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The hyperglycemic byproduct methylglyoxal impairs anticoagulant activity through covalent adduction of antithrombin III

Richard Jacobson^{a,b,d}, Nicholas Mignemi^{a,c}, Kristie Rose^f, Lynda O'Rear^a, Suryakala Sarilla^c, Heidi E. Hamm^d, Joey V. Barnett^d, Ingrid M. Verhamme^c, and Jonathan Schoenecker^{a,b,c,d,e,*}

^a Vanderbilt University Medical Center, Department of Orthopaedics, 2200 Children's Way, Nashville, TN 37232-9565 United States

^b Vanderbilt University Medical Center, Department of Center for Bone Biology, 2200 Children's Way, Nashville, TN 37232-9565 United States

^c Vanderbilt University Medical Center, Department of Pathology, Microbiology and Immunology, 2200 Children's Way, Nashville, TN 37232-9565 United States

^d Vanderbilt University Medical Center, Department of Pharmacology, 2200 Children's Way, Nashville, TN 37232-9565 United States

^e Vanderbilt University Medical Center, Department of Pediatrics, 2200 Children's Way, Nashville, TN 37232-9565 United States

^f Vanderbilt University, Mass Spectrometry Research Center, Proteomics Laboratory, Nashville, TN 37232 United States

Abstract

Introduction—The blood coagulation system is a tightly regulated balance of procoagulant and anticoagulant factors, disruption of which can cause clinical complications. Diabetics are known to have a hypercoagulable phenotype, along with increased circulating levels of methylglyoxal (MGO) and decreased activity of the anticoagulant plasma protein antithrombin III (ATIII). MGO has been shown to inhibit ATIII activity in vitro, however the mechanism of inhibition is incompletely understood. As such, we designed this study to investigate the kinetics and mechanism of MGO-mediated ATIII inhibition.

Methods—MGO-mediated ATIII inhibition was confirmed using inverse experiments detecting activity of the ATIII targets thrombin and factor Xa. Fluorogenic assays were performed in both PBS and plasma after incubation of ATIII with MGO, at molar ratios comparable to those

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* Corresponding author at: Vanderbilt University Medical Center, Department of Orthopaedics, 2200 Children's Way, Nashville, TN 37232-9565. Tel.: +1 615 936 3080. jon.schoenecker@vanderbilt.edu (J. Schoenecker).

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observed in the plasma of diabetic patients. LC-coupled tandem mass spectrometry was utilized to investigate the exact mechanism of MGO-mediated ATIII inhibition.

Results and conclusions—MGO concentration-dependently attenuated inhibition of thrombin and factor Xa by ATIII in PBS-based assays, both in the presence and absence of heparin. In addition, MGO concentration-dependently inhibited ATIII activity in a plasma-based system, to the level of plasma completely deficient in ATIII, again both in the presence and absence of heparin. Results from LC-MS/MS experiments revealed that MGO covalently adducts the active site Arg 393 of ATIII through two distinct glyoxalation mechanisms. We posit that active site adduction is the mechanism of MGO-mediated inhibition of ATIII, and thus contributes to the underlying pathophysiology of the diabetic hypercoagulable state and complications thereof.

Keywords

Antithrombin III; Hypercoagulability; Diabetes; Methylglyoxal

Introduction

Cardiovascular complications are the leading cause of mortality in patients with diabetes mellitus [1]. The pathogenesis of these complications is multifactorial - but a significant contributor is the diabetic hypercoagulable state, the etiology of which is incompletely understood [2]. It is currently known that baseline hypercoagulability in diabetic patients promotes thrombosis, resulting in both macro- and microvascular complications [2].

Chronic hyperglycemia in diabetes increases basal rates of nonenzymatic glycosylation and subsequent loss of function of plasma proteins [3]. Glucose and its metabolites are known to alter proteins in discrete patterns dependent on the properties of both the protein and the metabolite in question [4]. This study focuses on the hyperglycemic byproduct methylglyoxal (MGO) and its interaction with the anticoagulant protein antithrombin III (ATIII). In normal physiology, ATIII bound to a heparin scaffold inactivates the procoagulant proteases thrombin and factor Xa, inhibiting coagulation of blood. Interference with this process by the byproducts of hyperglycemia may contribute to the diabetic hypercoagulable state.

