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Post-translational oxidative modification of fibrinogen is associated with coagulopathy after traumatic injury

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Abstract

Victims of trauma often develop impaired blood clot formation (coagulopathy) that contributes to bleeding and mortality. Fibrin polymerization is one critical component of clot formation that can be impacted by post-translational oxidative modifications of fibrinogen after exposure to oxidants. In vitro evidence suggests that A α -C domain methionine sulfoxide formation, in particular, can induce conformational changes that prevent lateral aggregation of fibrin protofibrils during polymerization. We used mass spectrometry of plasma from trauma patients to find that fibrinogen A α -C domain methionine sulfoxide content was selectively-increased in patients with

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AUTHORSHIP

Contribution: N.J.W., X.W., A.E.S.J., E.B.L., K.R.W., X.F., Y.W., and D.C., performed experiments; N.J.W analyzed results, made the figures, and wrote the paper; N.J.W, J.C., C.W., S.A.S., J.L., E.J.M, D.F.B and D.C., designed the research and made critical paper revisions.

Potential Conflicts of Interest

N.J. White, Y. Wang, X. Fu, J.A. López, and D. Chung hold intellectual property regarding detection methods for oxidative modifications of fibrinogen. J.C. Cardenas, E.J. Martin, D.F. Brophy, C.E. Wade, X.Wang, A.E. St. John, E.B. Lim, and S.A. Stern declare no conflicts of interest associated with this manuscript. K.R. Ward has a number of invention disclosures and patents pending for coagulation monitoring technologies through the University of Michigan and no direct conflicts with the information contained in this manuscript.

All other authors declare no competing financial interests.

coagulopathy vs. those without coagulopathy. This evidence supports a novel linkage between oxidative stress, coagulopathy, and bleeding after injury.

Graphical Abstract

Keywords

Oxidative stress; fibrinogen; coagulopathy; methionine sulfoxide; hemorrhage; trauma

INTRODUCTION

Hemostasis is often impaired after severe traumatic injury leading to pathological bleeding that is known as trauma-induced coagulopathy (TIC). TIC is present in one-quarter to one-third of severely injured patients almost immediately after injury, carries a 4-6 times increased risk of mortality, and is associated with increased incidence of multi-organ failure, intensive care utilization, and increased need for blood transfusion [1-4]. Early hallmarks of TIC include extrinsic coagulation pathway dysfunction identified by a prolongation of prothrombin time (PT) ratio and/or International normalized ratio (INR) and decreased viscoelastic clot strength [1-3]. Those patients with abnormally elevated PT ratio's recorded in the Emergency Department are twice as likely to die compared to those with normal PT ratios [4]. The cause of TIC is felt to be multifactorial as a result tissue hypoperfusion and hypoxia, activation of the anticoagulant Protein C (aPC), thrombin deficiency, and fibrinogen proteolysis and dysfunction [5].

Perhaps the most significant component of TIC is increased enzymatic clot breakdown, or hyperfibrinolysis. After injury with blood loss producing hemorrhagic shock, the concentration of tissue plasminogen activator (tPA) is increased in blood, which increases activation of plasminogen to plasmin [5-7]. Plasmin is the primary proteolytic enzyme for fibrin, causing rapid degradation of fibrin clots by cleaving fibrin strands at lysine residues. Impaired fibrin polymerization can also directly contribute to hyperfibrinolysis by increasing susceptibility to enzymatic degradation [8,9]. Hyperfibrinolysis is strongly associated with trauma patient mortality and when significant, often predicts imminent death [10]. In addition, antifibrinolytic therapy using the lysine analogue tranexamic acid is the only therapy found to reduce mortality when given early after injury in a large randomized controlled trial [11].

The hemostatic plasma protein fibrinogen is a key determinant of bleeding after injury. Fibrinogen becomes fibrin clot by cleavage of short peptides by thrombin from the A α (FpA) and B β (FpB) chains. Fibrin polymerization and subsequent fibrin clot structure is

then governed by specific interaction of complementary binding sites exposed after fibrinopeptide cleavage. Loss of fibrinogen to hemorrhage, consumption, and hyperfibrinolysis are associated with decreased survival after trauma [12]. Fibrinogen is also the first hemostatic protein to decrease to critically low functional concentrations in surgical patients with significant blood loss [13]. Therefore, fibrinogen is an important and labile hemostatic protein that is affected by trauma.

