an expanding body of evidence implicates neutrophils and HNE in the development of arterial wall inflammation. Macrophage and endothelial cell localisation of HNE mRNA was identified by *in situ* hybridisation within human atheroma [102]. HNE colocalised with macrophages at the plaque shoulder. This region is characterised by a paucity of smooth muscle cells and is prone to rupture through mechanisms involving excess proteolytic activity. This work demonstrated HNE production by human endothelial cells and peripheral blood-derived monocytes suggesting the intriguing possibility that these cell types may contribute directly to elastase activity within the arterial wall. Neutrophils are activated in patients the acute coronary syndromes [19] and are observed infiltrating ruptured plaques and areas of endothelial erosion responsible for thrombosis in human coronary specimens [103]. It is possible that HNE is assimilated into the plaque from degranulating neutrophils. The phenomenon of neutrophil-derived proteins accumulating in the subendothelial space of atheromatous vessels has been demonstrated for myeloperoxidase, which is taken up by endothelial cells through transcytosis [104,105].

Rabinovitch's group have demonstrated that augmentation of elafin by delivery of recombinant protein or overexpression using transgenic mice or vectors has therapeutic benefit in models of inflammatory vessel wall injury [106]. Neutrophil recruitment and elastase activity increase within the arterial wall following balloon angioplasty [107,108]. Two episodes of balloon angioplasty 3 weeks apart were used in a double injury model in rabbit carotid arteries [107]. There was an 8-fold increase in elastase activity peaking 1 week after the second balloon injury. The elastase activity was associated with a 25 kDa protein that was inhibited by elafin suggesting neutrophil elastase. Using a liposome vector, human elafin cDNA was transfected into the carotid arteries following the second balloon injury. Elafin transgene expression was evident at 48 h and suppressed the rise in elastase activity by 90% compared with controls. This was associated with reduced restenosis and a 43% reduction in intimal cross-sectional area inelafin-transfected arteries. Wire induced carotid injury in transgenic mice expressing elafin produced similar findings [106]. Elafin transgenic animals exhibited almost complete suppression of elastase activity in response to wire injury. Wild-type mice had a doubling in the cross sectional arterial medial area at 10 days. This was taken as a measure of restenosis and reflects matrix deposition, cellular migration and proliferation. There was no significant difference in the medial area between non-injured wild-type and injured elafin transgenic mice. These changes were seen in association with reduced neutrophil infiltration and lower deposition of the matrix protein tenascin-C. Tenascin-C signals vascular smooth muscle cell proliferation [109], a key feature of arterial restenosis following angioplasty. Venous grafts are used as conduits in coronary bypass surgery. They are susceptible to rapid atherosclerotic degeneration and this was modelled in the rabbit with an interposition jugular venous graft inserted into the carotid artery [110]. Liposomal transfection of elafin cDNA resulted in reduced elastase activity, neutrophil infiltration within the graft and a 50% reduction in neointimal formation compared to mock transfected control grafts. This model was used to study the effects of elafin on chronic atherosclerotic plaque development. Following 3 months of high cholesterol feed, the rabbit interposition venous grafts developed marked intimal plaque with high lipid and macrophage content. The plaque size was reduced in elafin-transfected grafts by 40% and lipid content was reduced by 50%.

These findings indicate a prominent role for neutrophil elastase in acute arterial inflammation. Overexpression of human elafin cDNA in the arterial wall (rodents do not produce elafin) was associated with a reduction in elastase activity, neutrophil infiltration and injury response in the form of restenosis in response to mechanical injury. The observation that elafin overexpression reduced atherosclerotic plaque development in a chronic model of hyperlipidemic rabbits raises the possibility that additional anti-inflammatory actions of elafin may be at work reducing inflammatory cytokine production.

