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Attenuation of Thrombin-Mediated Fibrin Formation via Changes in Fibrinogen Conformation Induced by Reaction with *S*-nitroso-*N*-acetylpenicillamine, but not *S*-nitrosoglutathione

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Abstract

Previous work in a 4 h rabbit thrombogenicity model has shown that a nitric oxide- (NO) generating polymer extracorporeal circuits (ECC) with infusion of *S*-nitroso-*N*-acetylpenicillamine (SNAP) preserved platelets even though platelets were activated as shown by an increase in the glycoprotein, p-selectin. The platelet preservation mechanism was shown to be due to a changing fibrinogen structure leading to attenuation of platelet aggregation. Understanding the effects that SNAP, another RSNO, *S*-nitroso-glutathione (GSNO) as well as the non-RSNO, sodium nitroprusside (SNP), may have on human fibrinogen polymerization, this *in vitro* study evaluated the released NO effects on the thrombin-mediated fibrin formation and fibrinogen structure. Thrombin-induced fibrin formation at 300 μ M SNAP ($50 \pm 11\%$ of baseline) was significantly reduced compared to SNAP's parent, *N*-acetyl-penicillamine (NAP) ($95 \pm 13\%$) after 1 h of RSNO exposure. GSNO, its parent, glutathione (GSH) and 1000 ppm NO gas did not attenuate the thrombin-mediated fibrin formation. SNAP, NAP and SNP exposure for 1 h, however, did not decrease thrombin activity by directly inhibiting thrombin itself. Changes in fibrinogen conformation as measured by intrinsic tryptophan fluorescence significantly decreased in the 300 μ M SNAP (38057 ± 1196 mean fluorescence intensity (MFI) and SNP (368617 ± 541 MFI) groups versus the NAP control (47937 ± 1196 MFI). However, infused 1000 ppm NO gas had no direct effect on the ITF after 1 h incubation at 37°C. High performance liquid chromatography (HPLC) showed that fibrinogen degradation by 0.03 U/ml thrombin was concentration-dependently reduced after 1 h with SNAP but not with NAP or SNP. Western blotting showed RSNOs, SNAP, NAP and the non-RSNO, SNP-incubated fibrinogen solutions showed that the percent level of the A γ dimer to total A γ dimer + γ monomer was significantly reduced in the case of the SNAP group when compared to SNP group. These results suggest that NO donors such as SNAP and SNP induce fibrinogen conformational changes by potentially nitrosating fibrinogen tyrosine residues. These NO-mediated fibrinogen changes induced via NO donors may provide another mechanism of NO for improving thromboresistance in ECC.

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Keywords

Nitric oxide; Fibrinogen; S-nitroso-acetylpenicillamine (SNAP); S-nitroso-glutathione (GSNO); Thrombin

1. Introduction

Thrombin-induced fibrin formation is highly dependent on the cleavage of the fibrinopeptides A and B from circulating fibrinogen and initiating fibrin assembly. The soluble fibrinogen is converted by thrombin into an insoluble fibrin polymer via intermolecular interactions. The fibrin monomers polymerize via these specific and tightly controlled binding interactions to make oligomers that lengthen into protofibrils. The protofibrils aggregate laterally to make fibers, which then branch to yield a three-dimensional fibrin clot which is essential for hemostasis^{1–7}. Protofibril aggregation (i.e., fibrin monomer polymerization) after interaction with thrombin is dependent upon the inherent structure or conformation of the original fibrinogen monomers. Evidence for decreased thrombin-induced fibrin formation due to mutations or chemical modifications of soluble fibrinogen has been extensively studied^{8–20}. Of the chemical modifications that has been shown to have a marked alteration of fibrin formation is the interaction of nitric oxide (NO) with fibrinogen. The effects of NO on fibrinogen has been shown to attenuate the thrombin-induced fibrin formation but these previous works^{21–30} have unclear conclusions if NO or the various donors themselves are altering the fibrinogen conformation.

Nitric oxide is a diatomic free radical gas that has a plethora of biological effects^{31–54}. For the biological effects of NO to manifest and be preserved, most NO molecules are transnitrosated to endogenous thiol-containing proteins^{46, 55–58} or binding to heme cofactors of intracellular proteins such as soluble guanylate cyclase or hemoglobin^{59–65}. Endogenous thiol-containing proteins such as glutathione, albumin and possibly fibrinogen have been shown to cause significant reduction in platelet function and thus attenuate thrombotic events^{66, 67}. Our previous work incorporated a S-nitrosothiol, S-nitroso-acetyl penicillamine (SNAP), as a coating into extracorporeal circulations (ECC) such as extracellular membrane oxygenation (ECMO) to reduce thrombogenicity without systemic anticoagulation. SNAP coatings have shown that preventing fibrin formation via NO release reduced thrombus formations in various ECCs that were measured in a rabbit model of thrombogenicity^{26, 68, 69}. SNAP, when present in the rabbit model at a high level, caused significant reduction in platelet aggregation²⁶. However, this aggregate reduction was not due to NO-induced P-selectin attenuation because flow cytometry showed a normal increase in P-selectin expression in circulating rabbit platelets. A change in the fibrinogen conformational state was found and hypothesized to be the mechanism of the reduction in platelet aggregation²⁶. Nitric oxide has also been shown to change the conformational structure of circulating fibrinogen^{21, 70–72}. Circulating fibrinogen is a crucial complex protein in maintaining hemostasis^{1–3, 6, 10, 13, 73–82}. However, the effects of the NO produced from circulating RSNOs, after contact with NO generating polymer coatings, that possess immobilized Cu(II) or organoselenium species^{83, 84} and catalyze NO release from RSNOs), on preserving platelet count and function remains unclear.

