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Carbon Monoxide and Nitric Oxide Induced-Heme-Based Modification of Alpha-2-Antiplasmin and Plasmin Activity

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Abstract

CARBON MONOXIDE INDUCED/HEME-BASED MODIFICATION OF ALPHA-2-ANTIPLASMIN AND PLASMIN ACTIVITY

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Objectives: Carbon monoxide (CO) derived from cigarette smoke or released from carbon monoxide releasing-molecule 2 (CORM-2), diminishes fibrinolysis. The primary purpose of this study was to determine if CO diminished fibrinolysis by means of enhancing α_2-antiplasmin via an alleged heme group.

Methods: Plasma, isolated α_2-antiplasmin and isolated plasmin were exposed to CO released from CORM-2 (tricarbonyldichlororuthenium (II) dimer) and nitric oxide (NO) via a NO donor to induce carboxyheme and metheme states. Exposed, isolated enzymes were placed in either α_2-antiplasmin deficient or normal plasma. Effects of CO and NO on tissue-type plasminogen activator initiated fibrinolysis were determined by thrombelastography. Liquid chromatography-mass spectrometry (LC-MS/MS, see Table 1) was used to identify heme released from α_2-antiplasmin and plasmin.

Results: CO significantly enhanced α_2-antiplasmin activity but decreased plasmin activity. NO decreased both α_2-antiplasmin and plasmin activity. While

insufficient LC-MS/MS data was obtained with α_2-antiplasmin (secondary to glycosylation), a putative plasmin-associated heme was identified.

Conclusion: CO causes hypofibrinolysis by enhancing α_2-antiplasmin activity and decreasing plasmin activity. Based on responses to NO and LC-MS/MS data, it is highly likely that both enzymes are modulated by attached heme groups. Attempts to develop methods to detect CO-mediated hypercoagulability are ongoing, with the goal of identifying populations at risk of thrombotic morbidity secondary to cigarette smoking.

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Chapter 1: Introduction

The health problems associated with cigarette smoking have been thoroughly researched and are well known. Cigarette smoking afflicts nearly every organ in the human body and decreases the overall general health of smokers (1). Adverse health effects of cigarette smoking account for an estimated 443,000 deaths, or nearly 1 in 5 deaths in the U.S. each year (2, 3). The yearly death count caused by tobacco use is more than all deaths from human immunodeficiency virus (HIV), illegal drug use, alcohol use, motor vehicles injuries, suicides and murders combined (3). A smoker's risk factors are increased 2 to 4 times for coronary heart disease and stroke, 23 times for men in developing lung cancer, 13 times for women in developing lung cancer and 12 to 13 times for dying from chronic obstructive lung diseases compared with nonsmokers (1).

In the U.S., cigarettes are regulated to 599 additives, carbon monoxide (CO) being an important poisonous component of tobacco smoke (4)**.** Carbon monoxide inhalation while smoking results in increased levels of carboxyhemoglobin (COHb, see Table 1), causing decreased oxygen delivery to cells (5-7). This occurs because carbon monoxide competes with oxygen (O_2) in binding with hemoglobin. Excess quantities of CO impair oxygen delivery to cells because hemoglobin preferentially binds CO compared to O_2 at a ratio of approximately 240:1 (8). Tobacco smoke inhalation has been linked to numerous diseases, an

important one being thrombotic disease (9-11). Studies have demonstrated that CO influences the hemostatic system causing increased coagulation and decreased fibrinolysis (see Table 1) (12-21). Tobacco smoke, which contains CO, can be considered a significant risk factor for the pathogenesis of thrombotic diseases (9-11).

Normal hemostasis is a phenomenon caused by a tightly controlled process to (i) maintain blood in a fluid state in normal vessels, (ii) provide a means of rapidly forming a hemostatic clot at a site of vascular injury, and then (iii) restore normal flow through fibrinolytic processes (22, figure 1). Thrombosis is the formation of a blood clot in intact vessels, involving three components: reactions at the vascular wall, the actions of platelets, and the coagulation cascade (22).

At a site of vascular injury, the vascular wall vasoconstricts, primarily due to the molecule endothelin. Next platelets are activated and adhere to the site of vascular injury. Platelets aggregate together to form a hemostatic plug at the site of injury. This process is known as primary hemostasis. Circulating Tissue Factor (TF, see Table 1) is exposed to the site of injury and along with FVII, is the primary initiator of the coagulation cascade. A chain-reaction occurs of various factors activating the next one sequentially until thrombin (see Table 1) is generated and cleaves fibrinogen to produce the insoluble fibrin (see Table 1) (22). A fibrin meshwork is created which recruits more platelets and induces cross-linking via FXIII to create a stronger plug. The production of this fibrin meshwork plug is known as secondary hemostasis, eventually forming a more permanent plug (22). This more permanent plug prevents further hemorrhage at

the site of vascular injury. To restore the balance of hemostasis, vascular repair occurs and tissue plasminogen activator (tPA, see Table 1) activates plasmin (see Table 1), the primary enzyme of fibrinolysis. Plasmin cleaves the fibrin meshwork thus breaking down the more permanent plug. An important fibrinolytic regulatory enzyme which inhibits the activity of plasmin is α_2-antiplasmin (see Table 1). For a detailed explanation of the sequential events of the coagulation cascade as well as the counter-regulatory actions of the fibrinolytic cascade see figure 1.

While the mechanisms and sequential effects of the coagulation cascade are well established, the complete structural and mechanistic biochemistry of the many molecules participating in the coagulation cascade remain to be discovered.

Previous studies by Dr. Vance Nielsen's research group used the carbon monoxide releasing molecule-2 (CORM-2; tricarbonyldichlororuthenium (II) dimer, Figure 2) to deliver CO to plasma-based systems and study the effect of CO on coagulation and fibrinolysis (12-21). The degradation of heme (see Table 1) by heme oxygenase produces CO, as does cigarette smoking. Carbon monoxide can also be produced pharmacologically by carbon monoxide releasing molecules (CORMs). Studies have established that CORMs have the ability to cause vasodilation, decrease inflammation, ease ischemia-reperfusion injury, diminish apoptosis and prevent arterial thrombosis (12, 23-27). CORMs are metal carbonyls containing CO bound to a variety of heavy metals such as iron, cobalt, nickel and ruthenium (25, 26). The release of CO from these metals can be modulated by photoactivation, temperature, pH and the nature of the ambient

environment (e.g. organic solvent or physiological aqueous solution) (25, 26). The CO released from CORMs has been viewed as a modulator or cell signaling molecule, comparable to nitric oxide (NO) in its effects of the above mentioned circumstances (23-27).

Dr. Nielsen discovered that CO was found to enhance the speed of clot growth and final clot strength via the modification of fibrinogen, the substrate of thrombin. He found that CO also decreased tPA-induced fibrinolysis (13). Diminished fibrinolysis was found to be accomplished by CO increasing the effect of the antifibrinolytic serine protease inhibitor; α_2-antiplasmin (Figure 3), suggesting that the effect of CO on fibrinolysis is α_2-antiplasmin-dependent (13, 18, 28).

Similar results through independent studies have used blood obtained from smokers, documented via thrombelastography (see Table 1). These studies showed enhanced clot strength, antifibrinolytic responses to tPA-mediated lysis, and denser matrix of thin fibrin polymers (13, 29). These studies led to the conclusion that CO affects the coagulation cascade by causing faster-growing, stronger thrombus clots which take longer to lyse, and result in a hypercoagulable state.

The optimal determined concentration of CO used in these studies was 75μM, which in turn is expected to raise whole blood COHb concentration <0.5% (12-21). This is rather significant data, since smokers using a half-pack to two-pack per day have COHb concentrations of 3-7% (5), compared to smoke inhalation victims with CO concentrations around 7-20% (1000-3000μM) (30). These

studies indicate that chronic smokers and smoke inhalation victims are exposed to CO concentrations significantly higher than those studied and shown to cause increased coagulation and decreased fibrinolysis (5-7, 12-21, 30, 31). This may strengthen the theory of a hypercoagulable/hypofibrinolytic state associated with smoking.

There are many scenarios in which the hypercoagulable/hypofibrinolytic effects of CO released by CORMs may potentially be utilized both clinically and therapeutically. In preclinical rat and rabbit plasma, CORM-2 has been shown to display procoagulant and antifibrinolytic effects, with response in rabbit plasma being most similar to that of humans (32). CORM-2 also has the same procoagulant/antifibrinolytic effects in the plasma of chronically anticoagulated patients on Warfarin (see Table 1), thus indicating a possible therapeutic treatment for bleeding patients at risk for thrombosis if their anticoagulation was reversed significantly with blood products or Recombinant Activated Factor VII (see Table 1) (i.e. mechanical heart valve or left ventricular device) (17). CORM-2 shows promise in hemophiliac human plasma as well as human plasma diluted with resuscitative fluids (i.e. trauma-associated bleeding) (14, 19). There are also studies showing the efficacy of CORM-2 as a procoagulant/antifibrinolytic in plasma exposed to heparin or Argatroban (see Table 1), hypothermic conditions, and in the attenuation of Protamine-mediated hypocoagulation/hyperfibrinolysis (see Table 1) (20).

