Supplemental Material

Methods

Fibrinogen isolation: Fibrinogen was isolated from human plasma by glycine precipitation as described by Kazal et al with minor modifications[1]. Specifically, 0.75-1 ml plasma aliquots were used and glycine was added to a final concentration of 2.1 M. The solutions were incubated for 1 hour at 4 ^{o}C rocking end-over-end and then centrifuged at 10,000 g for 30 min. The supernatants were discarded and the pellets were reconstituted in 0.5 ml 10 mM phosphate, 140 mM NaCl pH 6.4. Next, glycine was added to 2 M, the solution was incubated at 4 ^{o}C for 1 hour and then centrifuged at 10,000 g for 30 min. Finally, the pellets were reconstituted with 200 µl 50 mM Tris, 140 mM NaCl pH 7.4 and fibrinogen concentration was determined by BCA protein assay (Pierce, Rockford, IL) using fibrinogen as the standard (American Diagnostica, Stamford, CT).

Peptide capture: Isolated fibrinogen (500 µg) was treated with dithionite (DTT), Tris-2carboxyethyl phosphine (TCEP) and iodoacetamide and then tryptic digested overnight in 10 kD molecular weight cutoff (MWCO) filters (Millipore, Billerica, MA) as described elsewhere[2]. The solution was spun to separate trypsin from the peptides and then anti-nitrotyrosine antibodies were added to the peptide mixture in a ratio 1:25 (antibody:protein). The mixture was incubated overnight at 4 ^{o}C and then transferred into a 10 kD MWCO filter, washed with 5 volumes (500 µl each volume) of Dulbecco's modified PBS, next with 5 volumes of 0.5 M NaCl and finally with 3 volumes of water. The bound peptides were eluted from the antibody with 500 µl of 1 M formic acid/10% ACN, concentrated to a small volume (~10 µl) in a speed vac and analyzed by liquid chromatography-tandem mass spectrometry.

Mass spectrometry and ms-ms spectra evaluation: Samples were analyzed in a LTQ linear ion-trap instrument as described elsewhere[3,4] with minor modifications. Briefly, MS/MS spectra were acquired using a full MS scan, which was followed by data-

dependent MS/MS scans on the four most intense precursor ions. Precursors that were detected twice within 15 seconds were put on a dynamic exclusion list for a period of 60 seconds. MS/MS spectra were matched with TurboSequest (Thermo Electron, San Jose, CA) to human protein sequences using a fasta-formatted database extracted from the UniRef100 protein sequence database version. The database includes both forward and reversed sequences for estimation of false-positive identification rates. S-carboxymethylation of Cys (+57 amu), oxidation of Met (+16 amu) and nitration of Tyr (+45 amu) were specified as dynamic modifications.

Peptide sequences matched to MS/MS spectra by Sequest were accepted based on the selection criteria described previously[3,4]. Briefly, each assigned peptide should match the following: Sequest Xcorr (X_C) scores: $X_C > 2.5$ for doubly charged and > 3.5 for triply charged ions; $\Delta Cn > 0.1$; $RSp < 5$; and preliminary score (Sp) >350. Assigned spectra that met the above criteria were then manually reviewed. For peptide assignments to be accepted they must have (i) a continuous *b* or *y*-ion series of at least 5 residues and (ii) the top 3 most intense fragment peaks assigned to either an *a*, *b*, *y*-ion, to an *a*, *b*, *y*-ion resulting from a neutral loss of water or ammonia, or to a multiply protonated fragment ion.

Fibrinogen polymerization assays and fibrinolysis: Clotting assays were performed in 100 µl glass cells. Fibrinogen concentration was adjusted to 0.5 mg/ml with 50 mM Tris, 140 mM NaCl pH 7.4 and the solution was equilibrated at 37 ^{o}C. Polymerization was initiated by the addition of human α-thrombin (American Diagnostica, Stamford, CT) to a final concentration of 1 NIH U/ml and clot formation was monitored by the increase in absorbance at 350 nm using a Hewlett-Packard diode array spectrophotometer. The initial velocity V_0 of clot formation was calculated by the slope of the curve immediately after the lag phase. Polymerization in the presence of calcium was performed with 1 mg/ml fibrinogen in TBS containing 2.5 mM $CaCl_2$ after addition of 1 NIH U/ml human α-thrombin. The clotting assays after non-specific and 3-nitrotyrosine immunodepletion

and those in the presence of non-specific and anti-nitrotyrosine antibody were performed in 96 well plates. For these assays, 100 µg of fibrinogen were diluted in 180 µL TBS, followed by the simultaneous addition of 20 µl α-thrombin to a final activity of 0.1 NIH U/ml. Plasma clotting experiments were also performed in 96 well plates. Plasma fibrinogen was adjusted to 1.5 mg/ml with TBS and clotting was initiated by the simultaneous addition of 0.1 U/ml human α-thrombin and 10 mM $CaCl_2$. Clotting in the presence of mimetic peptides Gly-His-Arg-Pro^{am} and Ala-His-Arg-Pro^{am} (synthesized by CPC scientific, San Jose, CA) was performed in 96 well plates with 1 mg/mL fibrinogen 250 µM peptide, and 0.1 U/ml thrombin.

