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# Human Neutrophil Elastase Mediates Fibrinolysis Shutdown Through Competitive Degradation of Plasminogen and Generation of Angiostatin

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## Abstract

**Background—**A subset of trauma patients undergo fibrinolysis shutdown rather than pathologic hyperfibrinolysis, contributing to organ failure. The molecular basis for fibrinolysis shutdown in trauma is incompletely understood. Elastase released from primed/activated human neutrophils (HNE) has historically been described as fibrin(ogen)olytic. However, HNE can also degrade plasminogen (PLG) to angiostatin (ANG), retaining the Kringle domains but not the proteolytic

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function, and could thereby compete for generation of active plasmin by tPA. We hypothesized that HNE can drive fibrinolysis shutdown rather than fibrinolysis.

**Methods—**Turbidometry was performed using light scatter (λ=620nm) in a purified fibrinogen + PLG system and in healthy citrate plasma clotted with $Ca^{2+}$/thrombin-/+tPA, -/+HNE, and -/+ANG to evaluate HNE effects on fibrinolysis, quantified by time to transition midpoint ($T_m$). $\Delta T_m$ from control is reported as percent of control ±95%CI. Purified HNE coincubated with PLG or tPA was analysed by western blot to identify cleavage products. Exogenous HNE was mixed ex-vivo with healthy volunteer blood (n=7) and used in TEG -/+tPA to evaluate effects on fibrinolysis.

**Results—**HNE did not cause measurable fibrinolysis on fibrin clots, clotted plasma, or whole blood as assessed by turbidometry or TEG in the absence of tPA. Upon tPA treatment, all 3 methods of evaluating fibrinolysis showed delays and decreases in fibrinolysis due to HNE relative to control: fibrin clot turbidometry $\Delta T_m$=110.7% (CI 105.0%-116.5%), clotted citrate plasma (n=6 healthy volunteers) $\Delta T_m$ =126.1% (CI 110.4%-141.8%), and whole blood native TEG (n=7 healthy volunteers) with ΔLY30=28% (p=0.043). Western blot analysis of HNE-PLG co-incubation confirmed that HNE generates angiostatin K1-3, and plasma turbidity assays treated with angiostatin K1-3 delayed fibrinolysis.

**Conclusions—**HNE degrades PLG and generates angiostatin K1-3, which predominates over HNE cleavage of fibrin(ogen). These findings suggest that neutrophil release of elastase may underlie trauma-induced fibrinolytic shutdown.

### Keywords

fibrinolysis; shutdown; elastase; plasminogen; angiostatin

## Introduction

Trauma patients presenting to the emergency department have a spectrum of fibrinolysis activity. Patients with excessive activity (hyperfibrinolysis) often die from exsanguination while those with impaired function (shutdown) are at increased risk of mortality from organ failure (1). Hyperfibrinolysis is mediated by increased levels of tissue plasminogen activator (tPA)(2, 3) and platelet inhibition (4). Animal work indicates that this process is driven by hemorrhagic shock (5). However, the mechanisms driving fibrinolysis resistance and shutdown remain elusive. Animal experiments have demonstrated that tissue injury, by itself, does not increase fibrinolysis(6) and may, in fact, inhibit fibrinolytic activity (5). Suppression of fibrinolytic activity can occur through multiple mechanisms including direct inhibition of pro-fibrinolytic proteins(7) and differential fibrin clot structure (8). The central protein in this process is plasminogen (PLG).

PLG is a multi-domain inactive zymogen consisting of a pan-apple domain, five kringle domains (K1-5) and a serine protease domain (9). Following docking of the kringle domains onto lysine residues of fibrin, PLG undergoes a conformational change that allows it to be cleaved by tPA, generating an active C-terminal protease fragment that remains tethered to the N-terminal kringle domain fragment through disulfide bonds (10). PLG can also undergo alternative cleavage events leading to release of smaller protein fragments consisting of K1-3 and K1-4, denoted as angiostatin (ANG) (11, 12). ANG inhibits angiogenesis through a

series of incompletely understood interactions with the endothelium(12), is thought to play a crucial role in preventing tumor metastasis(13), and has also been proposed to inhibit tPA activity (14). Furthermore, ANG can also competitively displace PLG from fibrin and thereby potentially prevent the conversion of PLG to plasmin, since the K1-3 form has a similar affinity for fibrin as glu-PLG, and a higher affinity than K1-4 (15). In our previous animal model comparing tissue injury to hemorrhagic shock, we observed that both types of trauma caused a reduction in PLG compared to control uninjured animals, with an increase in ANG only observed in the tissue injury group (5).