Fibrinogen is also extremely sensitive to oxidation relative to other plasma proteins [14]. We have previously demonstrated *in vitro* that exposure of fibrinogen to hypochlorous acid is associated with preferential oxidation of Methionine 476 in the A α -C domain to methionine sulfoxide (A α -Met476(SO)), inhibition of lateral aggregation of fibrin protofibrils during fibrin polymerization, and formation of thin-fibered fibrin clots that are mechanically weak [15]. *In silico* modelling has also revealed that specific A α -Met476 oxidation to A α -Met476(SO) plausibly mediates the inhibition of fibrin lateral aggregation by disruption of a key beta hairpin structure involved in the fibrin lateral aggregation mechanism [16]. HOCl is predominantly generated in the plasma as a product of neutrophil lysosomal myeloperoxidase, chloride, and hydrogen peroxide and is a key leukocyte-specific host-defense mechanism mediating bacterial killing by the formation of methionine sulfoxide in bacterial membrane proteins [17]. Leukocytes upregulate the same oxidative enzymes after blunt trauma and oxidation has been implicated in vascular responses to hemorrhagic shock [18-20].

Despite evidence that oxidants are generated after trauma and fibrinogen is highly sensitive to these oxidants, fibrinogen oxidation has not been detected in trauma patients with coagulopathy. Having established a plausible *in vitro* link between A α -Met476(SO) content and altered fibrin clot formation, the purpose of this study was to test the hypothesis that the same methionine residue of fibrinogen is selectively-oxidized in trauma patients with coagulopathy.

MATERIALS AND METHODS

Trauma Patients

Plasma was obtained from three de-identified human Emergency Department trauma repositories. Human subjects approvals were obtained from local institutional review boards for repository and medical records access according to the guidelines set forth by the declaration of Helsinki. The first cohort was used to identify selectively-vulnerable methionine residues on fibrinogen that were oxidized to methionine sulfoxide in coagulopathic trauma patients. Nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) approach was used to identify these residues in this cohort from glycine-purified trauma patient fibrinogen. For the second cohort, we used an ultra-performance LC (UPLC)-MS/MS with Multiple Reaction Monitoring (MRM) mode to confirm increased fibrinogen A α -Met476(SO) content directly in plasma without purification. We then used LC (UPLC)-MS/MS and viscoelastic clotting measurements to examine associations between fibrinogen A α -Met476(SO)% and clot formation after injury in a third cohort.

Trauma Derivation Cohort

Samples of platelet-poor plasma were obtained directly from a trauma plasma biorepository. Blood samples were drawn from trauma patients at Harborview Medical Center, a U.S. Level-I trauma center in Seattle WA, on arrival to the Emergency Department. Leftover citrated plasma was obtained from these samples after all clinical tests were performed. Leftover plasma was immediately frozen at -80°C and given a unique study number by the clinical laboratory for inclusion in the biorepository. Patient medical records were then reviewed and vital signs, laboratory data, injury patterns, and clinical outcomes were abstracted and matched to plasma samples after removal of all protected health information. To detect fibrinogen oxidation, fibrinogen was first purified from plasma by 4 rounds of glycine purification yielding highly-purified fibrinogen. Purified fibrinogen was then subjected to nanoLC-MS/MS to identify sites of increased methionine sulfoxide content. Samples were grouped for comparison by the presence or absence of coagulopathy as defined by an INR >1.2 reported by the hospital laboratory [4].

Trauma Confirmation Cohort

The specific MS signatures identified in the first cohort were then used to quantify fibrinogen methionine sulfoxide content directly in plasma without purification using UPLC-MS/MS-MRM. Blood was sampled from trauma patients presenting to Virginia Commonwealth University Medical Center, a U.S. Level I trauma center in Richmond VA. Blood was obtained in the Emergency Department and any subjects known to have received blood product transfusions prior to the blood sample were excluded. A subset of $N=25$ de-identified plasma samples with matched clinical laboratory data were submitted for UPLC-MS/MS-MRM for specific AcMet476(SO) signatures as detected directly in plasma without purification. MS personnel were blinded during measurement and analysis. Samples were again grouped for comparison by the presence or absence of coagulopathy as defined by an INR >1.2 reported by the hospital laboratory. To examine for selectivity of oxidation of fibrinogen over other plasma proteins, the M353(SO) content of albumin, a similar solvent-exposed methionine residue, was simultaneously measured in the same plasma.