While the effects of CORM-2-released CO on human plasma are known to induce coagulation and attenuate fibrinolysis, the exact mechanism of action is

unknown. An insight into the structural changes of fibrinogen caused by CORM-2-released CO occurred when it was discovered that CO causes a decrease in thick diameter fiber formation and an increase in the production of "stronger" thin diameter fibrin fibers (15). Interestingly a separate and unrelated study using thrombelastography and electron microscopy to study whole blood of smokers revealed similar results; smokers' blood had increased clot strength and produced significantly denser thin fibrin clot fibers than nonsmokers' blood (33). However, the mechanism by which CO directly modifies fibrinogen was still undetermined.

An investigation into CO modification of fibrinogen was then completed using liquid-chromatography-mass spectroscopy (LC-MS). Previous studies examined the modification of enzymatic function, using the mechanism of O_2, NO, or CO binding to a heme molecule attached to a protein of interest. From these studies, it was proposed that a heme group could be associated with fibrinogen. LC-MS found no direct modifications of the amino acids in fibrinogen, but small portions of both the α and γ chains could not be detected following exposure to CORM-2 and endoproteinase digestions with trypsin and Glu-C. Interestingly, an ion with the same mass-to-charge ratio (m/z) and retention time as heme was found in purified fibrinogen. Heme has a molecular weight of 616.18 in the reduced Fe^{2+} state. During mass spectrometry, heme binding proteins are known to liberate free heme which can be detected at m/z 617.18 in the $^{+1}$ charge state. The molecular ion identified by mass spectrometry of a purified fibrinogen sample had an m/z of 617.18 identical to that of heme.

In addition, exposure of plasma to NO by a NO-donating molecule (sodium nitroprusside) and a heme oxidizing agent (hydroquinone) together decreased CORM-2 mediated enhancement of coagulation, as compared to the coagulation kinetics of plasma not exposed to CORM-2. The combination of sodium nitroprusside and hydroquinone decreased CORM-2 mediated increases in the speed of clot growth (by 40%) and strength (by 50%).

The result of these preliminary two studies was that CO derived from CORM-2 likely modifies fibrinogen via modulation of a heme group(s) associated with fibrinogen. Hence the conclusion of these studies is that fibrinogen is a CO-sensing molecule (34).

The proposed mechanism was discovered in which CORM-2 modulates fibrinogen with respect to CO-mediated hypercoagulation, thus describing half of the observed effects of CORM-2 on hemostasis. Fibrinogen affects coagulation, and CO-modulated fibrinogen forms a faster growing and stronger clot. However, fibrinogen *per se* doesn't necessarily increase the duration of a formed clot or decrease its resistance to fibrinolysis. This led to investigations into the hypofibrinolytic effects of CORM-2, which hypothesized that CORM-2 affects fibrinolysis either by promoting an inhibitory regulatory fibrinolytic protein, or by inhibiting a fibrinolytic promoter. A study was conducted studying the effects of CORM-2 on the inhibitory anti-fibrinolytic enzymes: Plasminogen Activating Inhibitory-1 (PAI-1), Thrombin-Activable Fibrinolysis Inhibitor (TAFI), and α_2-antiplasmin. This study concluded that clot formation and disintegration kinetics were more dependent on α_2-antiplasmin activity than any other antifibrinolytic

enzyme tested (13). Deducing that α_2-antiplasmin is the primary enzyme induced by CO to produce hypofibrinolysis, the next question to be answered is whether α_2-antiplasmin is modulated in a similar heme-dependent manner like fibrinogen, or by an entirely new mechanism.

The objective of the present study was to mechanistically assess CO and NO-induced-heme-based modification of α_2-antiplasmin mediated hypofibrinolysis. It is hypothesized that CO-mediated modification of α_2-antiplasmin may play a major role in acquired hypofibrinolysis.

In order to determine the most efficient way of creating experiments to test the hypothesis, the following preliminary studies were performed:

α2-antiplasmin is CO-modulated enzyme

Purified α_2-antiplasmin was exposed to 0 or 100μM CORM-2 and then diluted into α_2-antiplasmin deficient plasma. CORM-2 was diluted to 10-fold less than the concentrations associated with no detectable response, in order for there to be no fibrinogen-associated enhancement of clot growth or strength. Purified α_2-antiplasmin exposed to CORM-2 resulted in a 42% increase in the time to maximum speed of lysis, a 31% decrease in the maximum speed of lysis, and a 39% prolongation in clot lysis time. The results suggested that α_2-antiplasmin is a CO-sensing molecule; further experiments were proposed to determine CO and/or

heme's influence on α_2-antiplasmin. The following preliminary studies were performed to confirm α_2-antiplasmin as a potential CO-sensing molecule.

1. Define CO and/or heme structural modification of α_2-antiplasmin

It is known that α_2-antiplasmin activity is enhanced by CO but the exact mechanism, possibly involving heme, remains to be identified. It has been discovered that CO modification independent of heme involved the K^+ channel Slo1-BK (35). Purified α_2-antiplasmin was treated with 0-100μM CORM-2. LC-MS profile, post-proteolytic digestion, was used to identify regions of modification which change proteolytic enzymatic access such as direct modification by CO and/or presence of heme and/or altered proteolytic pattern. The results indicated heme binding is present, so experiments were reproduced using α_2-antiplasmin to determine how it uses heme to detect CO. Histidines are established mediators of direct CO regulation of protein function (35). If the experiments did not identify heme, histidines in recombinant α_2-antiplasmin would have been mutated to both arginine and alanine and tested for loss of CO responsiveness in order to determine site-specific roles of CO enhancement of α_2-antiplasmin activity (35).

2. **Mechanistically assess CO-induced/heme-based modification of α_2-antiplasmin mediated hypofibrinolysis.**

Using the structural knowledge gained during the first study, a thrombelastographic-based coagulation/fibrinolysis assay array was created to identify CO-exposed, hypercoagulable plasma. This procedure could be used as a diagnostic tool to determine the role CO-exposure has on tobacco smoke-associated hypercoagulability. Normal plasma was obtained and subjected to thrombelastographic analyses to determine coagulation and fibrinolysis (13, 17-21).

3. **Heme Group Modulation Experiments**

The following method was used to modulate heme binding and/or CO to mechanistically show the CO sensing mechanism in CO-mediated hypofibrinolysis.

Normal plasma and purified α_2-antiplasmin were exposed to a NO donor in a cell culture dish to competitively block CO access to heme (36, 37). Plasma/proteins were exposed to 0-100μM CORM-2. With fluid thickness kept to 1-2mm, CO was released from heme, and replaced by NO, thus allowing CO to be rapidly lost to the atmosphere. The results determined that the CO-sensing mechanism of hypercoagulability is mediated through heme binding.

These preliminary studies provided insight into the CO-heme-α_2-antiplasmin interaction and its influence on fibrinolysis with or without exposure to CO. The preliminary studies provided guidance towards designing the experiments to test the hypothesis that α_2-antiplasmin is a heme-modulated-CO and NO-sensing molecule, in which CO-induced-heme-based-modification of α_2-antiplasmin creates a hypofibrinolytic system.

Chapter 2: Materials and Methods

Plasma, enzymes and reagents

Pooled normal plasma (George King BioMedical, Overland Park, KS, USA) anticoagulated with sodium citrate was used for experimentation. Plasma deficient in α_2-antiplasmin used in experimentation (Affinity Biologicals, Inc., Ancaster, Ontario, Canada) had a prothrombin time of 14.6 sec, an activated partial thromboplastin time of 33.4 sec, a fibrinogen concentration of 329 mg/dl and an α_2-antiplasmin activity of <1% of normal. Purified α_2-antiplasmin and plasmin (Enzyme Research Laboratories, South Bend, IN, USA) were maintained at -80^{o}C prior to experimentation. Purified tissue factor (TF), dissolved in dH_2O (Instrumentation Laboratory, Lexington, MA, USA) and tissue plasminogen activator (tPA) at 580 IU/µg (Genentech, Inc., San Francisco, CA, USA; 100 IU/ml final concentration) were maintained at 2-8^{o}C prior to experimentation. CORM-2, dimethyl sulfoxide (DMSO) and the oxidant O-phenylhydroxylamine (PHA, figure 4) were obtained from Sigma-Aldrich, St. Louis, MO, USA. The nitric oxide (NO) donor used in experimentation was (Z)-1-[N-Methyl-N-[6-(N-methylammoniohexyl) amino]] diazen-1-ium-1, 2-diolate (MAHMA NONOate, Cayman Chemicals, Ann Arbor, MI, USA, figure 5).