Human Neutrophil Elastase (HNE) has historically been described as fibrin(ogen)olytic, capable of cleaving a “knob” required for thrombin-mediated fibrin polymerization as well as being able to generate a unique sub-type of Fragment D (16, 17). HNE can also degrade plasmin(ogen)to ANG K1-3 and K1-4, in contrast to plasmin generated from PLG by tPA (15, 18). Furthermore, HNE can cleave FXIII to a truncated form of FXIIIa that crosslinks fibrin (19) and may also be able to tether α2 anti-plasmin to fibrin, both of which would increase resistance to clot lysis. There is limited data in the trauma literature on the role of HNE in coagulation-related processes. Hayakawa and colleagues found that trauma patients with DIC had slightly elevated protein amounts of HNE in plasma, which they interpreted as evidence of HNE-driven fibrinolysis, although no direct measurements of HNE activity were performed (17).

A substantial body of previous work has demonstrated that injury primes neutrophils for ROS production (c.f. (20-22)) and is associated with HNE release (23). In addition, it has been observed that the prevalence of fibrinolysis resistance increases in trauma patients cared for in the intensive care unit one day after injury(24), and this time coincides with peak levels of HNE in patients who develop post-resuscitation organ failure (25). While HNE has proteolytic activity against both fibrin(ogen) and PLG, to our knowledge there has been no investigation of the relevance of these competing fibrinolytic and anti-fibrinolytic processes on the overall state of the coagulation system as a whole. Therefore, to better understand the implications of these competing HNE proteolytic events, we set out to test the hypothesis that shutdown of fibrinolysis may be mediated by degradation of PLG to fragments incapable of catalyzing fibrinolysis, such as ANG, through the action of the neutrophil-derived pro-inflammatory protease HNE.

## Methods

The study was designed to test the hypothesis that HNE contributes to fibrinolysis shutdown by performing biochemical, kinetic and functional coagulation and fibrinolysis assays using purified protein systems, human plasma, and human whole blood. Blood products used for this study were obtained from healthy volunteers via venipuncture in the antecubital fossa with a 21-gauge needle and collection of blood in to 3.2% citrate vacuum tubes with approval from the respective Institutional Review Boards at the Massachusetts Institute of Technology and the University of Colorado.

### Turbidity Assay Measurements of Fibrinolysis

Measurements of fibrinolysis were performed via the methods of Fredenburgh et al (26). Briefly, clot formation and fibrinolysis were measured on a microplate reader via turbidity changes that cause long wavelength light ($\lambda$ = 620nm) to scatter as the clot forms and transmit through as it lyses, giving a readout of absorption ($A_{620}$). Results of each assay condition were normalized to their maximal absorption value (i.e. maximal clot formation, $A_{620\text{-}max}$) to allow for comparison (26). Fibrinolytic activity was quantified by the "transition midpoint" ($T_m$), which is the time from $A_{620\text{-}max}$ until absorption has decreased to halfway between $A_{620\text{-}Max}$ and the baseline value of the un-clotted solution (26). Results in the tPA-challenged assays where $T_m$ was reached were reported as the relative change in mean time to transition midpoint ($\Delta T_m$) of the HNE containing groups compared to controls (no HNE), which is the ratio of their respective mean $T_m$ values. Assays were conducted in technical triplicate or duplicate using both a "purified system" using the principle molecular components of fibrin polymerization and fibrinolysis, as well as in a diluted citrate plasma based system using healthy human citrate plasma. In the latter system, blood was drawn in 3.2% citrate blood collection tubes to allow for study of both the individual components and the plasma-based coagulation system as a whole. The purified system used physiological ratios of fibrinogen to plasminogen, and consisted of 1mg/mL clottable human fibrinogen (Sigma-Aldrich, St. Louis, MO) dissolved in normal saline containing 1mM $CaCl_2$ and 62.5ug/mL glu-plasminogen ("PLG", Haematologic Technologies, Essex Junction, VT). Clotting was activated by addition of human alpha-thrombin (5 nM final concentration) (Haematologic Technologies), in the presence or absence of 5pM human single-chain tPA (Sigma-Alrich) with or without 20mU/mL purified human neutrophil elastase ("HNE", Bio-Rad Laboratories, Hercules, CA). Turbidity assays using healthy human citrate plasma were performed using 1/3 strength citrate plasma, clotted with 20mM CaCl2 and 5nM alpha-thrombin, with or without 330pM human tPA, with or without 20mU/mL HNE in excess of the inhibitory capacity of the native plasma HNE inhibitors. Human recombinant angiostatin K1-3 (Sigma-Aldrich) was used in weight-based ratios with PLG (1:10, 1:1, and 10:1) using a reference value of plasma PLG concentration of 2uM (~176ug/mL) (27).

### HNE Activity Assays

Assays for HNE activity were performed using a standard curve of known purified HNE concentrations and activities in the presence of 1/3 strength healthy human citrate plasma diluted with normal saline to determine the threshold for overcoming native plasma inhibitors of HNE using the specific HNE substrate N-methoxysuccinyl-Ala-Ala-Pro-Val *p*-Nitroanalide (Sigma-Aldrich) by measuring release of 4-nitroanaline through absorption at $\lambda$ = 400nm ($E^M$ = 12,300 $M^{-1}cm^{-1}$) according to manufacturer's instructions and methods described by Castillo (28).