Clot Formation Cohort

Given the importance of clot formation and fibrinolysis to trauma patient outcomes, we then examined for a direct association between fibrinogen Ac-M476(SO) content and viscoelastic clot formation in a third cohort of trauma patients. This cohort was obtained from a trauma biorepository of citrated plasma from Emergency Department trauma patients sampled on arrival to Memorial-Hermann Medical Center, a U.S. Level I trauma center in Houston TX. These patients also underwent Emergency Department point of care rapid thrombelastography of whole blood (rTEG, Haemonetics, Braintree MA, USA) at the time of initial blood draw as part of their clinical care. rTEG is a viscoelastic measurement of clot formation using tissue factor is useful to guide bleeding management after trauma [21]. rTEG reports the activated clotting time (ACT) and onset time (R), or time required to achieve detectable clot formation, representing coagulation factor activity; clot formation time (K) and alpha angle which represent fibrin polymerization and platelet activity; maximal amplitude of deflection (MA) which represents fibrin clot integrity and platelet-

induced clot contraction; and clot durability (LY30%) reported as percent clot breakdown, or lysis, at 30 minutes after MA, representing the activity of fibrinolytic enzymes. We examined n=50 citrated plasma samples, half of which demonstrated deficient clot formation by rTEG as indicated by increased clot breakdown of at least 3% LY30% to ensure a range of coagulopathy and clot formation phenotypes in the cohort [22-23]. Fibrinogen A α -M476(SO) content was again measured directly in these plasma samples using UPLC-MS/MS- MRM. Corresponding clinical data regarding injury severity, shock severity, and relevant outcomes were also abstracted from the repository.

Glycine Purification of Fibrinogen from Plasma

Fibrinogen was purified from plasma using glycine precipitation. Plasma was incubated with glycine in a ratio of 0.1ml plasma to 15.6 mg glycine for one hour on ice and the precipitate collected by microcentrifugation for 10 min at 4°C. The supernatant was removed and the pellet suspended in phosphate buffer at pH 6.4. This process was repeated 3 additional times for a total of 4 rounds of glycine precipitation and fibrinogen protein yield measured by BCA colorimetric assay.

Thrombin and Reptilase time Assays

The START-4 steel ball coagulation analyzer (Diagnostics Stago, Asnières, France) was used to measure fibrin polymerization by thrombin time and reptilase time. Thrombin time measures the time to clot formation for plasma at 37°C after addition of an excess of thrombin which activates fibrinogen to fibrin by cleavage of fibrinopeptides A and B. Reptilase time uses batroxobin, a viper venom, to measure fibrin polymerization induced by cleavage of fibrinopeptide A only. Thrombin time is sensitive to the presence of antithrombin anticoagulants such as heparin, while the Reptilase time is not.

Protein Digestion

Purified fibrinogen samples from patients (5 μ g) or patient plasma (20 μ g total protein) were reduced with 5 mM dithiothreitol (DTT) at 70°C for 20 min, alkylated with 12.5 mM iodoacetamide at room temperature for 15 min, and then digested with sequencing grade modified trypsin (Promega) (1:10 wt/wt, trypsin/protein) in 50 mM ammonium bicarbonate containing 5% acetonitrile at 37°C overnight. Digestion was halted by acidification with trifluoroacetic acid. The digested mixtures were either concentrated and desalted with C18 extraction disk cartridges (3M) or submitted directly for LC-MS analysis.

NanoLC-MS/MS Analysis

Proteolytic peptides derived from purified fibrinogen samples from patients (5 μ g) were analyzed by nanoLC-MS/MS with a Thermo Scientific LTQ Orbitrap Velos mass spectrometer coupled to a Waters nanoAcquity Ultra Performance LC system. Peptides were separated at a flow rate of 300 nL/min on a nanoUPLC BEH130 C18 column (100 $\times$ 0.075 mm, 1.7 μ m, Waters), using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Peptides were eluted using a linear gradient of 5%–35% solvent B over 90 min. The spray voltage was 3.0 kV, and the collision energy for MS/MS was 35%. Percent oxidation of individual Met residues was determined by dividing the peak area of

the peptide containing methionine sulfoxide by the sum of the peak areas of both oxidized and non-oxidized peptides.