Evaluation of MAHMA NONOate and PHA as antifibrinolytic agents in pooled normal plasma

In a similar matter as the heme-mediated modulation of fibrinogen (28), conversion of the putative α_2-antiplasmin associated heme to a metheme (see Table 1) with nitric oxide (NO) or a well characterized, rapid heme-specific organic oxidant was attempted. The nitric oxide donor, MAHMA NONOate, has a half-life of 2.7 min at 22^{o}C at pH 7.4 and releases 2 moles of NO per mole of donor molecule. MAHMA NONOate is stable for 24h after suspension in 0.01M NaOH. PHA was chosen for this study as it is the most rapid and effective metheme forming oxidant known (38) and effectively converts fibrinogen-bound heme to a metheme state (unpublished data). PHA was dissolved in dH_2O on the day of experimentation. The sample composition (sequentially added in this order) consisted of 316 µl of plasma; 10 µl of TF (0.1% final concentration), 10 µl tPA (final activity 100 U/ml), either 3.6 µl of MAHMA NONOate (final concentration 0, 1 or 2mM) or PHA (final concentration 0, 5 or 10 mM), and 20 µl of 200 mM $CaCl_2$, for a final volume for this experimental series of 359.6µl. Eight replicate experiments were performed per condition tested.

Plasma sample mixtures were placed in a disposable cup in a computer-controlled thrombelastograph® hemostasis system (Model 5000, Haemoscope Corp., Niles, IL, USA), with addition of $CaCl_2$ as the last step to initiate clotting. Data were collected until clot lysis time occurred. The following variables were determined at

37^{o}C: **clot growth time** (CGT, time from clot amplitude of 2mm [102 dynes/cm^{2}] until maximum strength is achieved, in min), **clot lysis time** (CLT, time from when maximum strength was observed to 2mm amplitude, in min) and **clot lifespan** (CLS, the sum of CGT and CLT). The nomenclature used to describe these phenomena is as follows: **Time to maximum rate of thrombus generation (TMRTG):** The time interval (min) observed prior to maximum speed of clot growth. **Maximum rate of thrombus generation (MRTG):** The maximum velocity of clot growth observed (dynes/cm^{2}/sec). **Total Thrombus Generation (TTG):** The total area under the velocity curve clot growth (dynes/cm^{2}), representing the amount of clot strength generated during clot growth. **Time to maximum rate of lysis (TMRL):** The time interval (min) measured from the time of maximum strength to the time when the velocity of clot disintegration is maximal. **Maximum Rate of Lysis (MRL):** The maximum velocity of clot disintegration observed (-dynes/cm^{2}/sec). **Area of Clot Lysis (ACL):** The total area under the velocity curve during clot disintegration. As this model involves complete fibrinolysis, TTG is equivalent to ACL, so ACL was not presented as it provides no additional information. For a graphical representation of the Clot Lifespan Model, refer to figure 6.

Isolated exposure of α_2-antiplasmin to MAHMA NONOate and PHA

Purified α_2-antiplasmin (154 μg per condition) was diluted into 5% human albumin (CSL Behring LLC, Kankakee, IL, USA), to a concentration of 50 μg /ml, and suspended in Isolyte® a calcium-free electrolyte solution with pH 7.4 (B. Braun Medical Inc., Irvine, CA, USA). Each aliquot of diluted α_2-antiplasmin was exposed to 1% by volume of one of the following: DMSO, MAHMA NONOate (final concentration 2mM), PHA (final concentration 5mM) or CORM-2 (final concentration 100 μM). Solutions were incubated at 37^{o}C for 5 min and then left at room temperature during experimentation. The sample composition consisted of 288 μl of α_2-antiplasmin deficient plasma; 32 μl of exposed, diluted, purified α_2-antiplasmin (50 μg/ml final activity); 10 μl of TF (0.1% final concentration), 10μl tPA (final activity 100 U/ml), and 20 μl of 200 mM $CaCl_2$ for a final volume for this experimental series of 360 μl. Plasma sample mixtures were placed in a disposable cup in a computer-controlled thrombelastograph®. Data were collected until clot lysis time occurred. Six replicate experiments were performed per condition tested.

Spectrophotometric antiplasmin activity assay

A commercially available plasmin inhibitor kit (diaPharma Group, Inc., West Chester, OH, USA) was utilized. This kit allows spectrophotometric assessment of the ability of plasmin to cleave an artificial substrate with subsequent release of p-nitroaniline which is measured at 405 nm. Pooled, normal, platelet poor plasma (George King Bio-Medical) anticoagulated with sodium citrate was utilized for experimentation. Plasma was serially diluted to generate a standard curve with a range of 0-124% of normal as per the manufacturer's instructions. Additional plasma samples diluted 50% with the buffer provided in the kit were exposed to 2 mM MAHMA NONOate or 2 mM PHA, and eight replicate reactions for each condition were recorded. Results with a greater than 124% normal activity represent inhibition beyond the range of the standard curve.

Isolated exposure of plasmin to MAHMA NONOate and CORM-2

Purified plasmin was suspended into 5% human albumin/Isolyte® and exposed to 1% volume of either 0.01 M NaOH, MAHMA NONOate (final concentration 2 mM) or CORM-2 (final concentration 100 μM). Plasmin solutions were incubated at 37°C for 5 min and then left at room temperature during experimentation. The final

volume for this experimental series was 350 µl. The sample composition consisted, in this order, of 297 µl of pooled normal plasma; 33 µl of 5% human albumin/Isolyte® with or without vehicle/NO/CO, purified plasmin (30 µg/ml final activity); 10 µl of TF (0.1% final concentration), and 20µl of 200 mM $CaCl_2$. Plasma sample mixtures were placed in a disposable cup in a computer-controlled thrombelastograph®. Data were collected for 15 min. Eight replicate experiments per condition were performed.

LC-MS/MS detection of heme associated with α_2-antiplasmin and plasmin

Stock solution of α_2-antiplasmin (50 µg /ml) and plasmin (209 µg /ml) or 50 µg of BSA (Sigma-Aldrich) were digested in 50 mM ammonium bicarbonate buffer, pH 8.0, using sequence grade modified trypsin (Promega, Madison, WI, USA). Prior to digest, samples were reduced by 10 mM dithiothreitol (Sigma-Aldrich) for 1 hour at room temperature, and alkylated by 50 mM iodoacetamid (Sigma-Aldrich) for 1 hour at room temperature in the dark. Acidified digests (<pH 4) were desalted with reverse phase C18 Zip-Tips (Millipore, Billerica, MA, USA), dried, and re-suspended in 0.1% trifluoroacetic acid (TFA).

LC-MS/MS runs were completed using an Eksigent Nano LC-AS1 auto-sampler (Eksigent, Dublin, CA, USA) coupled with an LTQ linear ion trap mass spectrophotometer (Thermo Fisher Scientific, San Jose, CA, USA). Samples were injected onto an Integra Frit Proteopep II (New Objective, Wobum, MA, USA) C18

sample trap column (300 Å x 75 μm internal diameter x 2.5 cm bed length) at flow rate of 3 μl/min (0.1% TFA) for 15 minutes. The inline capillary column for reverse phase was an Integra Frit Proteopep II (New Objective) C18 column (300 Å x 75 μm internal diameter x 10 cm bed length). Sample was eluted from the trap column by a 35 min 2-38% linear gradient of buffer B (100% acentonitrile.1% TFA), followed by an 8 min 38-90% linear gradient of buffer B to a 10 min isocratic elution of 90% of buffer B at a flow rate of 300 nL/min. Collision energy of collision-induced dissociation was 35.0, and data acquisition was performed using a full MS scan, a zoom scan for the most intense ion, and MS/MS fragmentation. Centroid data was acquired except in case of zoom scan, where profile data was acquired. The electrospray voltage was 1.6 kV, positive polarity. The mass range for MS scans was 400-2,000 Da. Data was acquired using an isolation width of 2 m/z and activation time of 30 ms for MS and MS/MS scan types. Dynamic exclusion was set such that if an ion was chosen for MS/MS twice, it would be ignored for 45 seconds. Sequence data analysis to identify peptides and proteins was performed using Sequest in Bioworks (Thermo Fisher Scientific) software. Both a non-redundant FAST-ALL (FASTA) human database and a custom made sub-database for human α_2-antiplasmin and plasmin were interrogated. A cross-correlation (X-Cor) score of 2.5 or better for doubly charged and 3 for triply charged precursors were set as threshold for identifications. BSA digests were also analyzed to control for enzyme digest and mass spectrometer identification consistency. All peptides of interest were analyzed manually via MS/MS spectra.

After adequate identification of each enzyme, samples were queried to determine if heme was present in α_2-antiplasmin and plasmin with BSA as a negative control. Heme has a molecular weight of 616.18 in the reduced Fe^{+2} state. During mass spectrometry, heme binding proteins are known to liberate free heme (39) which can be detected at m/z 617.18 in the $^{+1}$ charge state (34, 39).