### Western Blots

Western blotting was performed following Towbin et al (29). Human glu-PLG (250ug/mL)or human single-chain tPA (250ug/mL) were incubated with or without 80mU/mL HNE for 30 minutes or 6 hours at 37C and proteins were separated on 10% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membranes. The PLG blots were then

probed with mouse anti-human plasminogen/angiostatin antibody that recognizes kringle domains 1-3 (clone GMA-013, Merck Millipore, Billerica, MA) at 1/1000 dilution in phosphate-buffered saline containing 0.1% Tween-20 and Odyssey blocking reagent (LI-COR, Lincoln, NE). In a similar fashion, the tPA blots were probed with mouse anti-human tPA that recognizes the A-chain (clone T-1, Abcam, Cambridge, UK) at 1/1000 dilution. Bound antibody was detected with IRDye® 800CW goat anti-mouse IgM antibody (LI-COR) per the manufacturer's instructions and imaged on a LI-COR Odyssey CLx.

### Thromboelastography

Blood was collected in 3.5-mL tubes containing 3.2% citrate, and citrated native TEGs were performed in the presence or absence of HNE using the TEG 5000 Thrombelastograph Hemostasis Analyzer (Haemonetics, Niles, IL, USA). The following indices were obtained from the tracings of the TEG: Reaction time (*R*-time min.), angle (°), maximum amplitude (MA [mm]), time to MA (TMA min.) and lysis 30 min after MA (LY30). The effects of HNE on plasmin-mediated fibrinolysis were examined using a previously published and validated tPA-TEG assay (30, 31) in which samples were supplemented with 75 ng/ml tPA in the presence or absence of 80 mU/ml HNE.

An HNE activity of 20 mU/mL was measured as the threshold required to saturate endogenous inhibitors of HNE in 1/3 diluted citrate plasma. At an activity level of 80 mU/mL, the final effective functional HNE activity in whole blood should therefore be 20 mU/mL, an identical activity level as that used in the previously conducted turbidity assays.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 6.07 (GraphPad Software Inc, San Diego, CA) and SPSS version 23 (IBM, Armonk, NY, USA). Turbidity assay data is plotted as mean ± SEM on kinetic graphs of $A_{620}$ vs time, and expressed as mean ± 95% confidence intervals (CI) on scatter plots evaluating changes in $T_m$ ($\Delta T_m$) and $A_{620\text{-}Max}$ ($\Delta A_{620\text{-}Max}$) in HNE groups relative to controls. TEG variables were non-normally distributed and were therefore evaluated using non-parametric tests between groups, with results shown as the median and $25^{th}$-$75^{th}$ percentile range. Alpha was set at the 0.05 level.

## Results

### HNE Does Not Promote Measurable Fibrinolysis in Turbidity Assays of Clotting

We first set out to directly examine whether HNE-mediated reductions in fibrin clot formation, reductions in clot strength, or increased clot lysis could be observed using turbidity assays and TEG where thrombin is simultaneously present and cleaving fibrinogen to generate fibrin clots. Initial experiments were performed using a purified biochemical system containing fibrinogen and thrombin in the presence or absence of HNE without (Figure 1A) or with (Figure 1B) PLG. Tm, the time to reach 50% lysis after maximal clot has formed, was not reached in either group over a range of increasing HNE activities from 200uU/mL to 20mU/mL (shown) over a 6 hour time period, in either the absence or presence of PLG, indicating that HNE neither degrades fibrin(ogen) or generates enzymatically active fragments of plasminogen.

We next examined the effect of HNE on lysis of clots generated by calcium and thrombin reconstitution of healthy human citrate plasma. As shown in Fig. 1C, the addition of HNE similarly failed to promote clot lysis in these turbidity assays.

### HNE Delays Clot Lysis in tPA-challenged Turbidity Assays

Given that HNE did not promote fibrinolysis in clotted fibrinogen -/+ PLG or in citrate plasma, we next investigated whether fibrinolysis was effected by HNE in the purified thrombin-fibrinogen system containing PLG,or plasma, when challenged with tPA. In the purified system, HNE addition significantly delayed clot lysis relative to control ($\Delta T_m$ = 110.7%, CI 105.0%-116.5%) (Fig. 2A,B). Interestingly we also observed a small but significant increase in the $A_{620\text{-}Max}$ of the HNE group relative to control ($A_{620\text{-}Max}$ = 106.0%, CI 103.2%-108.8%) (Fig 2B). In the plasma based system, the effect of HNE on delaying clot lysis was even more profound ($\Delta T_m$ = 126.1%, CI 110.4%-141.8%) (n=6 healthy volunteers) (Fig. 2C,D). Similar to what was observed in the purified system, a small but significant increase in the $A_{620\text{-}Max}$ of the HNE group relative to control in all 6 healthy volunteers was observed in the plasma group ($A_{620\text{-}Max}$ = 104.5%, CI 102.1%-106.9%) (Fig. 2D).