UPLC-MS/MS-MRM Analysis

UPLC-MS/MS-MRM analyses of tryptical peptides obtained directly from patient plasma were performed with the QTRAP® 6500 mass spectrometer (AB SCIEX Inc, Framingham, MA USA) in positive electrospray ionization mode (ESI+) with multiple reaction monitoring (MRM) coupled to an ACQUITY UPLC I-Class system (Waters Corp, Milford, MA, USA). Peptides were separated at a flow rate of 300 µL/min on a CORTECS C18 UPLC column (2.1 × 100 mm, 1.6 µm, Waters Corp. Milford, MA USA), using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Peptides were eluted using a linear gradient of 5%–35% solvent B over 12 min.

Statistical Analysis

Normally-distributed variables were described using mean and standard deviation or standard error of the mean (SEM) and were compared using standard T-test. Those variables having significantly skewed distributions by normal quantile plot were described using median and interquartile range and were compared using the nonparametric Wilcoxon Rank Sum test. Simple bivariate associations were tested using Pearson Product Moment Correlation, and linear regression was used to test for multivariate associations. Categorical outcomes were tested using Chi Square analysis. A retrospective post-hoc power analysis was performed using the results of the derivation cohort to prospectively calculate the number of samples needed to achieve adequate power to detect differences between coagulopathic and noncoagulopathic groups by INR in the second cohort, and differences in thrombelastography clot formation parameters according to Aα-Met476(SO) quartile in the final cohort. All differences were considered significant at $p < 0.05$. Statistical analysis was performed using JMP 9.0 (SAS Corp, Cary, NC USA).

RESULTS

The mean (SD) age of subjects enrolled was 51.5 (20.4) years for Seattle, 39.7 (19.0) years for Richmond, and 48.8 (20.8) years for Houston cohorts. The majority of subjects were male in Seattle (67%), Richmond (83%), and Houston (68%). In Seattle 90% were injured by blunt vs. penetrating trauma, while in Richmond 57% were injured by blunt trauma and 43% by penetrating injury. In Houston 68% were injured by a blunt mechanism, 16% were injured by penetrating injury, and 16% sustained burns. In Seattle, 43% of subjects were transfused with blood products within the first 24 hours of hospitalization and hospital mortality was 33%. In Richmond, 13% of subjects received blood products and all subjects survived to hospital discharge (0% mortality). In Houston, 46% of subjects received blood transfusions acutely and in-hospital mortality was 32%. Emergency Department blood pressure, Glasgow Coma Score as a clinical estimate of brain injury, blood hemoglobin concentration as an estimate of blood loss, and base excess as an estimate of metabolic shock severity measured at the time of blood sample are given in **Table 1**.

Derivation Cohort

In the first Seattle cohort, samples from 22 hospitalized trauma patients were selected from the plasma biorepository. One-half of these samples (N=11) were selected from trauma patients meeting clinical criteria for coagulopathy (INR > 1.2)) for comparison to a randomly selected non-coagulopathic control group with having INR 1.2 (N=11).

Methionine sulfoxide content of fibrinogen was quantified in glycine-purified fibrinogen using nanoLC-MS/MS. Methionine sulfoxide was detected in 7 different methionine positions was subtly-increased in coagulopathic subjects at positions B β -M314 and A α -M476 compared to controls. (**Table 2.**) A α -M476(SO)% was significantly increased in the coagulopathy group and was the only modification associated with INR, thrombin time, and Reptilase time. (**Figure 1.**) Multivariate linear regression was used to evaluate for a possible confounding effect of resuscitation fluids and blood transfusions received prior to sampling. A α -M476(SO)% remained significantly associated with INR after adjusting for either total volume of crystalloid fluid administered at the time of sample draw (whole model $R^2=0.59$, $p=0.0005$; A α -M476(SO) effect $p=0.042$), or total units of blood products transfused (sum of packed red blood cells, fresh frozen plasma, platelets) prior to the time of sampling (whole model $R^2=0.72$, $p=0.0001$; A α -M476(SO) effect $p=0.046$). A significant effect of A α -M476(SO)% on reptilase time remained after adjusting for the possible confounders D-dimer concentration, fibrinogen concentration, and volume of plasma transfused at the time of measurement (whole model $R^2=0.77$, $p=0.003$; A α -M476(SO)% effect $p=0.035$). Post-hoc power analysis revealed that only N=17 total samples were required to detect the same absolute 0.63% (SD=0.43) difference in A α -M476(SO) content with $\alpha=0.05$ and 80% power to detect differences. Therefore, the sample size of N=22 was adequate and we prospectively recruited the same number of samples for comparison in the second confirmation cohort assuming a similar absolute difference in A α -M476(SO) content.