Statistical Analyses

Thrombelastographic data are presented as either median and 1^{st}-3^{rd} quartiles or mean ±SD as appropriate following analysis of normality and variance. Analyses of the effects of MAHMA NONOate and PHA thrombelastographic parameter values in normal pooled plasma were conducted with Kruskal-Wallis one way analysis of variance (ANOVA) with the Student-Newman-Keuls *post hoc* test (SigmaStat 3.1, Systat Software, Inc., San Jose, CA, USA). Analyses of the effects of MAHMA NONOate or PHA on α_2-antiplasmin were conducted with one way ANOVA with the Holm-Sidak *post hoc* test. A separate, confirmatory analysis of the effects of CORM-2 on isolated, purified α_2-antiplasmin was performed with an unpaired Student's t-test. Analyses of the effects of MAHMA NONOate and CORM-2 on plasmin activity were conducted with one way ANOVA with the Holm-Sidak *post hoc* test. Graphical representation of the data was generated with commercially available software (origin 7.5, OriginLab Corp., Northampton, MA, USA; and, CorelDRAW 12.0, Corel

Corporation, Ottawa, Ontario, Canada). A P value of <0.05 was considered significant.

Chapter 3: Results

Evaluation of MAHMA NONOate and PHA as antifibrinolytic agents in pooled normal plasma

MAHMA NONOate and PHA's antifibrinolytic effects on normal, pooled plasma are displayed in tables 2 and 3. In samples exposed to 1 mM MAHMA NONOate, there was a small but significant attenuation in coagulation kinetics, with a mild profibrinolytic effect seen by a decrease in TMRL. However at a concentration of 2 mM, coagulation kinetics were normal, with clear antifibrinolytic effect represented by significant increases in TMRL, CGT, CLT, and CLS. PHA at a 10 mM concentration had a significant attenuation in coagulation kinetics, consistent with the formation of methemefibrinogen as previously reported with organic oxidants and unpublished, ongoing investigation (28). There was a smaller antifibrinolytic effect with PHA, with a significant decrease in MRL at 5 and 10 mM concentrations, and only a significant prolongation of CGT at 10 mM.

Isolated exposure of α_2-antiplasmin to MAHMA NONOate, PHA and CORM-2

α_2-antiplasmin was exposed to MAHMA NONOate and PHA; the effects on fibrinolysis are displayed in table 4. MAHMA NONOate and PHA significantly decreased TMRL, and shortened CLT and CLS. Additionally, MAHMA NONOate significantly increased MRL. These substantial profibrinolytic effects of the two compounds after exposure of isolated α_2-antiplasmin are opposite of that observed in normal plasma (tables 2 and 3). To rule out that this contradictory result was possibly due to using a different source of α_2-antiplasmin, as previously reported (28), we compared unexposed purified α_2-antiplasmin with α_2-antiplasmin exposed to CORM-2. Comparable to our lab's previous report [38], CORM-2 significantly increased MRTG (4.8±0.5 dynes/cm^2/sec), increased TTG (121±10 dynes/cm^2), increased TMRL (11.5±3.7 min), decreased MRL (-0.7±0.1 dynes/cm^2/sec), increased CLT (22.4±2.0 min) and increased CLS (26.3±2.2 min). Overall, NO or PHA mediated conversion of a putative heme to metheme state associated with α_2-antiplasmin results in increased fibrinolysis, whereas a carboxyheme state decreases fibrinolysis. The thrombus growth/disintegration curves representative of this data is presented in figure 7.

Spectrophotometric antiplasmin activity assay

Normal plasma diluted by 50% that was exposed to PHA had a 76.8±5.3% α_2-antiplasmin activity, whereas plasma exposed to MAHMA NONOate resulted in 8 replicates with >124% normal activity (inhibition beyond the range of the standard curve).

Isolated exposure of plasmin to MAHMA NONOate and CORM-2

MAHMA NONOate and PHA decreased α_2-antiplasmin activity in isolation. While both compounds appeared to enhance overall inhibition of plasmin activity in the spectrophotometric-based experiments, the possibility that plasmin was directly inhibited by either NO or CO was investigated. A previous study demonstrated that unlike tPA, plasmin addition did not result in delayed fibrinolysis but instead decreased MRTG and TTG (40). This demonstrated in a thrombelastographic model that plasmin, a non-propagating fibrinolytic agent, could have changes in its activity determined by changes in thrombus velocity of growth and final strength (40). NO and CO's effects on plasmin activity are depicted in table 5. Plasmin significantly decreased MRTG and TTG, whereas exposure of plasmin to either MAHMA NONOate or CORM-2 significantly attenuated the plasmin mediated decrease in

coagulation kinetics to similar extents. In sum, both NO and CO decreased plasmin activity. Thrombus formation curves representative of these data are presented in figure 8.

LC-MS/MS detection of heme associated with α_2-antiplasmin and plasmin

We were unable after two attempts to recover more than 10% of α_2-antiplasmin's amino acid sequence, likely secondary to extensive glycosylation (34). It is our expectation that ongoing work to deglycosylate and adequately proteolytically digest α_2-antiplasmin will result in identification of an α_2-antiplasmin associated heme. In the case of plasmin, approximately 70% of the enzyme's amino acid sequence could be identified following digestion with trypsin. Regarding the pursuit of the presence of a heme group released from plasmin, an ion with an approximate m/z of 617.15 similar to the characteristic m/z 617.18 $[M+H]^{1+}$ heme ion was detected as base peak from the acetonitrile gradient at the appropriate elusion time of 62.22 minutes (34, 39) in plasmin samples but was not found in BSA samples (negative control). A typical example of such spectral data is displayed in figure 9.

Chapter 4: Conclusion

The effects of α_2-antiplasmin and plasmin were diminished by NO or PHA; however α_2-antiplasmin's antifibrinolytic effects remained more dominant indicating that plasmin's activity is the crucial biochemical event in this process. The importance of this study is to show that, when plasma is exposed to NO or a heme-binding oxidant, the hypofibrinolytic effects exhibited are most likely secondary to inhibition of plasmin or by α_2-antiplasmin enhanced by conversion of an attached heme to a metheme state. This builds on our lab's previous investigations demonstrating that CO-influenced antifibrinolytic effects are due to both the augmentation of α_2-antiplasmin and inhibition of plasmin activity (13, 28). The molecular mechanism responsible for inhibition of plasmin activity by NO or CO is most likely conversion of the associated heme to either a metheme or carboxyheme state, respectively. It is also likely that the up/down regulation of α_2-antiplasmin activity by CO or NO involves an associated heme, as in the case of the enhancement of fibrinogen as a substrate by CO (34). In summary, the effects of CO and NO on the fibrinolytic/antifibrinolytic system are complex, involve α_2-antiplasmin-plasmin interactions, and are likely mediated by attached heme groups.

Given the novelty of the proposed idea that diatomic gases modulate heme groups attached to fibrinogen, α_2-antiplasmin, and plasmin causing changes in coagulation and fibrinolysis, skepticism can be expected. Hemoglobin, myoglobin, peroxidases and porphyrins are just some of the biological proteins and enzymes

known to contain a heme functional group. Hemoproteins have diverse biological functions including the transport of diatomic gases, chemical catalysis, diatomic gas detection, and electron transfer. Heme groups reacting with diatomic gases such as O_2, NO, and CO resulting in modulation of the substrate and/or signal transduction (enhancement/inhibition) of the substrate has been thoroughly investigated and is a widely accepted concept (41). It is well know that iron in the Fe^{2+} state is available to bind diatomic gases, whereas iron in the Fe^{3+} is unable to bind with diatomic gases (41-44). Lastly, gas sensing molecules containing heme groups have been shown to have distinct affinities for specific diatomic gases, while at the same time having no affinity to others. So while there may be skepticism towards this new phenomenon, there are many accepted biochemical principles which together provide evidence to support the hypothesis.

In our experiments, 4000 μM NO was required to modulate α_2-antiplasmin or plasmin, as compared to only 75 μM CO (NO:CO molar ratio 53:1). NO and CO are both components of cigarette smoke, albeit in different amounts (45). Combustion of a single cigarette releases approximately 0.2 mg of NO (30 Da) and 12 mg of CO (28 Da), with a NO:CO molar ratio of 1:64 (45). If only 50% of expected CO derived from combustion of two cigarettes (12 mg) were dissolved into the plasma volume of a 70 kg subject (approximately 3 L), then the consequent CO concentration would be more than 140 μM – an amount that meets/exceeds the 75-150 μM CO required to effect maximum hypofibrinolysis *in vitro* in plasma (13). Therefore it would be expected that cigarette smoke exposure would essentially guarantee the hypofibrinolytic state reported after smoking two cigarettes (29).Taken as a whole,

our present and previous *in vitro* results mechanistically account for the majority of procoagulant/hypofibrinolytic responses to smoking, given the gas mixture resulting from the combustion of cigarettes (28, 33, 34, 46).