### HNE Causes Resistance to Fibrinolysis when Assayed by Thromboelastography

To further characterize the functional effects of HNE, whole blood assessment of fibrinolysis was conducted using citrated whole blood native TEGs. The addition of HNE alone to whole blood did not statistically significantly alter clot parameters (Fig. 3 and Table 1). In marked contrast, when TEGs were performed in the presence of tPA, the median LY30 was reduced by 28% in the HNE-treated group(p=0.043) (Fig. 3D and Table 1)

### HNE cleavage of PLG and Generation of Angiostatin K1-3 Drive Fibrinolysis Shutdown

Given that HNE was not observed to cause measurable fibrinolysis in either turbidity assays or TEG, but instead caused significant delays in fibrinolysis in response to tPA-challenge, we next examined whether direct HNE cleavage of significant amounts of PLG could potentially explain these observations. Top-normal physiological levels of PLG (250ug/mL) were incubated with HNE for varying times and cleavage fragments identified by immunoblotting using an N-terminally directed antibody that recognizes uncut PLG, and both the K1-3 and K1-4 fragments. As shown in Fig 4A, ~50% PLG cleavage was observed by 30 minutes (lane 2) with the appearance of both angiostatin K1-3 and angiostatin K1-4, and nearly complete cleavage of PLG to angiostatin K1-3 and K1-4 by 6 hours (lane 4).

We next examined whether any of the HNE-derived cleavage fragments of PLG were able to generate lytic activity in response to tPA stimulation using turbidity assays. As seen in Figure 4B, PLG pre-incubation with HNE for 6 hours prior to fibrinogen addition caused a marked prolongation of $T_m$ relative to HNE-untreated controls ($\Delta T_m$ = 253%, CI 243.5%-263.5%).More extended HNE incubation times completely abrogated the ability of tPA to lyse clots, consistent with completion of the PLG degradation that was near-complete at the 6 hour time point. We then examined whether HNE could also degrade tPA, which would cause resistance to fibrinolysis by preventing formation of plasmin from PLG. After performing 30 minute and 6 hour incubations of tPA with or without HNE, we performed an

immunoblotting assay using an antibody against the N-terminal A-chain of tPA. As seen in Figure 4C, no cleavage of tPA occurred by 30 minutes in the presence of HNE (lane 2) and very little occurred by 6 hours in the presence of HNE (lane 4). Finally we investigated whether, beyond a simple competition between tPA and HNE for the PLG substrate, the angiostatin K1-3 fragment itself contributed to inhibition of fibrinolysis, possibly by competing with tPA and/or PLG for lysine binding sites on fibrin clots, thereby preventing the lytic protein machinery from co-localizing on fibrin. To test this hypothesis, in the presence of a more complete milieu of plasma coagulation proteins, we added angiostatin K1-3 in increasing concentrations to citrate plasma immediately prior to performing tPA-challenged turbidity assays (Figure 4D). While lower concentrations of angiostatin K1-3 did not significantly effect the Tm, when angiostatin K1-3 was present at much higher concentrations than PLG (10:1 angiostatin K1-3 to PLG ratio), there was a marked delay in fibrinolysis ($\Delta T_m$ = 179.8%, CI 172.4%-187.1%).

## Discussion

In this study we examined the potential role of HNE as a fibrinolysis-promoting or inhibiting factor in the context of functional clotting assays with direct relevance to patients with trauma or sepsis in whom elevated levels of neutrophil activation have been reported. Although HNE is capable of cleaving fibrinogen and fibrin, which could theoretically promote post-clot fibrinolysis, we observed instead that the major function of HNE was to promote resistance to fibrinolysis in both a reconstituted clotting system and in plasma. We showed that this effect was the direct result of HNE cleavage of PLG to enzymatically inactive degradation products, thereby depleting the free PLG pool available to tPA for conversion to plasmin. These findings implicate neutrophil priming and activation in fibrinolysis shutdown and bring into question the historical description of HNE as a fibrin(ogen)olytic enzyme in the setting of trauma and sepsis. In turbidity assays with HNE at physiologically relevant concentrations/activities, we did not observe either significant clot lysis or a reduction in light scattering (and actually observed a slight increase in scattering) suggesting that in this relatively high thrombin environment the fibrin clot forms and is not broken down to any meaningful degree by HNE. This was further validated on functional assays by citrated whole blood native TEG, where HNE did not lead to any significant changes in R-time, MA, angle, or LY30. In the tPA-challenged turbidity assays, we instead observed that HNE caused a delay in fibrinolytic activity in both a purified and plasma based system, and prolonged exposure of HNE to PLG led to further resistance against fibrinolysis. This observation held true in functional clotting assays as well, where HNE treatment led to a significant reduction in LY30 on tPA-challenged whole blood TEGs relative to control (no HNE).