Effect of low-level A α -M476(SO)% on fibrin polymerization

We then confirmed experimentally that the A α -M476(SO) content found in coagulopathic trauma patients could affect fibrin polymerization when initiated by Reptilase. Fibrinogen was first purified by glycine precipitation from pooled normal human plasma and incubated with HOCL to induce maximal A α -M476(SO)% formation according to previously published methods [14]. Oxidized fibrinogen was then mixed at low concentrations with non-oxidized fibrinogen purified from the same plasma ranging from 2-8% of the total fibrinogen, at a ratio similar to those found in coagulopathic trauma patients. Reptilase time was then determined for each concentration. LC/MS/MS was used to confirm the fibrinogen A α -M476(SO)% in the final preparations. We found that even at very low A α -M476(SO)%, there remained a significant positive linear association between A α -M476(SO)% and Reptilase time. (**Figure 2**).

Confirmation Cohort

The Richmond cohort was used to confirm that A α -M476(SO)% was increased in coagulopathic trauma patients. N=23 plasma samples were submitted for MS analysis in a blinded fashion and underwent post-hoc analysis after stratifying into two groups by the same INR>1.2 coagulopathy criteria. Trauma subjects (N=12) were assigned to the control

group (INR 1.2) and were compared to subjects assigned to the coagulopathy group (N=11) (INR> 1.2). Mean (SD) A α -M476(SO)% was again subtly, but significantly, increased in the group with coagulopathy compared to control. (**Figure 3.**) In contrast, albumin M353(SO)% present in the same plasma was not increased with coagulopathy.

Fibrinogen A α -M476(SO)% was also associated with increasing anatomical injury determined by Injury Severity Score (ISS), and base excess as a marker of metabolic shock.

Fibrinogen oxidation and clot formation

We examined changes in multiple TEG clot formation parameters when stratified by A α -Met476(SO) quartile in the third trauma patient cohort from Houston TX. Based upon the derivation cohort power analysis, and assuming that TEG clot formation parameters would behave somewhat similarly to INR, N=11 samples were obtained for each A α -Met476(SO) quartile (a total of N 44 required) so that individual quartiles could be compared with adequate power to detect differences.

We obtained 50 plasma samples and N=49 plasma samples had adequate volume for full analysis. Median (IQR) fibrinogen A α -M476(SO)% for the entire cohort was 2.4% (2.1, 2.7) ranging from a minimum of 1.4% to a maximum of 5.1%. The median Albumin M353(SO) % content was 4.1% (3.4, 5.4) ranging from a minimum of 2.7% to a maximum of 9.2%. rTEG parameters were grouped and compared for differences according to fibrinogen A α -M476(SO)% quartile. (**Figure 4.**) There was a strong trend towards decreased MA in the highest fibrinogen A α -Met476(SO) quartile compared to the next highest quartile (Wilcoxon p=0.06). LY30% was significantly increased in the highest A α -M476(SO)% quartile compared to all other quartiles (Wilcoxon p 0.041 for all comparisons). There were no significant differences in rTEG parameters when stratified by Albumin M353(SO)% (**data not shown**). There were also a limited number of INR values measured for these samples, as INR is not routinely measured in this trauma center. Of 12 available measurements, there was a significant positive linear association between fibrinogen A α -M476(SO)% and INR (Pearson R=0.7, p=0.01). There was no significant association between Albumin M353(SO) % and INR (Pearson R=0.5, p=0.12).

DISCUSSION

This is the first report of selective oxidation of fibrinogen being associated with impaired blood coagulation after traumatic injury. This result suggests that oxidation of fibrinogen may contribute to coagulopathy, specifically a reduction in clot strength and increase in fibrinolysis, after trauma.