To test the hypothesis that NO from either SNAP, GSNO, the non-RSNO, sodium nitroprusside (SNP) as well as direct NO gas, mediate fibrinogen conformational changes, the present *in vitro* study was designed to evaluate if thrombin-induced fibrin formation is reduced by a mechanism that NO alters the structure of fibrinogen. The conformational changes in fibrinogen was determined using the quenching of the intrinsic tryptophan fluorescence, high performance liquid chromatography (HPLC) retention time shift and western blotting of fibrinogen A α γ dimer expression which is normally increased after thrombin activation.

2. Materials and Methods

2.1. Materials

Human plasma fibrinogen containing $\geq 90\%$ clottable proteins was obtained from Calbiochem (San Diego, CA) and bovine thrombin was purchased from Siemens Healthcare (Plainfield, IN). The 96-well microtiter plates (clear, polystyrene) used for fluorescence measurements were obtained from Corning Incorporated (Corning, NY). S-nitroso-acetyl penicillamine (SNAP), N-acetyl penicillamine (NAP), S-nitroso-glutathione (GSNO), glutathione (GSH) and sodium nitroprusside (SNP) were purchased from Sigma Aldrich (Milwaukee, WI). NO gas at 100 or 1000 ppm (balance nitrogen gas) was purchased from Praxair (Detroit, MI). The mouse anti-human fibrinogen alpha, rabbit anti-human fibrinogen beta and goat anti-human fibrinogen gamma antibodies were obtained from Santa Cruz (Dallas, TX). The secondary antibodies chicken anti-mouse-AF488, donkey anti-goat-AF555 and chicken anti-rabbit-AF647 were from Invitrogen (Life Tech. Eugene, OR). All other reagents were of the highest purity available.

2.2. Fibrin Formation and Antithrombin Assays

Fibrin formation was determined *in vitro* using a kinetic turbidity assay that measured fibrin production at 405 nm for 60 min every 1 min interval. Human plasma fibrinogen was prepared at a working concentration of 301.9 $\mu\text{g/ml}$ and bovine thrombin was prepared at a working concentration of 0.5 IU/ml. In a 96-well microtiter plate (Corning Inc., Corning, NY), 52.1 μl Hank's Balanced Salt Solution (HBSS) with calcium and magnesium (Life Technologies, Grand Island, NY) was added to each well. The RSNOs (100 $\mu\text{l/well}$), SNAP, NAP, GSNO, GSH as well as the non-RSNO, SNP, ranging in final concentration from 1 to 1000 μM , were added to the wells. The working solution of human fibrinogen (58.8 μl) was then added to each well giving a final fibrinogen concentration of 75.5 $\mu\text{g/ml}$. The plate was placed into a 37° C incubator for 1 h. Thrombin (14.1 μl) was then added to the wells with a final concentration of 0.03 IU/ml and the plate was immediately read in a Cytation 3 microplate reader (Biotek, Winooski, VT) at 405 nm every minute for 60 minutes.

The direct effects of nitrogen, 100 ppm or 1000 ppm NO gas was evaluated for their ability to reduce thrombin-induced fibrin formation. All three gases were slowly bubbled into the fibrinogen solution for 1 h at 37°C and resulted in no fibrin formation even with the nitrogen gas control and is presumed due to the denaturation of fibrinogen^{85, 86}. The gases were then flushed through gas-diffusible silicone tubing (i.e., not bubbled but infused) in the fibrinogen solution for 1 h at 37°C in order to mitigate the potential fibrinogen denaturation.

To rule out any direct thrombin effect of the RSNOs, SNAP and NAP, as well as the non-RSNO, SNP, on the formation of fibrin *in vitro*, their antithrombin activity was determined. The assay utilizes a chromogenic substrate which mimics fibrinogen, the natural substrate for the serine protease, thrombin [16]. Briefly, the chromogenic substrate (i.e., N-p-tosyl-Gly-Pro-Arg-p-nitroanilide acetate) (Sigma-Aldrich, St. Louis, MO) is cleaved by thrombin (BC thrombin, Siemens, Newark, DE) into a residual peptide (i.e., Tosyl-Gly-Pro-Arg-OH) and free p-nitroanilide, which is measured at 405 nm in a Biotek Cytation 3 microplate reader. Briefly, the various RSNOs (1 to 1000 μ M) were first incubated with 0.03 IU/ml thrombin for 60 min at 37° C. The chromogenic substrate (24 μ M) was then added to each well at 58.8 μ l. The plate was immediately read in the plate reader.