Our suspicion that the underlying mechanism of fibrinolysis shutdown by HNE is mediated through degradation of PLG was supported by verifying observations made in the oncology literature that HNE degrades PLG (18) to angiostatin K1-3 and K1-4 (amongst other species), which in-vitro would deplete the pool of available PLG substrate for tPA to generate plasmin, and similarly in-vivo could potentially deplete the circulating pool of PLG and render patients unable to generate sufficient plasmin to lyse clots for maintenance of vascular and microvascular patency. We simulated this by using a pre-incubation of HNE

with PLG for 6 hours at 37C, where we found only trace amounts of PLG remaining by western blot and profoundly delayed lysis in turbidity assays. Extending the HNE pre-incubation with PLG to12 hours resulted in undetectable amounts of PLG by immunoblotting, and a complete failure of tPA-challenged samples to undergo clot lysis using turbidity assays.

We further postulate that clinical organ failure in combination with fibrinolysis shutdown on functional assays (TEG, ROTEM)may provide a unifying molecular mechanism between inflammation and organ failure, where to date there is no clear pathophysiological basis for explaining exactly how inflammation actually leads to a non-functioning organ (e.g. the liver). In support of this hypothesis, we observed that exogenous addition of angiostatin K1-3 (one of the main degradation products generated by the action of HNE on PLG) to citrate plasma from healthy volunteers was sufficient to significantly prolong time to lysis in tPA-challenged plasma turbidity assays. Importantly, co-incubation of HNE and tPA showed only minimal tPA cleavage by HNE, especially when compared to HNE cleavage of PLG. These data strongly argue that HNE does not mediate fibrinolysis shutdown by degrading tPA.

There are several limitations to this study. First, the study is limited to ex-vivo and in-vitro methods using purified proteins and blood/plasma from healthy volunteers, which does not necessarily reflect the in-vivo phenomena that occur in trauma patients. Second, there are multiple components that have direct and indirect effects on fibrinolysis phenotypes in trauma patients, where the present study does not directly address the cross-talk between these other regulators (e.g. alpha 2-antiplasmin) and HNE-mediated resistance to fibrinolysis. Furthermore, causality between fibrinolysis phenotypes and trauma patient outcomes remains speculative and further study is required to more clearly define these relationships.

## Conclusions

In summary, we have shown that HNE is capable of causing fibrinolysis shutdown through proteolytically depleting the pool of PLG available to tPA for plasmin generation while simultaneously causing inhibition of fibrinolysis through generation of angiostatin K1-3 (depicted in Figure 5). These findings suggest that neutrophils primed/activated by trauma can drive fibrinolysis shutdown in a subset of trauma patients and that PMN-mediated PLG degradation to ANG may be a critical link between the inflammatory response and the coagulation system following major trauma. In lieu of these findings, it is not appropriate to describe HNE as being singularly fibrin(ogen)olytic. We believe that further studies in clinical samples, and evaluation of whether concomitant measurements of the levels of thrombin, PLG, tPA, HNE, and inhibitors can be used to define functionally relevant states of clot dynamics, and identify the subset of trauma patients who are at risk for HNE-mediated fibrinolysis shutdown, are merited.

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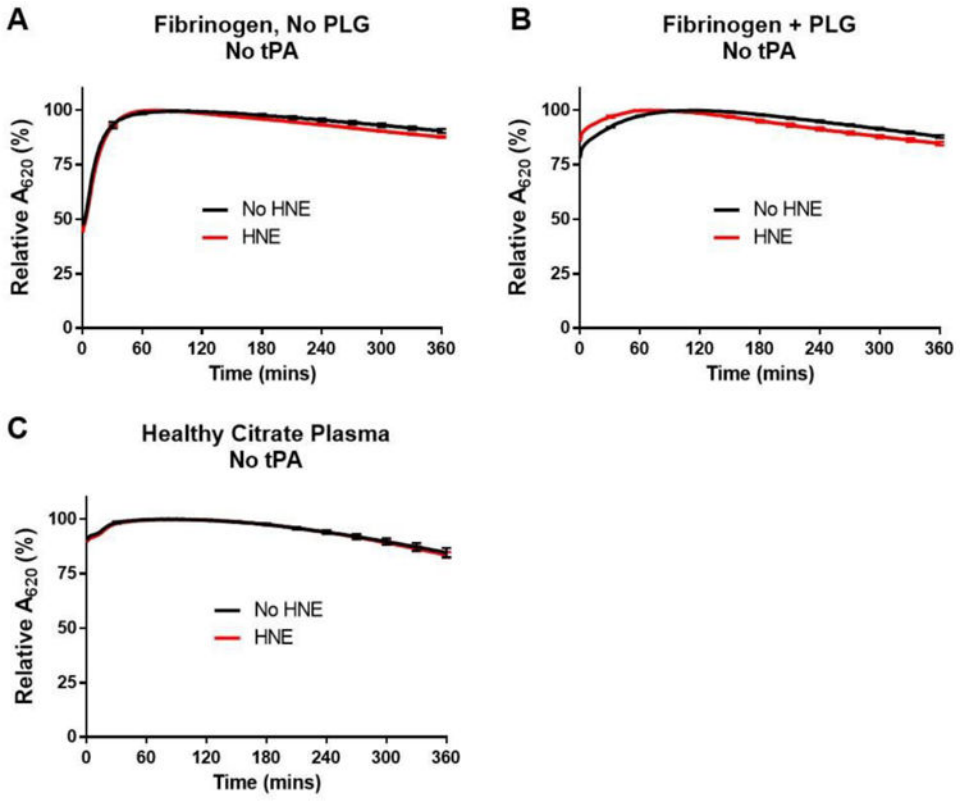