Even though tobacco smoke produces a hypercoagulable state, it has also been shown that CORM-released CO inhibits platelet aggregation *in vitro* in both humans and rats (47, 48). However, it should be observed that human (47) and rat (48) platelet aggregation is decreased approximately 17% and 35%, respectively, by 75 μM CO. Further, the bleeding time of a rat tail-tip amputation model (48) was increased by only 30% following a 30 mg/kg administration of CORM-3 (tricarbonyldichloro (glycinato) ruthenium (II); 295 Da) – this concentration of CORM-3 would be expected to release >1400 μM CO. CO released at this concentration would be 10 times greater than the concentration required to maximally enhance coagulation and attenuate fibrinolysis *in vitro* (13) following exposure of plasma to CORM-2 or released after smoking two cigarettes (46). In addition, the 30% prolongation of bleeding time in response to such a large administration of CO is far less significant than the 300% increase in bleeding time after administration of Clopidogrel (see Table 1) in the same animal model (48). So, even though the antiplatelet effects of CO have been well-documented (47, 48), clinically it has been demonstrated that the encountered concentrations of CO elicit protein-mediated hypercoagulability, without discernable loss of (indeed, enhancement of) platelet activity following exposure to tobacco smoke (33).

There are still many important issues to be determined; the duration of CO mediated hypercoagulability *in vivo* is undetermined, as well as the extent of CO

influence on smoking-associated perioperative thrombophilia (49, 50). Currently, CO-mediated hypercoagulability and hypofibrinolysis is being examined in rabbit models, in order to evaluate the coagulant effects of CO *in vivo*, and to determine dose efficacy. Future studies involving CORM-2 may include: evaluating the plasma of smokers and the effect CO has on the coagulation kinetics of their plasma, case studies on the intra-operative course of patients with a history of smoking (how a chronically increased COHb concentration may increase the risk of thrombotic events intra/post-operatively), and ultimately designing a diagnostic tool to assist in identifying patients at risk of spontaneous thrombotic events, or other events associated with trauma, surgery, smoke inhalation etc. CORM-2-mediated-release of CO should also be evaluated as a possible therapeutic procoagulant treatment or as an adjuvant to currently used procoagulants such as recombinant activator human factor VII, fresh frozen plasma, thrombin/fibrin glue, and zeolites.

In conclusion, the effects of α_2-antiplasmin and plasmin are modulated by CO and NO. This likely due to the secondary conversion of an attached heme group(s) to carboxyheme and metheme states, respectively. Given the known amount of CO in cigarettes (46) and the positive correlation between chronic smoking and a procoagulant state (33, 51-55), this study supports the hypothesis that CO modulation of fibrinogen (34), α_2-antiplasmin (28) and plasmin play a critical role in tobacco-associated thrombophilia (56).

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Appendix A

List of Figures

Figure 1. The Coagulation and Fibrinolytic Cascades

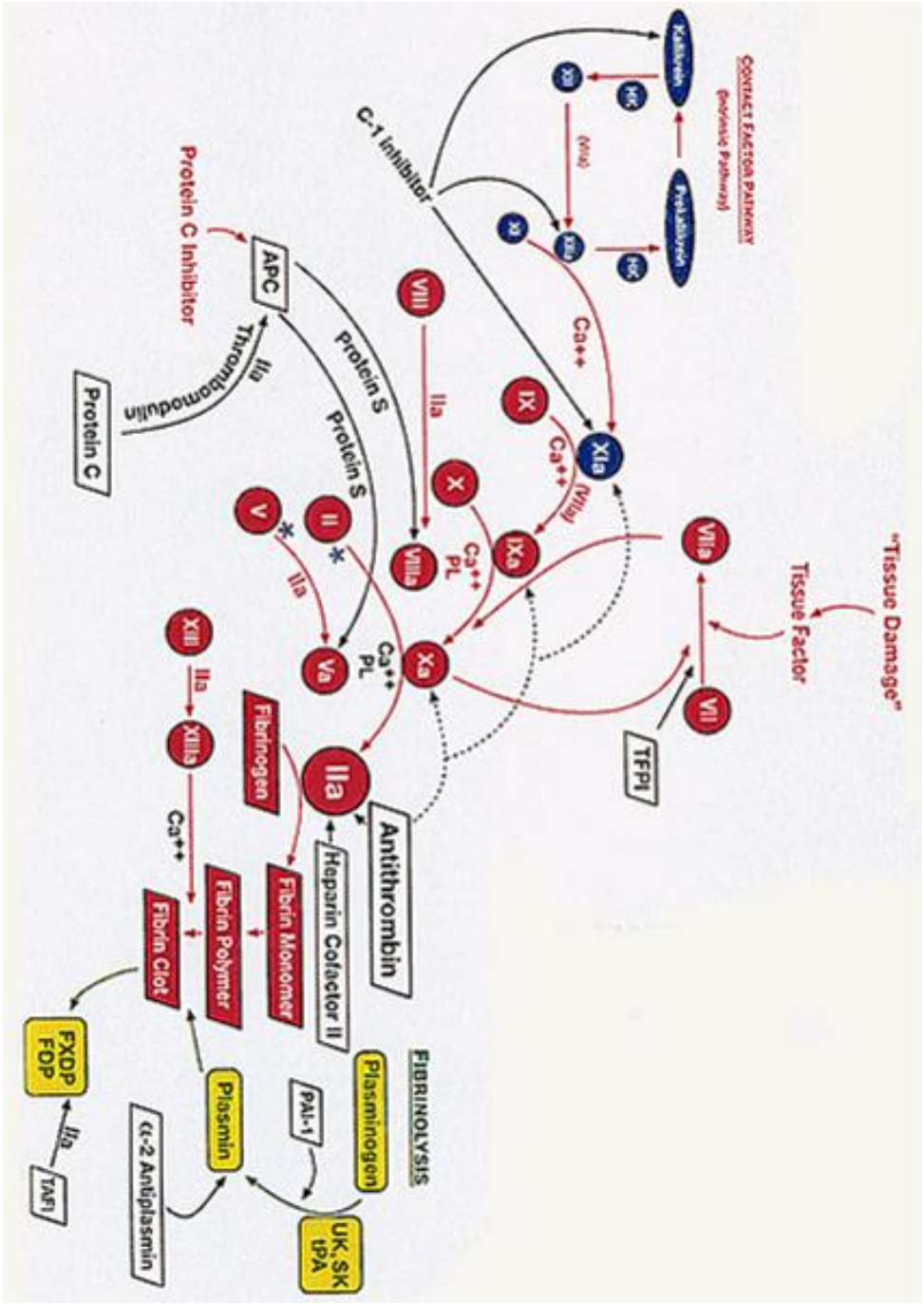
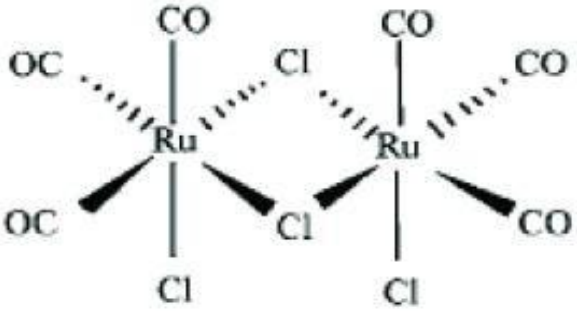


Figure 2. Chemical Structure of CORM-2 Molecule



Tricarbonyldichloro ruthenium (II) dimer

$[Ru(CO)_3Cl_2]_2$

Figure 3. Structure of α_2-antiplasmin

Figure 4. Chemical Structure of Phenylhydroxylamine

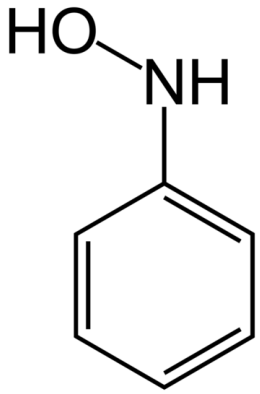


Figure 5. Chemical Structure of (Z)-1-[N-Methyl-N-[6-(N-methylammoniohexyl) amino]] diazen-1-ium-1, 2-diolate (MAHMA NONOate)