For fibrinolysis, 1.0 mg/mL fibrinogen was polymerized with 0.1 U/mL α-thrombin in 96 well plates. Seven hundred (700) nM glu-Plasminogen (American Diagnostica, *Stamford, CT*) and 70 nM tissue plasminogen activator (EMD Chemicals, Gibbstown, NJ) in TBS were added on the top of the clots and absorbance at 350 nm for 3 hours was recorded. Lysis rate was calculated by the slope in the latter part of the curve. The plasmin-induced fibrinogen degradation products were collected and analyzed in 10% SDS-PAGE under non-reducing conditions.

Viscoelastic measurements: Clots with a volume of 0.115 ml were formed between 12-mm diameter glass coverslips in a Plazek torsion pendulum at ambient temperature. A momentary impulse was applied to the clot in the pendulum causing free oscillations with strains of less than a few percent. The dynamic storage modulus (G'), loss modulus (G"), and loss tangent ($\tan\delta$ = G"/G') were calculated from recordings of these oscillations[5,6]. The storage modulus G' is a measure of the elastic properties or stiffness of the clot. In general, an increase in G' could arise from an increase in the number of branch points or an increase in the stiffness of the fibers. The loss modulus G'' represents the energy dissipated by non-elastic, viscous processes, e.g. slippage between protofibrils in the fibers. The loss tangent $\tan\delta$ is a measure of the energy dissipated by viscous processes relative to the energy stored by elastic processes.

Fibrin clot structure examined by scanning electron microscopy: The fibrin clots were prepared for scanning electron microscopy as described in the *fibrinogen polymerization assay* section. After fixation, the specimens were rinsed 3X with phosphate buffer and dehydrated in a series of ethanol concentrations up to 100%. The clots were critical point dried with CO_2 for 45 min in a Denton apparatus, mounted and sputter coated with gold palladium. These methods work well for preserving the structure of the lysis front in clots being digested[5]. Samples were examined in a Philips XL2O scanning electron microscope.

References

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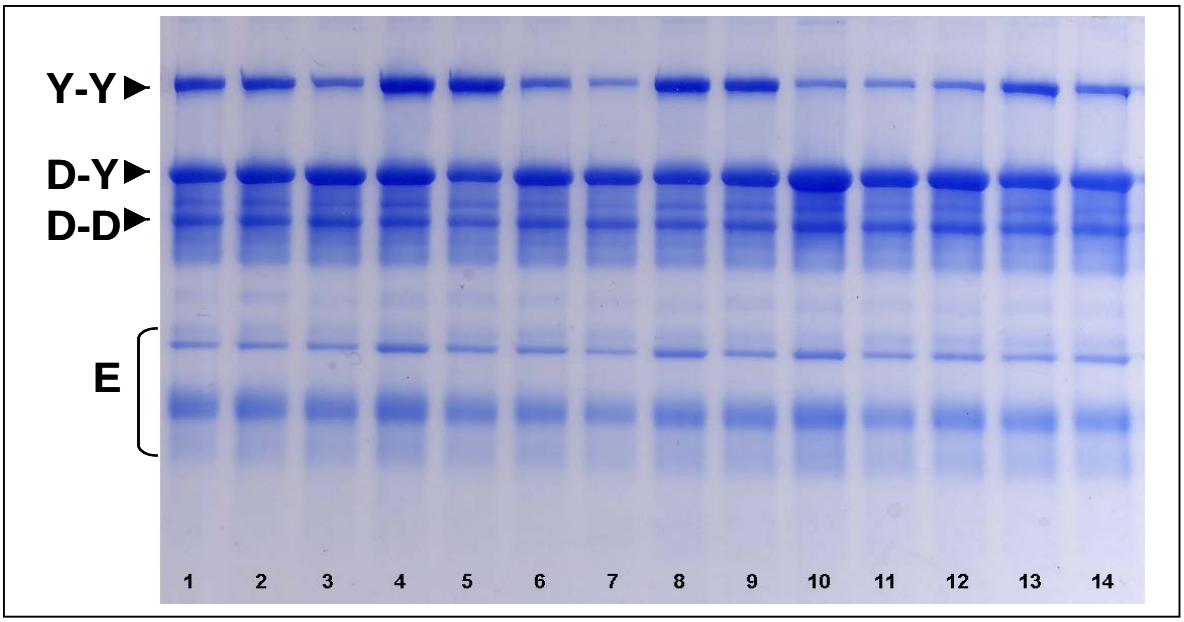
Supplemental figure 1:



Supplemental figure 1: Fibrinogen degradation products from the 14 samples (increasing levels of nitration from 1 to 14) used for fibrinolysis assays were collected and analyzed in 10% SDS-PAGE (7.5 µg/lane) under non-reducing conditions. There are no apparent differences in the generation of predicted products including Y-Dimer (Y-Y) and D-dimer (D-D).