2.3. Intrinsic Tryptophan Fluorescence (ITF)

Changes in fibrinogen structure induced by the RSNOs, SNAP and NAP as well as the non-RSNO, SNP, were determined using an assay that measured the fluorescence intensity of intrinsic tryptophan in fibrinogen which fluoresces around 350 nm^{87–92}. This assay has been described previously²¹. Briefly, ITF spectra of fibrinogen/RSNO solutions in HBSS were measured with the use of the Cytation 3 microplate reader in fluorescence mode. The samples in a 96-well plate were excited at 290 nm. The emission spectrum between 300 and 460 nm was monitored at each concentration of RSNO (1–1000 μ M). To determine the fluorescence quenching of the internal tryptophan moieties in fibrinogen, Stern-Volmer plots were used to analyze the quenching effects of the RSNOs on fibrinogen. The Stern-Volmer equation was used to describe the fluorescence quenching⁸⁹,

$$I_0/I = 1 + K_{SV}[Q] \quad (1)$$

Where I_0 and I represent the fluorescence intensity in the absence and presence of each RSNO concentration, respectively, and K_{SV} is the Stern-Volmer constant. As changes in the Stern-Volmer relationship occur then it is hypothesized that changes in the tertiary conformational state of fibrinogen is occurring.

To determine the effects of 100 or 1000 ppm NO gas bubbled or infused through silicone tubing on the ITF, the fibrinogen solutions were incubated for 1 h at 37°C. The measured ITF between 300 and 460 nm was monitored as described above.

2.4. Fibrinogen Levels measured by HPLC

To confirm conformational state changes in fibrinogen after exposure with RSNOs, HPLC was performed on the solutions from the *in vitro* fibrin formation assay. The solutions containing fibrinogen and RSNOs were analyzed after 1 h at 37°C without thrombin. Briefly, human fibrinogen was diluted to 75.5 μ g/ml with Hank's buffered salt solution (HBSS) (Gibco Invitrogen, Grand Island, NY) per well in a 96-well microplate. After 1 h at 37°C, 42.9 μ l of a solution containing 5% phosphoric acid, 71.4% acetonitrile and water to each well and then 100 μ l of this solution was put into HPLC vials. Standard curves for the various RSNOs were obtained in the range from 0 to 1000 μ M. All samples were separated with the Alliance 2695 HPLC (Waters Corp. Milford, MA) and a dual wave-length UV

detector (2487, Waters Corp. Milford, MA). Briefly, separations were performed using a reversed-phase Waters Xbridge ethylene bridged hybrid (BEH) C4 column with 3.5 μ m packed-particles and 4.6 mm x 100 mm dimensions. Solvent A was 0.1% trifluoroacetic acid (TFA) in HPLC grade water. Solvent B was 0.075% TFA in 71.4% acetonitrile and 28.6% water. The column temperature was set to 40° C and sample volumes were injected at 50 μ l. Gradient conditions were as follows: 60 min method, a linear gradient of 10–100% solvent B over 50 min at a flow rate of 0.96 ml/min, followed by 10 min return to 90% solvent A and 10% solvent B. The UV detection was done at 333 nm for the RSNOs and 222 nm for the non-nitrosylated thiols. Changes in the fibrinogen peak with and without RSNOs were measured which included both retention time and the area under the curve.

2.5. Western Blotting Assay

To further confirm conformational state changes in fibrinogen after exposure with RSNOs, Western blot analysis of plasma fibrinogen was performed on the solutions from the *in vitro* fibrin formation assay. Since human fibrinogen, which was used in the *in vitro* fibrin polymerization assay, has been shown that the α C domain interactions appear critical for the enhancement of lateral aggregation during fibrin polymerization^{1, 7, 93}, changes in α C domain interactions such as $\text{A}\alpha\gamma$ dimer expression were assessed. Briefly, after the 1 h incubation of the RSNO with 75.5 μ g/ml fibrinogen, the fibrinogen sample (200 μ l) was then mixed with 200 μ l of Laemmli sample buffer containing containing 2% β -mercaptoethanol and then boiled for 5 minutes. The cooled samples were then frozen and stored at –20° C. After thawing, the samples (15 μ l) were run on a 12% sodium dodecyl sulfate-polyacrylamide gel using a Bio-Rad miniblot electrophoresis system (Criterion Hercules, CA). Proteins were transferred onto poly(vinyl-difluoride) membranes for 2 h at 1 amp. For evaluating the alpha, beta and gamma subunits of fibrinogen, membranes were blocked with Tris-buffered saline containing 0.05% Tween-20 and 3% nonfat dry milk (Bio-Rad, Hercules, CA) for 1 h at room temperature followed by an overnight incubation at 4° C with mouse anti-human alpha fibrinogen, rabbit anti-human beta fibrinogen and goat anti-human gamma fibrinogen antibodies (all diluted 1:200). The membranes were washed twice for 10 min each and then incubated with the secondary antibodies chicken anti-mouse-AF488, chicken anti-rabbit-AF647 and donkey anti-goat-AF555 (all diluted 1:500) for 1 h. After two 10 min washes the blots were imaged in a fluorescence imager (Bio-Rad VersaDoc MP4000 Hercules, CA). The alpha, beta and gamma subunit expressions in fibrinogen were quantified using Quantity One software (Bio-Rad).

Statistical Analysis

Data are expressed as mean $\pm$ SEM (standard error of the mean). Comparison between the various concentrations of RSNOs and control groups were analyzed by the one-way ANOVA with a nonparametric multiple comparison of means using Wilcoxon Method. All statistical analyses were performed using the statistical program SAS JMP (SAS Institute Cary, NC). Values of $P < 0.05$ were considered statistically significant for all tests.