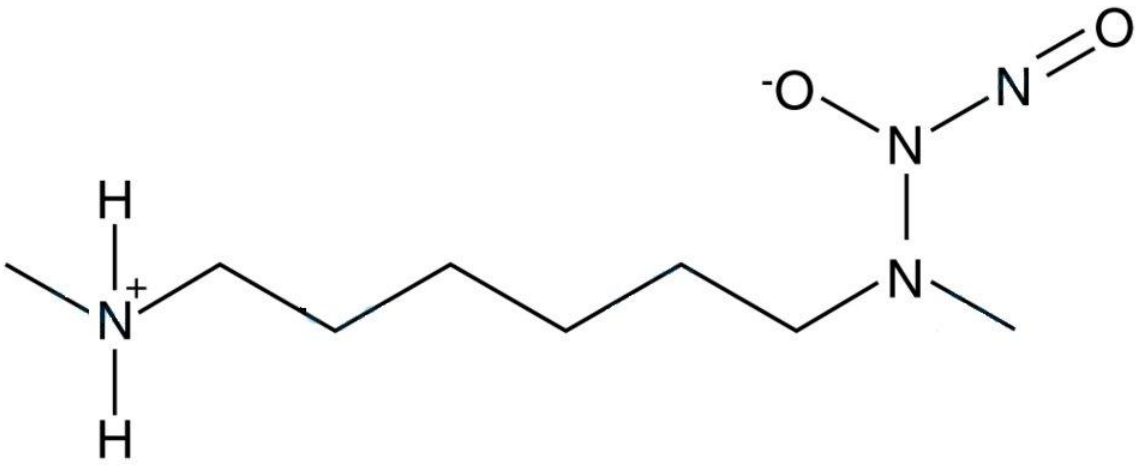
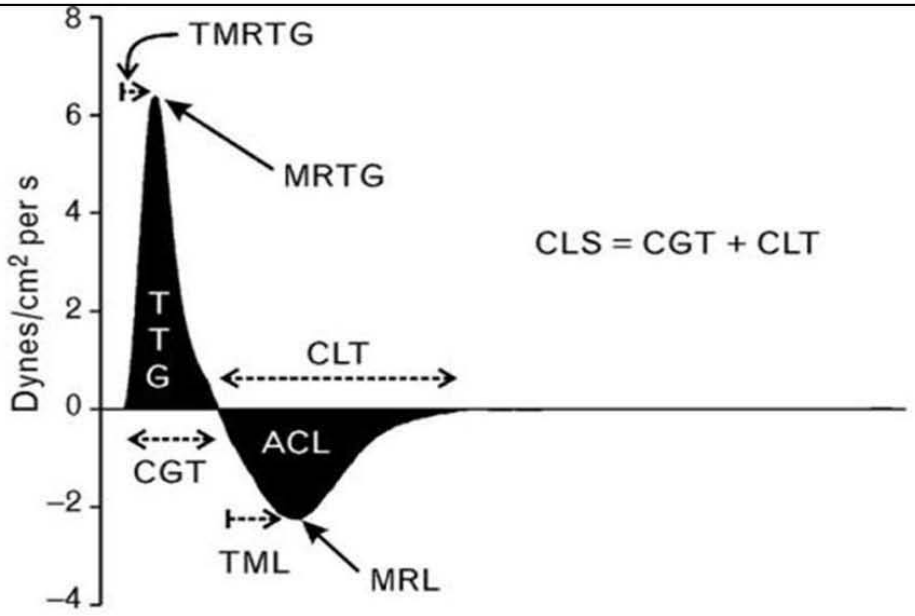
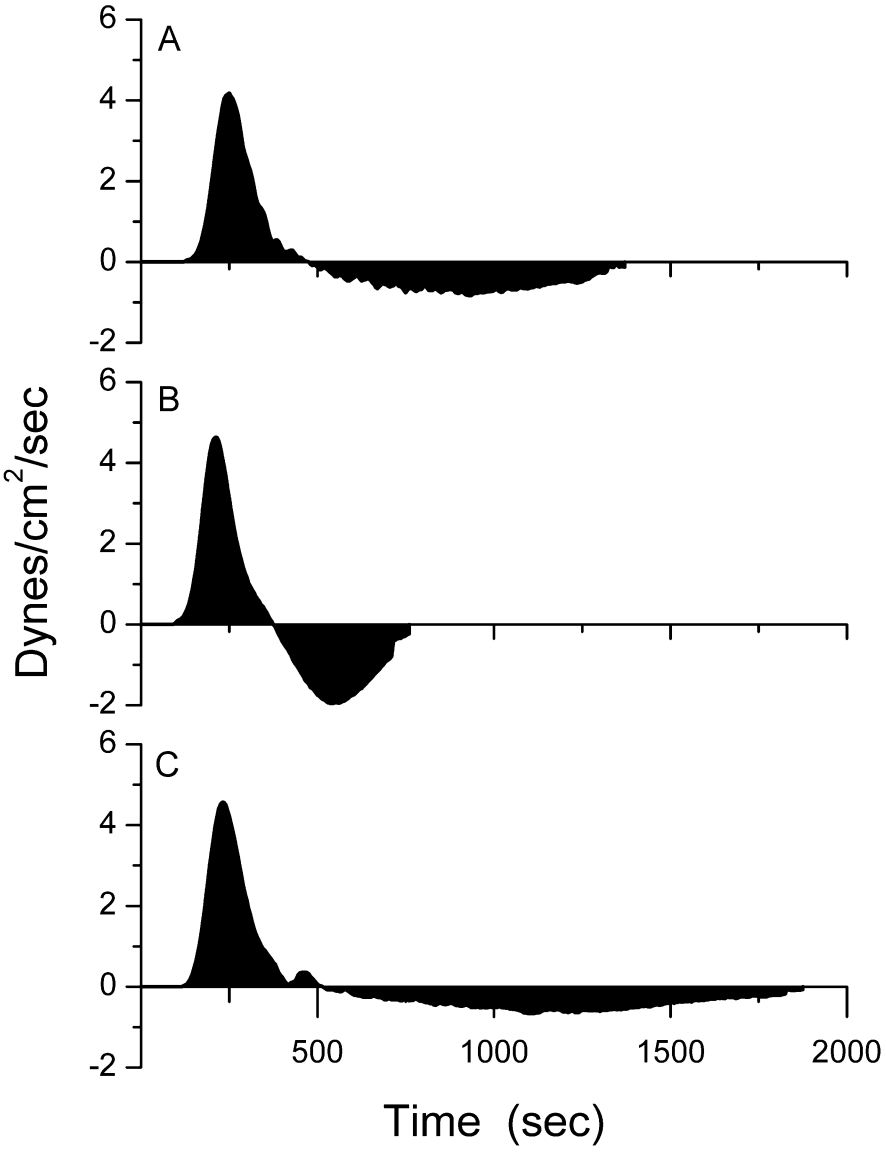


Figure 6. Clot Lifespan Model



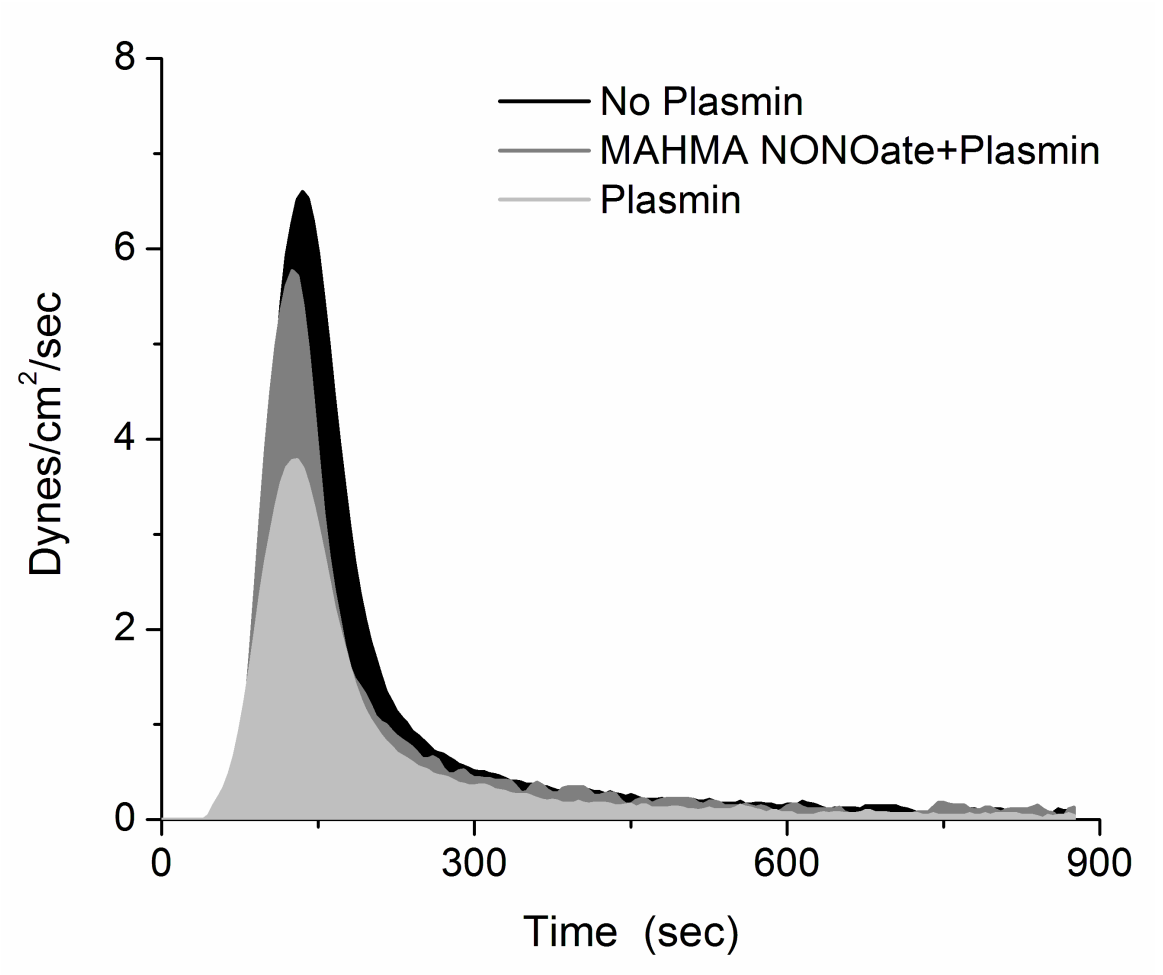
Clot lifespan model variable definitions. CGT, clot growth time (s): defined as the time when clot formation commences [clot strength of 102 dynes/cm^2 (2 mm amplitude)] to when maximum clot strength is observed; ACL, area under the curve of lysis (dynes/cm^2); CLS, clot lifespan (s) is the sum of CGT and CLT; CLT, clot lysis time (s) begins when maximum clot strength is observed and continues until lysis renders clot strength equal to 102 dynes/cm^2; MRL, maximum rate of lysis (dynes/cm^2 per s); MRTG, maximum rate of thrombus generation (dynes/cm^2 per s); TML, time to maximum rate of lysis (s); TMRTG, time to maximum rate of thrombus generation (s); TTG, total thrombus generation (dynes/cm^2).