**Figure 1. Human neutrophil elastase does not cause measurable fibrinolysis in turbidity assays**

*Panel A:* Turbidity assay of purified system containing fibrinogen, thrombin, and -/+ HNE demonstrating no significant differences or fibrinolysis between control and HNE groups. *Panel B:* Turbidity assay of purified system containing fibrinogen, glu-plasminogen, thrombin, and -/+ HNE demonstrating no significant differences or fibrinolysis between control and HNE groups. *Panel C:* Turbidity assay of healthy human citrate plasma clotted with 20mM calcium and 5nM thrombin -/+ HNE demonstrating no significant differences or fibrinolysis between control and HNE groups. Results reported as mean ± SEM.

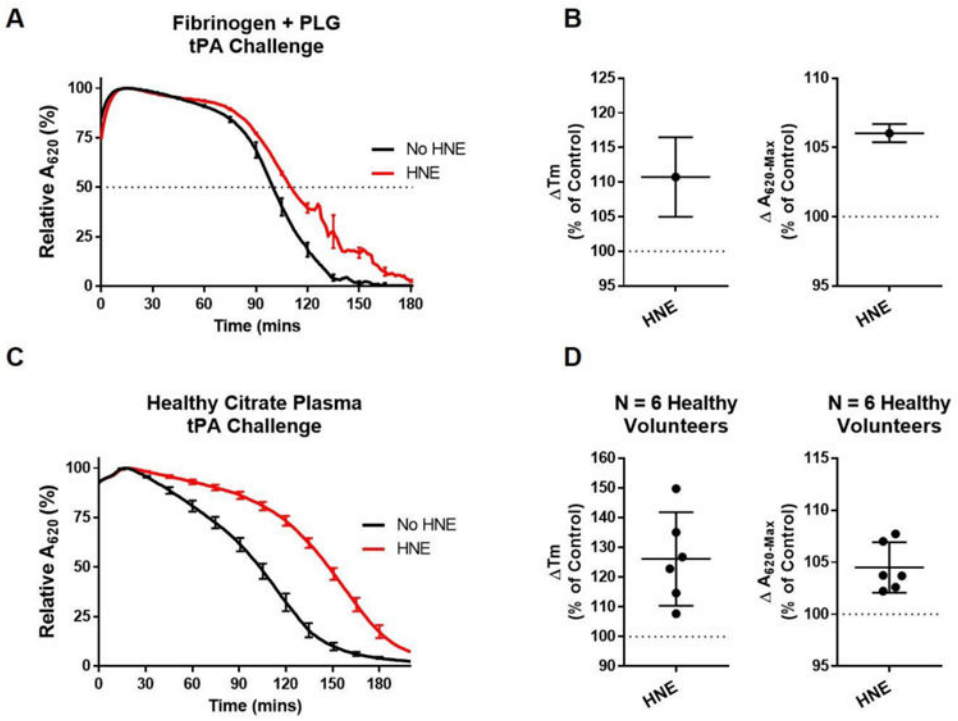


**Figure 2. Human neutrophil elastase delays fibrinolysis in tPA-challenged turbidity assays**

Panel A and B: Turbidity assay of purified system containing fibrinogen, thrombin, glu-plasminogen, tPA and -/+ HNE demonstrating significant prolongation of fibrinolysis due to HNE compared to the control group as well as increased $A_{620\text{-}Max}$ in the HNE group relative to control. Panel C and D: Turbidity assays of healthy human plasma (n = 6) challenged with tPA and -/+ HNE demonstrating significant prolongation of fibrinolysis due to HNE compared to the control group (representative curve in Panel C) as well as increased $A_{620\text{-}Max}$ in the HNE group relative to control. Results are reported as mean ± SEM on kinetic graphs of $A_{620}$ vs time, and as mean ± 95% confidence intervals (CI) on scatter plots evaluating changes in $T_m$ ($\Delta T_m$) and $A_{620\text{-}Max}$ ($\Delta A_{620\text{-}Max}$).