3. Results

3.1. Effects of RSNOs and NO gas on *in vitro* fibrin formation.

It is well known that thrombin catalyzes the exposure of specific polymerization sites of the central hemostatic plasma protein, fibrinogen, to begin the clotting process. Hence, any alteration in the conformational structure of fibrinogen (Figure 1A) can reduce the effectiveness of thrombin to mediate the formation of fibrin, an essential component of a developed thrombus. Previous work hypothesized that high plasma concentrations ($> 100 \mu\text{M}$) of RSNOs could alter the polymerization of fibrinogen possibly through conformational changes in fibrinogen (Figure 1B). The actual SNAP concentration in the previous work after 4 h of intravenous infusion into rabbits was determined to be approximately $405 \mu\text{M}$ in the blood which falls within the SNAP concentration range used in the current study ($0\text{--}1000 \mu\text{M}$)²⁶. To understand the interaction of the RSNO, SNAP, on thrombin-induced fibrin formation, the *in vitro* anti-thrombin assay was performed. As shown in Figure 2A, the non-nitrosylated form of SNAP, NAP, had no effect on the fibrin formation mediated by thrombin up to $1000 \mu\text{M}$. The RSNO, SNAP, did significantly reduce the thrombin-induced fibrin formation in a concentration-dependent manner between 30 and $300 \mu\text{M}$. SNP did significantly reduce fibrin formation at 300 and $1000 \mu\text{M}$ while SNAP reversed its reduction on fibrin formation at $1000 \mu\text{M}$ possibly due to a smaller effect or possible autofluorescence at this higher SNAP concentration. NO gas at 100 or 1000 ppm was bubbled through a fibrinogen solution for 1 h at 37°C and resulted in complete inhibition of the thrombin-induced fibrin formation (data not shown). Additionally, nitrogen gas was bubbled in a fibrinogen solution and it too inhibited the fibrin formation. The next step was to infuse the 100 or 1000 ppm NO gas through a silicone tube submerged in a fibrinogen solution (i.e., infused) and was now found not to inhibit the fibrin formation induced by thrombin (Figure 2B).

To rule out any direct effects of SNAP, NAP and SNP on thrombin activity, a chromogenic substrate that mimics fibrinogen is reacted with thrombin in an *in vitro* anti-thrombin assay. The RSNOs and SNP all had no effect on the conversion of the chromogenic substrate into an UV measurable product at 405 nm and 37°C over 1 h (Table 1).

3.2. Effects of SNAP, NAP, SNP and NO gas on intrinsic tryptophan fluorescence in fibrinogen.

Intrinsic tryptophan fluorescence can provide a structural and functional assessment of compound interactions with proteins especially proteins rich in aromatic rich domains, such as fibrinogen⁸⁹. Tryptophan residues are part of these aromatic rich regions and fluoresce around 350 nm when it is excited at 295 nm . Quenching of the peak fluorescence of fibrinogen at 350 nm by compounds such as RSNOs can be indicative of alterations in the conformational state. SNAP showed a marked reduction in peak fluorescence dose-dependently at 300 and $1000 \mu\text{M}$ (Figure 3A). The non-nitrosated form of SNAP, NAP, had no effect on the 350 nm -measured fluorescence. Interestingly, the non-RSNO, SNP, also did dose-dependently (300 and $1000 \mu\text{M}$) reduce the fluorescence of fibrinogen.

To ascertain if the reduction in the peak intrinsic tryptophan fluorescence correlated with the concentration of the RSNO, the data for SNAP, NAP and SNP were fitted to a Stern-Volmer equation as described above in the Methods section. As shown in Figure 3B both SNAP and SNP showed a strong linear relationship between peak fluorescence and RSNO concentration (SNAP $R^2 = 0.989$; SNP $R^2 = 0.995$, respectively). NAP, the non-nitrosated form of SNAP, had a significantly less correlation ($R^2 = 0.812$) than either SNAP or SNP.

Shown in Figure 3C, the resulting ITF of 100 or 1000 ppm NO gas bubbled or infused in a fibrinogen solution was obtained. The bubbled gas into the solutions resulted in significant quenching of the intrinsic tryptophan fluorescence of fibrinogen over the wavelengths of 300 to 460 nm. However, when 100 or 1000 ppm NO gas was infused through silicone tube which was in the fibrinogen solution, these NO gas infusions showed no effect on the ITF of fibrinogen.

3.3. Effects of SNAP, NAP and SNP on Fibrinogen Conformation Measured by HPLC.

In the early stages of fibrin polymerization, conformational changes in fibrinogen structure via equilibria between thiols and disulfide bonds have been observed and found to inhibit thrombin-catalyzed fibrinogen polymerization²¹. To confirm that NO binding to tryptophan as shown in the intrinsic tryptophan fluorescence caused fibrinogen structure changes, HPLC analysis of RSNO-mediated structural changes (i.e., molecular weight and/or hydrophilicity/hydrophobicity changes) were done on the 1 h incubated samples. Using a Xbridge BEH C4 column on a Waters Alliance system, protein standards of fibrinogen and thrombin as well as the RSNOs, SNAP, NAP and non-RSNO, SNP were analyzed (Figures 4A and B). The retention time on the column is mainly determined by the molecular weight and/or the hydrophilicity of the compounds. Thrombin and fibrinogen had retention times of 25.143 and 26.901 min, respectively. SNAP had a retention time of 5.332 min while NAP, the non-nitrosylated form of SNAP, had a time of 2.964 min. The lowest molecular weight compound, SNP, also had the shortest column retention time which was 1.726 min. With these times in mind, it was questioned how RSNOs affected the retention time of fibrinogen.