Fibrinogen Oxidation

There is evidence for similar post-translational modifications of fibrinogen in response to acute diseases when measuring Advanced Oxidative Protein Products (AOPP), which is predominantly made up of undifferentiated oxidized albumin and fibrinogen [24]. AOPP's become elevated in diseases where oxidative stress is acutely increased including; acute renal dysfunction, acute on chronic liver dysfunction, and in critical illness [25,26]. AOPP's are also increased in plasma exposed *in vitro* to HOCl and can be significantly elevated and

associated with MPO and plasma NADPH oxidase concentration and activity in hemodialysis patients [27,28]. Fibrinogen is also localized with leukocytes *in vivo* via integrin $\alpha M\beta 2$ /Mac-1 binding to the γ -chain (N390RLSIGE396) binding sequence and its binding supports inflammatory host responses to infection [29]. Such close spatial association may facilitate direct oxidation of fibrinogen sulfhydryl groups by leukocyte-derived HOCL *in vivo*.

Formation of methionine sulfoxide is not exclusive to HOCL. However, reactions of HOCL with methionine residues and other sulfur containing side chains are the fastest known, with 2nd order rate constants of up to 3.8×10^7 /M/sec at physiologic pH [30]. Sulfhydryl groups of human plasma proteins are the main target for PMN-derived HOCL oxidation, followed by thioether groups and amino groups [31]. Antioxidant mechanisms reversing fibrinogen oxidation are much-less efficient and may also become impaired after trauma. The rate of oxidation of methionine by HOCL is considerably faster than the rate constant of 37.0/M/sec reported for its subsequent reduction by its primary antioxidant enzyme methionine sulfoxide reductase [32]. The mechanism of reduction of methionine sulfoxide by methionine sulfoxide reductase is a three-step process of which, reduction by thioredoxin is the rate-limiting step, and is therefore dependent on the thioredoxin recycling process [33]. Thioredoxin cycling can also become inhibited during hemorrhagic shock by expression of a family of thioredoxin-interacting proteins that can directly inhibit the expression and reducing activity of thioredoxin [33,34]. Additional support for an unbalancing of blood redox balance after trauma is provided by Rael and colleagues who found that plasma oxidation-reduction potential was significantly elevated in trauma patients and could be used to discriminate between mild and severe injury profiles [35]. Therefore, our observation of subtly-increased methionine sulfoxide content of fibrinogen may suggest disruption of local sulfonyl redox balance either from inflammatory leukocyte activation (a common feature of trauma), a reduced antioxidant capacity, or both.

Relevance to Coagulopathy

Dissociation and interaction of fibrinogen αC domains from the E region enables fibrin protofibril lateral aggregation during clot formation [36-38]. Alpha C double hairpin structures interact with adjacent and complementary hairpin structures on adjacent fibrin monomers to form large beta sheet structures, eventually coalescing into fibers [39]. We have previously characterized the effect of A α -Met476(SO)% on local double hairpin structural conformations using molecular dynamics simulation [16]. The simulations suggest that oxidation of the single A α -Met476 site causes a hinge-like transition of the hairpin structure to a more linear and extended configuration. The functional consequences appear to be inhibition of fibrin protofibril lateral aggregation during fibrin polymerization producing thinner fibrin fibers and mechanically weak clots that are paradoxically less susceptible to fibrinolysis *in vitro* due to decreased clot porosity [15].

Our results suggest that fibrin polymerization may become impaired in trauma patients with increased fibrinogen A α -Met476(SO)%, manifesting as decreased clot strength and increased fibrinolysis after injury as determined by viscoelastometry. A similar disruption of the carboxyl-terminal region of the Aa chain can be found in the case of congenital

fibrinogen Caracas II. Fibrinogen Caracas II is characterized by prolonged thrombin time and translucent fibrin clots [40]. The mutation is described as a unique N-glycosylated Asn substitution for a Ser at position 434 of the mutant Aa chain that disrupts folding of the carboxyl-terminal region of the Aa chain back upon the central region of the molecule, disrupting its opportunity to participate in lateral aggregation events [40]. Woodhead and Weisel et al., used electron microscopy of individual molecules to find that most of the alpha C domains of fibrinogen Caracas II fail to interact with each other or with the central domain [41]. Caracas II fibrin was made up of thinner fibers that were less-ordered than normal fibers. However, whole clots contained large pores bounded by local fiber networks made up of thin fibers and permeation experiments suggested increased pore diameter, both of which are in direct conflict with decreased pore size and permeation measured after A α -Met476(SO) formation *in vitro* [15]. The viscoelastic properties of the Caracas II clot were also similar to control clots, leading the authors to conclude that this dysfibrinogenemia is relatively asymptomatic. Clearly, altering the A α -carboxy terminal region of fibrinogen can have important effects on fibrin clot structure and function that is sensitive to both pre and post-translational modification.