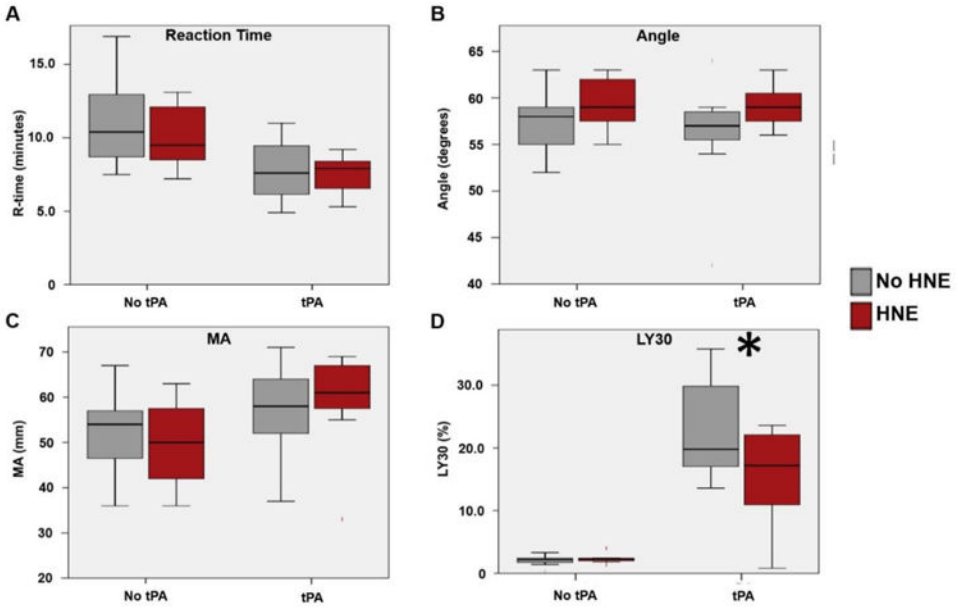


**Figure 3. Human neutrophil elastase causes resistance to fibrinolysis on citrated whole blood native TEG challenged with tPA**

Panel A-D: Citrated whole blood native TEG (n=7 healthy volunteers) demonstrating no significant differences in R time, angle, MA, or LY30 between control and HNE groups in the absence of tPA, but a significant reduction in median LY30 in the HNE group upon tPA-challenge compared to controls (no HNE). Results shown as the median and 25$^{th}$-75$^{th}$ percentile range.

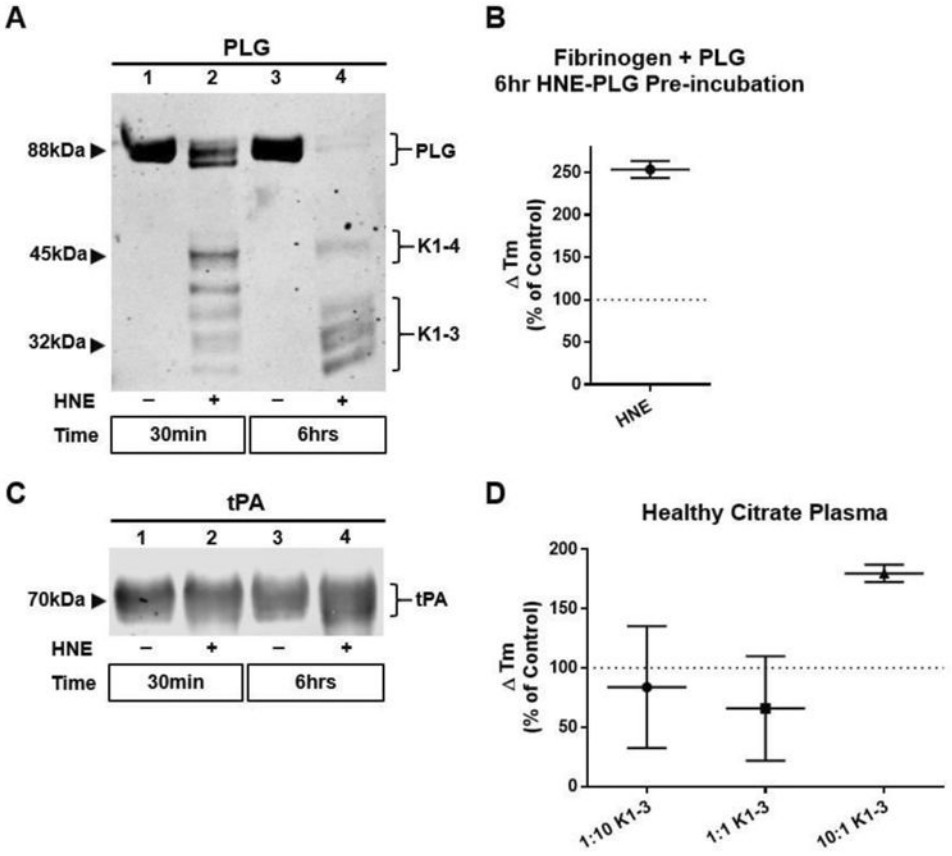


**Figure 4. Human neutrophil elastase causes fibrinolysis shutdown through plasminogen depletion and generation of the fibrinolysis inhibitor angiostatin**