As shown in Figure 5A, the representative chromatographs of SNAP, NAP and SNP effects on fibrinogen retention time are illustrated. SNAP was found to markedly shorten the fibrinogen retention time compared to its standard time (26.56 ± 0.10 min vs 27.01 ± 0.06 min, respectively). Quantification of the retention times of the effects of SNAP, NAP and SNP on fibrinogen retention times is shown in Figure 5B. SNAP at 1000 μ M significantly reduced the fibrinogen retention time while NAP and SNP had no effect on its retention time.

3.4. Effects of SNAP, NAP and SNP on Fibrinogen Conformation Measured by Western Blotting.

To confirm the fibrinogen structural changes that was observed via HPLC, the changes in the amount of A γ heterodimer was determined by Western immunoblot assay. Briefly, in the biosynthesis of fibrinogen, the formation of A γ heterodimers linked by disulfide bonds are suggested to be intermediates in the assembly of fibrinogen⁸⁰. Previous work has shown that conformational changes in fibrinogen structure via the change in formation of these A γ

heterodimers due to exposure to SNAP have been observed and found to inhibit thrombin-catalyzed fibrinogen polymerization²¹. Figure 6A shows the representative Western blots of the A α , B β , γ and A γ heterodimers in samples from RSNOs, SNAP, NAP and the non-RSNO, SNP-incubated fibrinogen solutions in absence and presence of thrombin for 1 h. When samples from RSNOs, SNAP, NAP and the non-RSNO, SNP-incubated fibrinogen solutions were run on a reduced SDS-PAGE gel, the percent level of the A γ dimer to total A γ dimer + γ monomer was significantly reduced in the case of the SNAP group when compared to SNP group (Figure 6B). In addition, the NAP group also reduced the percent level of the A γ dimer to total A γ dimer + γ monomer compared to SNP. SNAP releasing NO appeared to alter the A γ heterodimer formation which is consistent with the HPLC results which showed a change in fibrinogen structure.

4. Discussion

Altered structural conformation of the plasma protein, fibrinogen, has been reported to occur through a S-nitrosylation mechanism as one of several posttranslational modifications^{21, 42, 70}. Nitric oxide (NO) is an endogenous free radical gas produced and released from vascular endothelium cells and by binding to endogenous S-nitrosothiols (eg., glutathione, albumin and other cysteine containing proteins) much of its biological activity is extended over its typical half-life of seconds^{94–101}. NO released from S-nitrosothiols (RSNO) such as S-nitrosoglutathione (GSNO), S-nitrosoalbumin or the synthetic RSNO, S-nitroso-acetylpenicillamine (SNAP) can modify cysteine-containing proteins like fibrinogen and alter its ability to mediate thrombosis^{102, 103}. Previous work has shown that chemical affinities exist between various RSNOs and that their effects on thrombosis and in particular platelet aggregation can be selective^{46, 55, 58, 104–106}. SNAP was less selective in inhibiting platelet aggregation than GSNO but SNAP is more effective in releasing NO than GSNO due its dependence solely on presence of metal ions while NO release from GSNO requires an enzymatic interaction⁵⁵. However, the process in which NO bound to fibrinogen can alter platelet aggregation such as changing the fibrinogen structure is unclear, but in this paper several methods have been used to determine if fibrinogen conformational changes do occur when SNAP is present. Our previous work has reported that, even though overall platelet number was preserved, functional platelet aggregation was significantly inhibited with exogenous SNAP addition after the 4 h of blood exposure in a rabbit model of thrombogenicity¹⁰⁷. If platelet aggregation was inhibited but platelets still responded normally to exogenous stimuli such as collagen by increasing the glycoprotein, P-selectin, then it was hypothesized that structural changes in plasma fibrinogen, and not platelet inhibition, occurred due to the NO released from SNAP and caused the reduction in platelet aggregation. It was further shown that the fibrinogen structural alterations included the reduction in the heterodimer formation of A γ which is integral to fibrin polymerization. However, confirmation of the RSNO influence on fibrinogen structure still had to be elucidated by asking questions such as do all RSNOs or any NO donor cause changes in fibrinogen monomer polymerization.

As presented here, exogenous SNAP induce structural changes in fibrinogen that significantly reduce the enzymatic activity of thrombin-induced fibrinogen polymerization. We have provided evidence that supports our previous work²⁶ where a change in plasma