Figure 7. Effects of NO and CO on isolated, purified α_2-antiplasmin activity



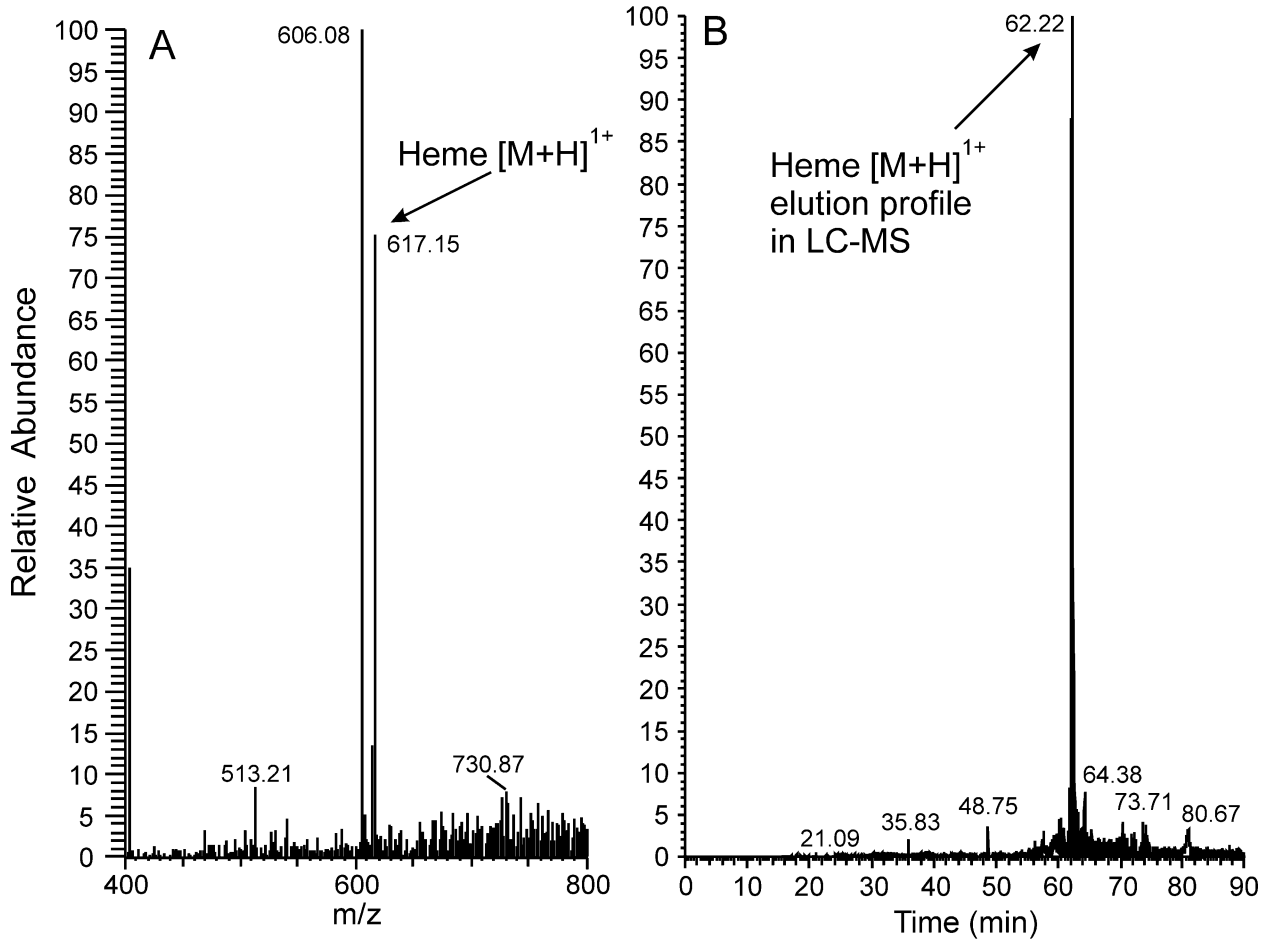
Panel A: Fifty μg/ml of α_2-antiplasmin was added to α_2-antiplasmin deficient plasma in the presence of tPA. Panel B: NO derived from MAHMA NONOate was added to α_2-antiplasmin prior to reaction, accelerating fibrinolysis. Panel C: CO derived from CORM-2 was added to α_2-antiplasmin prior to reaction, attenuating fibrinolysis.

Figure 8. Effects of NO on isolated, purified plasmin activity



Coagulation in normal plasma (black trace) was inhibited by addition of plasmin (light gray trace). Plasmin exposed to NO derived from MAHMA NONOate (gray trace) decreased coagulation to a significantly lesser extent than unexposed plasmin.

Figure 9. Evidence of free heme in tryptic digest of human plasmin.



Panel A: A reverse phase LC-MS/MS analysis of a tryptic digest of human purified plasmin; the full MS scan reveals a m/z 617.15, corresponding to singly charged reduced heme. Panel B: An extracted ion chromatogram of m/z 617.15 reveals the heme elution time of 62.22 minutes during LC-MS/MS.

Appendix B

List of Tables

Table 1. Abbreviations and Definitions

Terms	Explanation/Example
α_2-antiplasmin	A serine protease responsible for inactivating plasmin, inhibiting fibrinolysis
Argatroban	An anticoagulant which is a direct thrombin inhibitor, often used to treat thrombosis in patients with heparin-induced thrombocytopenia (HIT)
Carboxyhemoglobin (COHb)	A stable complex of carbon monoxide and the hemoglobin of red blood cells which forms due to the inhalation of carbon monoxide or by normal metabolism
Clopidogrel	An antiplatelet agent that irreversibly inhibits the $P2Y_{12}$ subtype of the ADP receptor; is important in the aggregation of platelets and cross-linking fibrin
Fibrin	The active form of fibrinogen which polymerizes to form a mesh which becomes a hemostatic plug or clot over a wound site

Fibrinolysis	The process of breaking down a fibrin clot; plasmin is the primary enzyme responsible for dissolving fibrin clots
Heme	A prosthetic group consisting of iron at the center of a large heterocyclic ring known as a porphyrin; among many biochemical functions, hemes transport diatomic gases (such as CO, NO, and O_2) when the iron is in the Fe^{2+} state, when chemicals such as PHA react with heme, it converts $Fe^{2+} \rightarrow Fe^{3+}$, inhibiting the ability of heme to freely bind diatomic gases
Heparin	A faster acting anticoagulant than Warfarin, it works by activating antithrombin III which inactivates thrombin
Liquid Chromatography (LC)	A laboratory technique using a machine to separate a mixture, so as to purify it or identify its contents
MAHMA NONOate	A chemical compound used as a nitric oxide (NO) donor for these experiments, in a phosphate buffer at pH 7.4, the $T_{1/2}$~3.5 minutes