## 5. Therapeutic application of elafin in cardiovascular disease

Elafin augmentation protects the cardiovascular system from a range of diseases characterised by neutrophil-mediated inflammation (Fig. 2). Elafin provides the endothelium and myocardium with protection against the damaging effects of neutrophil-derived elastases. This suggests that intravenous elafin administration or gene overexpression can provide inhibition by reaching elastase enzyme that is not suppressed by the high circulating concentrations of larger molecular weight elastase inhibitors. Elafin

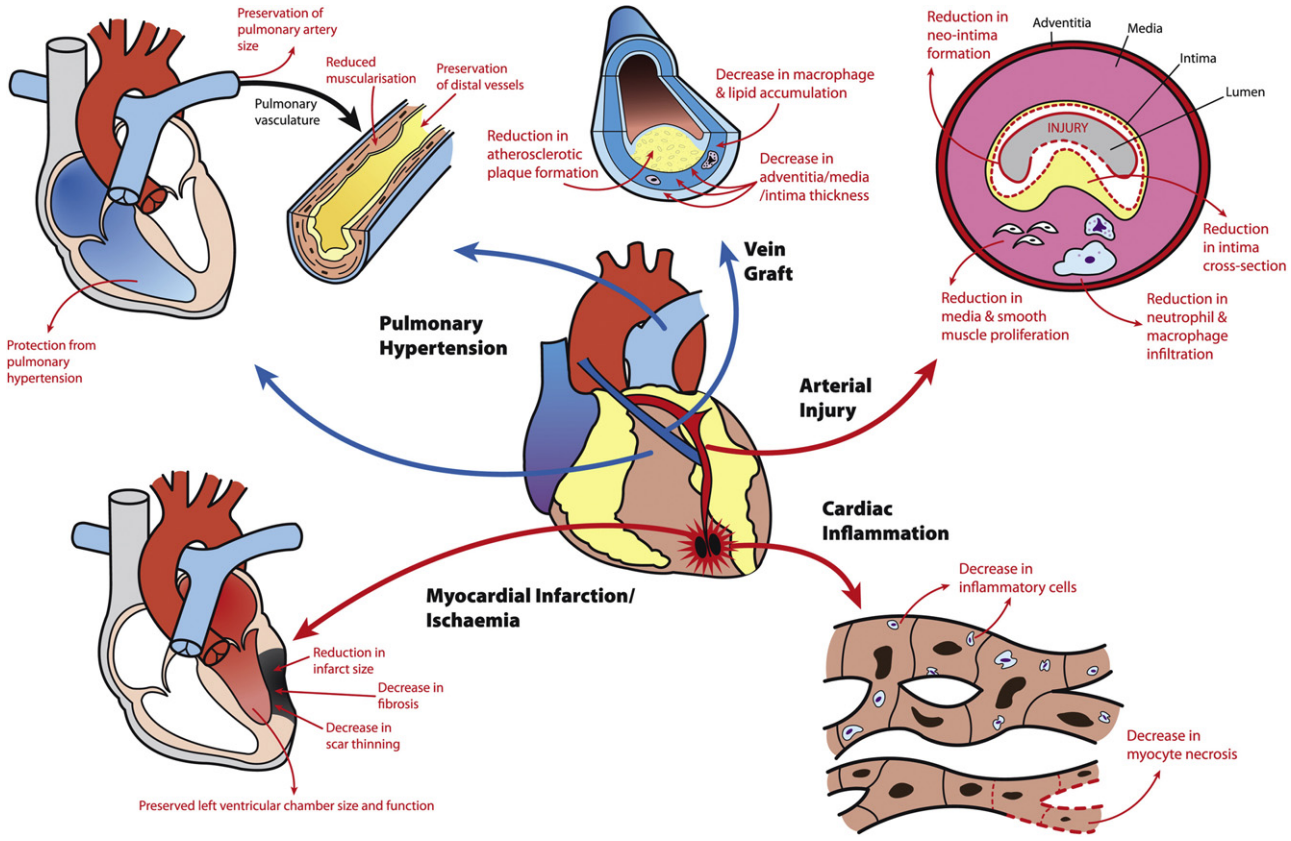


**Fig. 2.** Potential therapeutic effects of elafin on the cardiovascular system. Cardiovascular effects of elafin.
Elafin administration or gene overexpression has demonstrated beneficial effects in a series of vascular injury models. Clockwise from top-right these are arterial injury and balloon angioplasty, myocarditis, myocardial ischemia and infarction, pulmonary hypertension and vein graft degeneration. Elafin treatment is associated with consistent findings of reduced elastase activity, tissue injury and inflammatory cell infiltration in these models.

attenuates disease progression in chronic injury models including atherosclerosis and pulmonary hypertension [111] and provides survival benefit in a murine model of viral myocarditis [112]. The neutrophil does not have a prominent role in these pathologies indicating alternative mechanisms for elafin's effect beyond inhibition of elastase through suppressing NF-κB activation or modulating the adaptive immune response. Recombinant elafin has very low toxicity with short plasma and activity half-lives. It is possible to maintain circulating activity with intravenous infusion and this approach is used routinely with antithrombotic drugs in acute coronary syndrome patients. Translating the efficacy of elafin in pre-clinical models to diseases, such as acute myocardial infarction, will depend on achieving adequate concentrations of active protein at the site of tissue injury. In most pre-clinical models, elafin is delivered before or at the point of vascular injury. This advantageous position is not generally feasible in the clinic. The complexities of human disease over animal models have seen many promising therapies for ischemia reperfusion fail at translation [113]. The EMPIRE study (elafin myocardial protection from ischemia reperfusion: Eudra CT no. 2010-019527-58)[114] will recapitulate some of the conditions of pre-clinical