fibrinogen structure could prevent the functional aggregation of activated platelets. The evidence shows that the fibrinogen structure is altered if the SNAP concentration is 300 to 1000 μM in the presence of thrombin and this concentration matches the SNAP concentration (405 μM) that was observed in our previous work ²⁶. Intrinsic tryptophan fluorescence, HPLC and Western blotting all showed that alterations in fibrinogen conformation, in the most part, occurred in a concentration-dependent manner with exposure to SNAP and, to a small extent, SNP but not to the non-nitrosylated form of SNAP, NAP. These alterations were observed via three different methodologies. All three assays provided evidence that fibrinogen was structural changed that reduced its ability to form fibrin after thrombin interaction. Intrinsic tryptophan fluorescence (ITF) has been shown to indicate changes in protein tertiary structure ⁹². Changes in ITF can result in either an increase or decrease (i.e., quenching) of the tryptophan fluorescence within a protein. It was observed that the ITF in the current study was decreased significantly by both SNAP and SNP indicating that the released NO quenched the ITF of fibrinogen after 1 h exposure. The direct mechanism of this quenching by NO still needs proven but these results indicate that NO is binding to the cleaved disulfide bridges in fibrinogen and modifies the fluorescence of the intrinsic tryptophan molecules in the region of the previous disulfide bridges. Ishida et al. ¹⁰⁸ showed that oxidation of the tryptophan molecules in the D domains (i.e., the C terminus of the Aa monomer) by H_2O_2 resulted in a reduction in fibrinogen polymerization after thrombin exposure and a subsequent quenching of the ITF of fibrinogen ⁸¹. Based on this finding, it is reasonable to conclude that transnitrosation of the cleaved disulfides around the tryptophan molecules in the fibrinogen C terminus of the Aa monomer would also quench the ITF and result in reduce fibrin formation. Why SNP also had this strong quenching effect on fibrinogen ITF. SNP does release NO similar to SNAP, however, SNP is not a thiol and has not been shown to possess transnitrosating activity. This hypothesis for SNP on fibrinogen ITF needs further investigation.

HPLC evaluation of the fibrinogen structural changes was another assay used to demonstrate these changes. Reverse-phase HPLC has shown that fibrinogen in our study had a retention time on a C4 BEH HPLC column on average of 27 min. After 1 h exposure to SNAP and not to SNP, the retention time for fibrinogen decreased to around 26.52 min. Both an increase in molecular weight and hydrophilicity would decrease the time fibrinogen is retained on the column. Previous work has shown that fibrinogen oxidized by Fe^{+3} /ascorbate had an increased normal-phase HPLC retention time compared to native fibrinogen on a size exclusion column which has the larger proteins retained for a shorter time on column than smaller proteins. Oxidized fibrinogen was found to have longer retention time due to two small protein peaks while native fibrinogen remained as one peak ¹⁰⁹. In comparison with our results, the decreased retention time with the SNAP-exposed fibrinogen would match the longer retention time in a SEC HPLC column due to the reverse-phase versus normal-phase and the hydrophilicity/hydrophobicity interaction of the fibrinogen with the BEH column matrix. Since the leftward shift in the HPLC chromatograph was observed only with SNAP-exposed fibrinogen, it is concluded that SNAP can transnitrosate the sulfhydryls in fibrinogen while the non-RSNO, SNP, is not able to transfer its NO to fibrinogen. Further work is planned to investigate the transnitrosation of fibrinogen through SNAP or other RSNOs with HPLC methodology.

Another aspect of the RSNO effects on fibrinogen and its monomers was shown in the present study to decrease the interaction of the monomers to form dimers after thrombin exposure and visualized through Western blotting. As mentioned before, fibrinogen conformation consists of A α , B β and γ monomeric subunits¹¹⁰. As shown in Figure 1 thrombin catalyzes the fibrinopeptides A and B from the A α and B β subunits, respectively, and initiates the polymerization of the fibrinogen monomers into dimers and then into the final fibrin biopolymer. However, after 1 h of SNAP incubation, the formation of final fibrin was significantly reduced indicating an alteration in the dimer and/or final fibrin conformation. Our previous results showed a marked reduction in platelet aggregation and plasma fibrinogen A α γ dimer assembly which all have a strong dependency on the level of SNAP present²⁶. The current results also show that at 1000 μ M SNAP, but not NAP, showed a significant reduction in the fibrinogen A α γ dimer assembly. Since NAP did not show this reduction, transnitrosation of the fibrinogen monomers was hypothesized to be the possible mechanism of the dysfunction of fibrinogen dimer assembly. Geer et al. showed similar effects of the endogenous thiol, glutathione, on fibrinogen dimer assembly by altering the A α monomer structure via the removal of the C-termini of the α -chains which interact with the γ -chains⁷⁰. It was found that the C-termini of the A α monomer was the region of the γ -chain binding to form the dimer protofibril precursor to fibrin. Based on this and our results NO possibly transnitrosates the C-termini of the A α monomer and reduces the binding of the γ -chain and ultimately reduction of fibrin formation upon thrombin induction. It is hypothesized that NO is transnitrosated from SNAP to cysteines in the γ -chain binding region of the A α monomer in fibrinogen which explains our attenuated fibrin formation in response to thrombin stimulation. Further molecular studies are planned to verify this proposed mechanism of the A α γ dimer assembly.

S-nitrosothiols, biologic molecules that are sulfhydryl-containing plasma and cellular proteins that have reacted with and bound nitrosonium ions and have the ability to transnitrosate cysteine residues within a broad functional spectrum of proteins^{46, 111}. The posttranslational modifications and functional regulation of both plasma and intracellular proteins is mediated through the *S*-nitrosylation mechanism⁵⁶. However, another posttranslational modification and functional regulation of proteins such as fibrinogen can be mediated through oxidative processes^{81, 112, 113}. The interrelationship between transnitrosation and oxidation posttranslational processes on fibrinogen is seen most in the interaction of fibrinogen with oxidative agent, peroxynitrite, which is formed from the reaction of NO and O₂⁻ radicals¹¹⁴. As was seen in our Western results where SNAP reduced the A α γ dimer assembly in the beginning polymerization to form fibrin, Nowak et al.¹¹² observed supporting evidence that oxidation of fibrinogen by peroxynitrite also reduced A α γ dimer assembly. In the oxidative modification of fibrinogen, NO still plays a role in this modification by interacting indirectly through the effects of peroxynitrite. Nitrosation of tyrosine residues was also observed with peroxynitrite interaction with fibrinogen¹¹². Our reduced A α γ dimer assembly results may support Nowak et al. in that SNAP may induce peroxynitrite formation which could alter fibrinogen structure so that fibrin polymerization is reduced. This concept of a role of peroxynitrite in oxidative modifications of the proteins such as fibrinogen has been recently confirmed²⁴.