*Panel A:* Western blot under non-reducing conditions demonstrating that top-normal physiologic levels of PLG (250ug/mL) are rapidly cleaved by HNE at levels similar (or below) those reported in trauma (conc. 80ug/mL, activity 80mU/mL) with formation of angiostatin K1-3 and K1-4 by 30 minutes (lane 2) and near complete degradation of PLG to angiostatin K1-3 by 6 hours (lane 4) at 37°C relative to controls (lanes 1,3). Angiostatin K1-3 is visualized as 3 bands at ~32kDa, angiostatin K1-4 is visualized as a band at 45kDa, and an intermediate species of angiostatin K1-3 containing the N-terminal 76 amino acids prior to cleavage at 38kDa. *Panel B:* Turbidity assay using PLG pre-incubated with HNE for 6 hours prior to addition of fibrinogen, thrombin and tPA demonstrating profound delay in fibrinolysis relative to control. *Panel C:* Non-reducing western blot demonstrating that tPA (250ug/mL) undergoes very little cleavage by HNE (80mU/mL) after 30 minutes (lane 2) or 6 hours (lane 4) at 37°C relative to controls (lanes 1,3). *Panel D:* Turbidity assay of healthy human plasma + tPA treated with stepwise increases in angiostatin K1-3 and clotted with thrombin + calcium demonstrating significant prolongation of fibrinolysis when angiostatin K1-3 levels are increased beyond reference plasminogen levels.

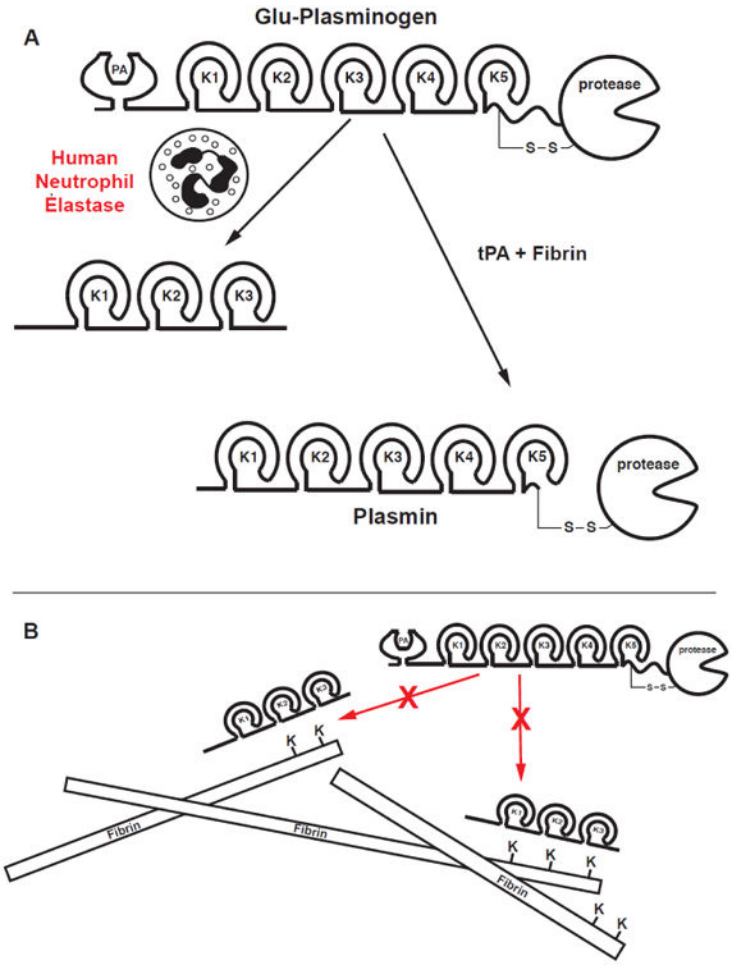


**Figure 5. Mechanistic model for the proposed mechanism of human neutrophil elastase-mediated fibrinolysis shutdown**

Elastase released by human neutrophils activated after traumatic injury cleaves PLG to angiostatin K1-3, depleting the pool of PLG substrate that is normally cleaved by tPA to generate plasmin for fibrinolysis (Panel A). Angiostatin K1-3 generated in this fashion then competes with the remaining PLG for lysine binding sites on fibrin clots (Panel B), where PLG binding to lysine residues on fibrin is a requisite step for tPA cleavage of PLG to plasmin. By depleting the pool of PLG and generating angiostatin K1-3, human neutrophil elastase released after traumatic injury may contribute to fibrinolysis shutdown.

**Table 1**

**Citrated Native TEG Indices in Whole Blood in Response to HNE and tPA Treatment**

| | R Time (min) | Angle (degrees) | MA (mm) | LY30 (%) |
|---|---|---|---|---|
| Whole Blood (WB) | 10.4 (8.7-13) | 54 (47-57) | 58 (55-59) | 2.2 (1.8-2.4) |
| + HNE p vs WB | 9.5 (8.5-12.1) p=0.093 | 50 (42-57) p=0.136 | 59 (58-62) p=0.056 | 2.3 (2.0-2.4) p=0.271 |
| +tPA | 7.6 (6.2-9.5) | 58 (52-64) | 57 (56-59) | 19.8 (17.1-29.9) |
| +HNE + tPA p vs tPA | 7.9 (6.6-8.4) 0.553 | 61 (58-67) 0.398 | 59 (58-61) 0.734 | 17.2 (11.0-22.1) 0.043 |