Mass Spectrometry (MS)	A laboratory technique using a machine to determine the mass-to-charge ratio of charged particles in order to determine the elemental composition of a sample or molecule
Methemoglobin (metheme)	A form of hemoglobin in which the iron of the heme group is in the Fe^{3+} state, inhibiting its ability to bind diatomic gases such as O_2 or CO
Phenylhydroxylamine (PHA)	An organic compound used as an oxidant in these experiments to convert the iron on heme molecules from a $Fe^{2+} \rightarrow Fe^{3+}$ state
Plasmin	The enzyme responsible for dissolving fibrin clots; it is converted from plasminogen to the active form plasmin by tPA
Protamine	A drug which reverses heparin by binding and inhibiting it
Recombinant Activating Factor VII (RAF VII)	Manufactured FVII used for initiating coagulation during uncontrollable hemorrhage, i.e. hemophilia patients
Thrombelastography (TEG)	A method of testing the efficiency of coagulation. A sample of blood or plasma is placed in a small cup and gently rotated to

	imitate sluggish venous flow to activate coagulation. A small pin sensor is placed into the cup and a clot forms between the pin and the cup. Parameters such as clot speed and strength are measured during this process
Thrombin	An enzyme whose major role is to convert fibrinogen to fibrin, initiating the formation of a hemostatic plug (clot)
Tissue Factor (TF)	The cell surface receptor for the serine protease Factor VIIa, which activates Factor X, the first step of the common coagulation pathway; TF is an activator of the extrinsic pathway
Tissue-type Plasminogen Activator (tPA)	An enzyme that catalyzes the formation of active plasmin from its inactive form plasminogen; plasmin is the major enzyme responsible for fibrinolysis
Warfarin	A slower acting anticoagulant used for patients requiring chronic anticoagulation; it acts by inhibiting vitamin K-dependent synthesis of calcium-dependent clotting factors II, VII, IX, X and proteins C, S, and Z

Table 2. Effects of MAHMA NONOate on tPA mediated fibrinolysis in normal, pooled plasma.

	Condition of MAHMA NONOate	**0 mM**	**1 mM**	**2 mM**
Coagulant	***TMRTG***	2.5(2.5, 2.6)	3.1(3.0, 3.2)*	2.7(2.5, 2.7)*†
	MRTG	5.8(5.2, 6.3)	4.4(4.1, 4.8)*	6.0(5.6, 6.5) †
	TTG	120(107,127)	94(86,101)*	118(109,131) †
	CGT	3.1(2.6, 3.4)	2.7(2.6, 2.9)	3.8(3.2, 4.5)*†
Fibrinolytic	***TMRL***	7.9(6.6, 9.4)	6.2(4.3, 6.9)*	13.4(9.2, 15.0)*†
	MRL	-1.1(-1.2,-1.0)	-1.2(-1.3,-1.0)	-1.2(-1.3,-1.0)
	CLT	13.0(12.6, 13.6)	11.3(9.7, 13.4)	18.2(16.0, 23.0)*†
CGT+CLT	***CLS***	16.2(15.56, 17.0)	14.2(12.1, 16.2)	22.6(19.1, 26.6)*†

Data are presented as median (1^{st}-3^{rd} quartiles). TMRTG = time to maximum rate of thrombus generation (min); MRTG = maximum rate of thrombus generation (dynes/cm^2/sec); TTG = total thrombus generation (dynes/cm^2); TMRL = time to maximum rate of lysis (sec); MRL = maximum rate of lysis (-dynes/cm^2/sec); CGT = clot growth time (min); CLT = clot lysis time (min); CLS = clot lifespan (min). *P<0.05 vs. 0 mM PHA, †P<0.05 vs. 1 mM MAHMA NONOate.

Table 3. Effects of PHA on tPA mediated fibrinolysis in normal, pooled plasma.

	Condition of PHA	*0 mM*	*5 mM*	*10mM*
Coagulant	***TMRTG***	3.0(2.2, 3.2)	2.9(2.9, 3.0)	3.6(3.5, 3.8)*†
	MRTG	5.0(4.4, 6.5)	4.2(4.0, 4.7)	3.5(3.2, 3.8)*†
	TTG	113(101,119)	96(90,102)	89(80, 96)*†
	CGT	3.0(2.6, 3.6)	3.8(2.9, 4.2)	5.0(4.2, 5.2)*†
Fibrinolytic	***TMRL***	7.5(5.0, 9.4)	6.9(6.1, 11.3)	9.7(6.6, 11.4)
	MRL	-0.9(-1.1,-0.8)	-0.7(-0.9,-0.6)*	-0.7(-0.8,-0.7)*
	CLT	14.6(12.5, 15.0)	16.7(12.5, 20.9)	17.9(14.2, 18.4)
CGT + CLT	***CLS***	17.4(15.4, 18.9)	20.0(15.8, 25.0)	21.8(19.4, 23.6)

Data are presented as median (1^{st}-3^{rd} quartiles). TMRTG = time to maximum rate of thrombus generation (min); MRTG = maximum rate of thrombus generation (dynes/cm^2/sec); TTG = total thrombus generation (dynes/cm^2); TMRL = time to maximum rate of lysis (sec); MRL = maximum rate of lysis (-dynes/cm^2/sec); CGT = clot growth time (min); CLT = clot lysis time (min); CLS = clot lifespan (min). *P<0.05 vs. 0 mM PHA, †P<0.05 vs. 5 mM PHA.

Table 4. Effects of exposure of purified α_2-antiplasmin to MAHMA NONOate or PHA on tPA mediated fibrinolysis in α_2-antiplasmin deficient plasma.

	Condition	*No Exposure*	*MAHMA NONOate (2mM)*	*PHA (5mM)*
Coagulant	***TMRTG***	4.0±0.2	3.6±0.1*	3.6±0.2*
	MRTG	4.1±0.6	4.8±0.4	4.5±0.5
	TTG	100±10	108±6	102±11
	CGT	3.4±0.6	3.4±0.7	3.1±0.6
Fibrinolytic	***TMRL***	7.5±2.5	4.4±0.8*	4.8±0.8*
	MRL	-0.9±0.2	-1.8±0.3*	-1.1±0.2†
	CLT	13.8±2.3	7.7±0.9*	11.0±1.9*†
CGT + CLT	***CLS***	17.1±2.2	11.1±1.4*	14.2±2.1*†

No Exposure = α_2-antiplasmin not exposed to MAHMA NONOate or PHA; MAHMA NONOate = α_2-antiplasmin exposed to 2 mM MAHMA NONOate; PHA = α_2-antiplasmin exposed to 5 mM PHA. Data are presented as mean±SD. TMRTG = time to maximum rate of thrombus generation (min); MRTG = maximum rate of thrombus generation (dynes/cm^2/sec); TTG = total thrombus generation (dynes/cm^2); TMRL = time to maximum rate of lysis (sec); MRL = maximum rate of lysis (-dynes/cm^2/sec); CGT = clot growth time (min); CLT = clot lysis time (min); CLS = clot lifespan (min). *P<0.05 vs. no exposure, †P<0.05 vs. MAHMA NONOate exposure.

Table 5. Effects of MAHMA NONOate and CORM-2 on plasmin-mediated decreases in coagulation in normal, pooled plasma.

	Condition	*No Plasmin*	*Plasmin*	*MAHMA NONOate*	*CORM-2*
Coagulant	***TMRTG***	2.2±0.1	2.0±0.1*	2.0±0.1*	2.0±0.1*
	MRTG	6.5±0.4	3.7±0.4*	5.7±0.6*†	4.9±0.5*†‡
	TTG	138±7	90±6*	116±10*†	111±11*†

No Plasmin = no addition of plasmin; Plasmin = 50 μg/ml plasmin addition; MAHMA NONOate = addition of plasmin exposed to 2 mM MAHMA NONOate; CORM-2 = addition of plasmin exposed to 100 μM CORM-2. Data are presented as mean±SD. TMRTG = time to maximum rate of thrombus generation (min); MRTG = maximum rate of thrombus generation (dynes/cm^2/sec); TTG = total thrombus generation (dynes/cm^2). *P<0.05 vs. no plasmin, †P<0.05 vs. plasmin, ‡ vs. plasmin exposed to MAHMA NONOate.

Table 6. Overview of Molecules with Hemes and Effects

Molecules	***CO***	***NO***	***PHA***
Normal Pooled Plasma	Enhances Coagulation, Inhibits of Fibrinolysis	Decreases Coagulation; prolongs onset of fibrinolysis	Decreases Coagulation; decreases rate of fibrinolysis
Fibrinogen	Enhances velocity of clot growth and strength	Decreases velocity of clot growth and strength	Decreases velocity of clot growth and strength
α_2-antiplasmin	Enhances enzyme activity, producing a carboxyheme state, and antifibrinolytic effects	Inhibits activity, producing a metheme state and profibrinolytic effects	Inhibits activity, producing a metheme state, and profibrinolytic effects
Plasmin	Inhibition	Inhibition	Inhibition