studies to demonstrate whether elafin can attenuate myocardial ischemia–reperfusion injury and inflammation in patients undergoing coronary bypass surgery. Patients will receive an elafin infusion prior to going onto cardiopulmonary bypass and the effects on myocardial injury will be quantified by troponin release within the first 48 h after surgery and infarct volume measured with cardiac magnetic resonance scanning. Post-operative inflammatory cytokine response will also be examined. This study aims to provide proof-of-principle for elafin's therapeutic potential in cardiovascular disease.

## 6. Conclusion

Elafin inhibits destructive and inflammatory neutrophil derived proteases. Beyond this elafin inhibits inflammatory cytokines and modulates the innate and adaptive immune systems. Elafin is expressed within epithelial tissues that have evolved mechanisms to adapt and repair from neutrophil mediated injury. Elafin administration in preclinical studies of inflammatory vascular injury limits tissue destruction and preserves organ function. As such it may provide a therapeutic option in the clinical setting. The EMPIRE trial is currently underway to investigate this.

Activated neutrophils release HNE and proteinase 3 leading to endothelial disruption and extracellular matrix degradation. These processes contribute to aneurysm development and disruption of vulnerable atherosclerotic plaques. HNE and proteinase-3 modulate the activity of TNF-α, IL-1β, IL-8 and IL-18 by proteolytic cleavage. HNE activation of protease activated receptors activation may contribute to changes in endothelial permeability and migration of neutrophils. HNE stimulates production of IL-8 production from endothelial cells and induces IL-6 release from necrotic myocardium. HNE cleavage of the CD14 receptor on macrophages impedes resolution of inflammation by preventing recognition and clearance of apoptotic cells. HNE also promotes thrombosis by inactivating tissue factor pathway inhibitor.

Elafin administration or gene overexpression has demonstrated beneficial effects in a series of vascular injury models as illustrated in Fig. 2. Clockwise from top-right these are arterial injury and balloon angioplasty, myocarditis, myocardial ischemia and infarction, pulmonary hypertension and vein graft degeneration. Elafin treatment is associated with consistent findings of reduced

elastase activity, tissue injury and inflammatory cell infiltration in these models.

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