5. Conclusions

This study has demonstrated that the RSNO, SNAP, but not its non-nitrated form, NAP, concentration-dependently reduced the ability of thrombin to form fibrin. Further, the NO donor, SNP, which binds NO in a non-thiol fashion, had minimal attenuation of fibrin formation compared to SNAP. The reduction in the thrombin-induced fibrin formation via RSNOs has been shown to be mediated by changes in fibrinogen structure through attenuation of the intrinsic tryptophan fluorescence, HPLC retention time shifts and the reduction in the A α - γ dimer assembly as shown with Western blotting. Studies investigating improvements of the SNAP polymer coatings for ECLS must make determinations of the 'proper' concentration of any RSNOs that contact plasma fibrinogen that avoid any of the potential fibrinogen structure alterations that can occur at higher concentrations. The hypothesized posttranslational modification of fibrinogen by the S-nitrosylated mechanism mediated by SNAP described here has been confirmed by these results and provide a potentially new regulation of hemostatic processes.

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Figure 1. Fibrinogen Structure and Fibrin Formation. (A) Schematic of human fibrinogen showing the three main subunits, alpha, beta and gamma. Also shown is the position of the fibrinopeptides A and B on fibrinogen which are cleaved by thrombin. (B) Schematic of fibrin formation after initiation by thrombin. A working hypothesis is that *S*-nitrosothiols (RSNO) negatively affect the formation of fibrin through structural changes in fibrinogen and not inhibiting thrombin activity.

Figure 2.

(A) Effects of S-nitroso-acetyl Penicillamine (SNAP), N-acetyl Penicillamine (NAP), Sodium Nitroprusside (SNP), S-nitroso-glutathione (GSNO) and glutathione (GSH) on Thrombin-induced Fibrin Formation. An ultraviolet (UV) fluorescence kinetic assay in a 96-well format that used 75.5 $\mu\text{g}/\text{ml}$ human fibrinogen in presence of 0.03 IU/ml bovine thrombin measuring fibrin formation was obtained over 1 h at 37° C. The effect of RSNOs on this formation was evaluated in a concentration-response manner between 0–1000 μM . The data are means $\pm$ SEM (n = 9–11). * = $p < 0.05$, SNAP vs NAP; # = $p < 0.05$, SNAP vs SNP; \$ = $p < 0.05$, NAP vs SNP. (B) Effects of 100 ppm and 1000 ppm nitric oxide (NO) on Thrombin-induced Fibrin Formation. Assay was same as described in (A) above but instead of RSNO incubated for 1 h the NO gas was infused, not bubbled, through a fibrinogen solution via a silicone tube at 37°C. The data are means $\pm$ SEM (n = 6–7). * = $p < 0.05$, 100 ppm NO gas vs 1000 ppm NO gas.

Figure 3.

(A) The effects of SNAP, NAP and SNP on Intrinsic Tryptophan Fluorescence within Fibrinogen. The emission spectra of fibrinogen solutions between 300 and 460 nm was monitored as a function of 300 or 1000 μM of the various RSNOs. Excitation wavelength was 295 nm. SNAP= green lines; NAP= yellow lines; SNP= red lines. (B) Stern-Volmer plots of fibrinogen fluorescence in the absence and presence of RSNOs. Quenching of intrinsic tryptophan fluorescence at the given concentrations of the various RSNOs was analyzed using the equation, $I_0/I - 1$, where I_0 is the fibrinogen fluorescence intensity in absence of RSNOs and I is the fluorescence intensity in presence of RSNOs⁹⁰. The data are means $\pm$ SEM ($n = 3$). Regression correlations were found to give $R^2=0.9885$, 0.8118 and 0.9951 for SNAP, NAP and SNP, respectively. (C) Effects of 100 ppm and 1000 ppm nitric oxide (NO) on Intrinsic Tryptophan Fluorescence within Fibrinogen. The emission spectra of fibrinogen solutions between 300 and 460 nm was monitored as a function of 100 or 1000 ppm NO gas infused or bubbled into the fibrinogen solution. Excitation wavelength was 295 nm. Fibrinogen control= blue line; 1000 ppm NO gas (not bubbled but infused) = yellow lines; 100 ppm NO gas (bubbled)= red line; 1000 ppm NO gas (bubbled)= green line. Both bubbled fibrinogen solutions showed significant quenching of the intrinsic tryptophan fluorescence^{85, 86, 115}.

Figure 4.