Relevance to Trauma Care

Fibrinogen oxidation might promote blood loss after trauma by directly inducing changes in fibrin clot structure via altered fibrin polymerization. The earliest documented change in clot formation in trauma patients is a reduction of clot strength or elastic modulus when measured using viscoelastic methods [3, 42]. Our previous work suggests a similar reduction in elastic and storage modulus of fibrin clot when fibrinogen was exposed to HOCl *in vitro* [15]. We found that rTEG maximal clot amplitude tended to decrease and fibrinolysis was significantly increased in the highest fibrinogen A α -Met476(SO)% quartile. In addition, a strong positive correlation between INR and A α -Met476(SO)% was preserved across three different trauma cohorts. These results suggest that oxidants present in plasma may also affect fibrinogen polymerization, clot strength, and resulting susceptibility to fibrinolysis. Further confirmatory work is needed to determine the contribution of fibrinogen oxidation to clot formation, bleeding, and outcomes after trauma.

Our results are also important because other plasma coagulation proteins are also susceptible to oxidation. Vulnerable methionine residues on both thrombomodulin and protein C have been identified that, when oxidized, result in drastic decreases of functional activity of these proteins [43,44]. The favored mechanisms of coagulopathy after trauma hinge upon increased thrombomodulin-thrombin interactions and activation of protein C in response to shock and tissue injury. Increased soluble thrombomodulin sequesters circulating thrombin, shifting its affinity from fibrinogen to protein C, thus forming activated protein C. Increased concentration of activated protein C then inhibits the activity of Factors V and VIII on the platelet surface and causes disinhibition of tissue plasminogen activator via inhibition of plasminogen activator inhibitor, resulting in both anticoagulation and hyperfibrinolysis [5]. Oxidation of a single methionine within the catalytic thrombin-binding pocket of thrombomodulin (Met388) decreases thrombin binding affinity *in vitro* [44]. The specific site of oxidation site of protein C has not yet been identified, but exposure to HOCl *in vitro* also severely reduces its activity [43]. Therefore, if significant *in vivo* methionine sulfoxide

formation is found on thrombomodulin and protein C after injury, their function may be altered from that expected and refinement of our current understanding of the traumatic coagulopathy mechanism would be required.

Recognizing the need for proper fibrinogen function to stop bleeding, some clinical guidelines are now prioritizing liberal fibrinogen replacement using fibrinogen concentrates guided by viscoelastic testing during severe bleeding management [45]. Our results suggest that antioxidant therapies may also be useful to improve other important outcomes after trauma. Thomson, and Ischiropoulos, et al. found that immunoglobulins recognizing the 3-nitrotyrosine post-translational modification were significantly higher in trauma patients who subsequently developed acute lung injury compared to control trauma patients [46]. Furthermore, antioxidant supplementation in critically-ill trauma and surgical patients has been associated with less multi-organ failure and acute respiratory distress syndrome [47]. However, it remains to be determined how antioxidant therapies might specifically impact the early course of coagulopathy after trauma.

Limitations

Our conclusions are limited only to associations between fibrinogen function, one specific methionine oxidation, and clot formation after trauma. Further focused study in experimental models is required to reveal mechanisms of action. Our results in no way indicate that A α -Met476(SO) content is the only oxidative modification of fibrinogen after trauma, or is solely responsible for the witnessed changes in coagulation function. Given the ease in which fibrinogen is oxidized by multiple oxidative and nitrative stressors, it is likely that other relevant modifications are also associated with coagulopathy. The human samples that we examined were also limited in number. We were also unable to obtain plasma prior to any clinical therapy. Therefore, we could not fully separate fibrinogen oxidation produced purely from injury and blood loss from the effects of treatment in the human cohorts. The majority of oxidized fibrinogen may also have been converted to fibrin at wound sites or converted to fibrin degradation products by increase of fibrinolysis during TIC. These alternative oxidized fibrin products would have been lost during glycine purification of fibrinogen from plasma and undetectable by our specific MS-based detection methods. Oxidized fibrinogen is also found in the plasma in two compartments, free and bound to apo(a) of Lp(a) which may have been lost during purification [48]. Therefore, even the subtle increase of A α -Met476(SO)% we found in intact fibrinogen sampled from the systemic circulation may represent even more significant oxidation taking place more locally at wounds.