HPLC Elution Patterns of Proteins involved with Fibrin Formation as well as Various RSNOs. (A) Retention times for 0.03 IU/ml thrombin and 75.5 $\mu\text{g/ml}$ fibrinogen. (B) Retention times of SNAP, NAP and SNP. () show times in minutes. Each RSNO (100 μl) was added to 52.1 μl Hank's balanced salt solution (HBSS) in a concentration-dependent manner (0–1000 μM). Human fibrinogen (58.8 μl of 301.9 $\mu\text{g/ml}$ = final 75.5 $\mu\text{g/ml}$) was added and then incubated at 37° C for 1 h. After incubation 42.9 μl of 5% phosphoric acid/71.4% acetonitrile/water solution was added to each well and then 100 μl aliquot was transferred to HPLC vials for analysis. A 50 μl sample was injected on a Xbridge BEH C4 column (300A, 3.5 μm , 4.6 mm x 100 mm) and the proteins were separated using a gradient flow over 1 h at 40° C. The mobile phase A was water + 0.1% trifluoroacetic acid (TFA) and B 0.075% TFA in 71.4% acetonitrile and water. The proteins were detected at 220 nm.

Figure 5.

HPLC Elution Patterns of fibrinogen and Retention Times of SNAP, NAP and SNP. (A) Representative chromatographs showing retention times for 75.5 µg/ml fibrinogen after 1 h incubation at 37° C in presence of 1000 µM of each RSNO. Note the leftward shift in the fibrinogen peak with SNAP compared to the fibrinogen peak with NAP, SNP or fibrinogen alone. (B) Retention times of SNAP, NAP and SNP. The data are means ± SEM (n = 3–7). * = p < 0.05, SNAP vs NAP and SNP. Each RSNO (100 µl) was added to 52.1 µl Hank's balanced salt solution (HBSS) in a concentration-dependent manner (0–1000 µM). Human fibrinogen (58.8 µl of 301.9 µg/ml = final 75.5 µg/ml) was added and then incubated at 37° C for 1 h. After incubation 42.9 µl of 5% phosphoric acid/71.4% acetonitrile/water solution was added to each well and then 100 µl aliquot was transferred to HPLC vials for analysis. A 50 µl sample was injected and proteins were separated on a Xbridge BEH C4 column, 300A, 3.5µm, 4.6 mm x 100 mm using a gradient flow over 1 h at 40° C. The mobile phase A was

water + 0.1% trifluoroacetic acid (TFA) and B 0.075% TFA in 71.4% acetonitrile and water. The proteins were detected at 220 nm.

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Figure 6.

Western immunoblot analysis of fibrinogen subunits, Aα, Bβ and γ after 1 h exposure with SNAP, NAP and SNP in absence and presence of thrombin. (A), Representative immunoblots. Aliquots (15 μl) of samples were separated on 12% polyacrylamide gels, protein transferred to poly(vinyl-difluoride) (PVDF) membranes and then immunostained with mouse anti-human alpha fibrinogen, rabbit anti-human beta fibrinogen and goat anti-human gamma fibrinogen antibodies (all diluted 1:200). After membrane washings, the membranes were incubated with the secondary antibodies chicken anti-mouse-AF488, chicken anti-rabbit-AF647 and donkey anti-goat-AF555 (all diluted 1:500) for 1 h. After two 10 min washes the blots were imaged in a fluorescence imager (Bio-Rad VersaDoc MP4000 Hercules, CA). Molecular weight markers are shown on left with positions of the relevant fibrinogen chains and Aαγ dimers indicated on the right. (B) Western analysis of samples in (A), after the 1 h incubation, were quantified by densitometry using Quantity One imaging

software (Bio-Rad Hercules, CA). The specific A α γ dimer formation was calculated as the ratio (density) of the A α γ dimer band over the sum of dimers plus monomers. The data was collected after 1 h in presence of thrombin. The ratio data are means $\pm$ SEM (n=3). * = p<0.05, SNAP or NAP vs SNP.

TABLE 1*In Vitro* Antithrombin Assay to Rule Out Direct Thrombin Inhibition.

Treatment	Baseline (OD)	RSNO (μ M)						
		1	3	10	30	100	300	1000*
SNAP	0.159 $\pm$ 0.002	98.2 $\pm$ 0.6	99.1 $\pm$ 0.5	94.7 $\pm$ 2.1	97.4 $\pm$ 0.6	94.1 $\pm$ 3.2	93.6 $\pm$ 1.8	94.1 $\pm$ 0.9
NAP	0.162 $\pm$ 0.002	98.9 $\pm$ 0.5	100.3 $\pm$ 0.7	98.4 $\pm$ 0.1	97.3 $\pm$ 0.9	96.6 $\pm$ 2.1	100.1 $\pm$ 6.1	97.2 $\pm$ 1.3
SNP	0.115 $\pm$ 0.038	99.9 $\pm$ 0.0	98.5 $\pm$ 1.9	99.8 $\pm$ 0.5	99.1 $\pm$ 1.0	98.1 $\pm$ 2.2	99.1 $\pm$ 1.0	95.0 $\pm$ 1.6
100 ppm								
NO gas	0.164 $\pm$ 0.009	128.1 $\pm$ 8.5 (14)						

* Values are % of baseline

Values are means $\pm$ SEM; n size=3 except for NO gas where n size= 14.

The 96-well plate assay utilized the chromogenic substrate, N-p-tosyl-Gly-Pro-Arg-p-nitroanilide acetate, which is cleaved by 0.03 IU/ml thrombin into a residual peptide, Tosyl-Gly-Pro-Arg-OH and free p-nitroanilide. The level of free p-nitroanilide is measured at 405 nm in a microplate reader. NS at p<0.05 vs baseline; ANOVA with Student's *t* multirange test.