CONCLUSION

Selectively-increased methionine sulfoxide content of fibrinogen in vivo after trauma may signal coagulopathy after injury. Definitive mechanistic study of fibrinogen oxidation after trauma, including identification of additional oxidative modifications, is warranted.

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Highlights

- Fibrinogen methionine sulfoxide is increased in coagulopathic trauma patients.
- Methionine sulfoxide is preferentially formed in the fibrinogen A α -C domain.
- Oxidation by inflammation may modulate blood coagulation responses to injury.

Figure 1.

Associations between fibrinogen Aα-M476(SO)% and international normalized prothrombin time ratio (INR), thrombin time, and Reptilase time measured in plasma. R=Pearson product moment correlation coefficient.

Figure 2.

Increased Aα-MSO476 sulfoxide content is associated with fibrin polymerization impairment by reptilase activation at low levels corresponding to those found in trauma patients. Other oxidative modifications of fibrinogen are also possible after exposure to HOCL, but were not investigated.

Figure 3. Fibrinogen methionine sulfoxide content is preferentially-increased in coagulopathic trauma patients and is associated with important clinical variables

(A) Mean Aα-M476(SO)% is increased under coagulopathic conditions (INR>1.2) in trauma patients when detected directly in plasma. (B) Albumin M353(SO)% is not increased in the same plasma sample. Fibrinogen Aα-M476(SO)% was also negatively associated with base excess as a clinical marker of metabolic shock severity (C), and positively associated with injury severity measured using the Injury Severity Score (D) Bars= Mean and StdDev. R=Pearson product moment correlation coefficient. (Richmond Cohort)

Figure 4. Viscoelastic clot formation parameters by Aα-M476(SO)% in Emergency Department trauma patients

Whole blood viscoelastic clot formation measured in the Emergency Department using rapid TEG are compared by Aα-M476(SO)% quartile measured by UPLC-MS/MS-MRM. Percent clot lysis (LY30%) was significantly increased in the highest Aα-M476(SO)% quartile compared to all other quartiles. Points are individual measurements and bars represent median and interquartile range. P values= nonparametric Wilcoxon Rank Sums test.

Table 1

Physiological parameters of trauma subjects in the Emergency Department at the time of blood sample collection.

	Seattle, WA			Richmond, VA			Houston, TX		
	N	Mean	Std Dev	N	Mean	Std Dev	N	Mean	Std Dev
Systolic Blood Pressure (mmHg)	21	136.0	39.2	23	135.5	29.7	50	129.8	35.2
Diastolic Blood Pressure (mmHg)	21	96.9	27.0	23	83.3	21.2	50	76.4	21.8
Glasgow Coma Score [*]	16	8	6	22	12	5	50	7	5
Blood Hemoglobin conc. (g/dl)	21	12.1	2.6	23	12.3	2.9	49	12.9	2.2
Base Excess (meq/L) [*]	11	-5.5	4.1	21	-2.1	5.6	48	-3.8	6.9

^{*} Glasgow Coma Score is an aggregate score of neurological function after injury that predicts neurological outcomes. The maximum score is 15, and points are deducted for verbal, eye opening, and movement deficits elicited during the clinical exam. Lower scores indicate increasingly severe brain injury. Base excess represents the number of milliequivalents of base required to return the blood to normal pH=7.4 and is an indicator of acidosis incurred by hemorrhagic shock. More negative numbers indicate greater blood loss and more severe hemorrhagic shock.

Table 2

Fibrinogen Methionine Sulfoxide Content (% of Total)

	Control (INR 1.2)			Coagulopathy (INR>1.2)			
Methionine Position(Chain)	N	Mean	SEM	N	Mean	SEM	T test P value
422(β)	11	0.22%	0.02%	11	0.18%	0.02%	0.199
305(β)	11	1.45%	0.12%	11	1.78%	0.12%	0.063
314(β)	11	0.73%	0.14%	11	1.15%	0.14%	0.046
367(β)	11	1.67%	0.20%	11	1.64%	0.20%	0.917
373(β)	11	0.36%	0.03%	11	0.39%	0.03%	0.496
78(γ)	11	1.08%	0.08%	11	1.21%	0.08%	0.228
476(α)	11	1.83%	0.13%	11	2.46%	0.13%	0.003

INR, International Normalized Ratio