Prior work in both humans and mice has revealed that hyperglycemia leads to reduced activity of circulating ATIII. Interestingly, hyperglycemic byproducts rather than glucose itself appear to have the largest role in adduction of plasma coagulation proteins [5,6]. In mice, this was attributable to decreased circulating ATIII antigen [7]; however, studies of human plasma illustrate decreased ATIII activity despite identical antigen levels between diabetics and normoglycemic patients [5]. Importantly, MGO is found in significantly elevated levels in the plasma of uncontrolled diabetics at baseline, reaching as high as 6 μM while nondiabetic control plasma had a concentration near 1 μM [8]. ATIII circulates at 1.3-5.2 μM on average [9]. Deficiencies in ATIII activity are strongly associated with increased risk of thrombotic events [10,11].

In order to phenocopy hypercoagulable diabetic conditions, we designed our plasma-based experiments with physiologic concentrations of ATIII and both physiologic and

supraphysiologic concentrations of MGO. Experimental conditions included the presence or absence of heparin, an endogenous and exogenous enhancer of ATIII activity [12]. Incubation with MGO is known to decrease ATIII's capacity to inhibit purified thrombin in a PBS-based system [13]. However, the exact mechanism of this interaction remains elusive. As such, determination of the molecular mechanism of loss of ATIII function in diabetics could lead to novel therapeutic pathways targeted at preventing thrombotic events in diabetics. To this end, prior *in vitro* work has revealed that certain natural extracts with proven human safety records and known antioxidant functions prevent loss of function in ATIII during incubation with MGO in dilute human plasma [14].

Prior studies have reported adduction of circulating plasma proteins by MGO [15]. As such, we developed the hypothesis that functionally critical residues on ATIII are covalently adducted during exposure to MGO, contributing to the loss of anticoagulant function. To test this hypothesis, we have investigated the interactions between MGO and ATIII using multiple approaches. Functional inactivation of ATIII after incubation with MGO was tested using kinetic assays in purified PBS-based systems as well as thrombin generation assays in human plasma. To investigate the biochemical mechanism of MGO-based inhibition of ATIII, tandem mass spectrometry was employed to explore covalent adduction at functionally significant ATIII residues.

Methods

Reagents

Purified human thrombin, ATIII and factor Xa were purchased from Haematologic Technologies, Inc (Essex Junction, USA). Purified methylglyoxal was purchased from Sigma Aldrich (St. Louis, USA). Fluorogenic thrombin substrate, Z-Gly-Gly-Arg-AMC, was purchased from Bachem (Torrance, CA). Fluorogenic Xa substrate Boc-Ile-Glu-Gly-Arg-AMC was purchased from Bachem (Torrance, CA). Human standard plasma and ATIII-deficient plasma were provided by Affinity biological (Ancaster, CAN). PBS with a pH of 7.4 and 9 g/L sodium chloride, 0.795 g/L disodium phosphate and 0.144 g/L potassium dihydrogen phosphate was purchased from Corning (Midland, MI). Heparin with an average molecular weight of 4500 Da was purchased from Sanofi (Bridgewater, NJ).

ATIII incubation and treatment

ATIII was incubated with MGO in PBS buffer, in a 1.7 mL micro-centrifuge tube at 37 °C, 5% CO₂ for 48 hours. Final concentrations were 25 µM ATIII, and MGO diluted in PBS buffer to final MGO:ATIII molar ratios of 2:1, 10:1, 20:1 and 54:1 yielding near-physiologic and supraphysiologic molar ratios. Control ATIII was incubated in PBS buffer.

Purified thrombin kinetic assay

All assays were read on a Synergy2 plate reader from Biotek (Winooski, VT) with an excitation wavelength of 390 nm and an emission wavelength of 460 nm. Reagents and substrates were diluted in PBS and assays were run on opaque-walled 96 well plates. Upon addition of the final reagent for each assay, plates were shaken for 5 seconds and kinetic reads were initiated. Fluorescence in each well was measured once every 3 seconds for a

total of 90 minutes. Initial rates of change were defined as the average change in fluorescence over time for the first 20 seconds of each reaction. Apparent first-order rate constants of thrombin inactivation were calculated from exponential analysis of the complete time traces [16,17].

Fluorogenic thrombin substrate was warmed to room temperature and added to wells at a final concentration of 420 μ M in PBS. MGO- or vehicle-treated ATIII were pipetted into each well at a final concentration of 250 nM. In a separate set of experiments, heparin was added at a final concentration of 250 nM. Purified thrombin was diluted and auto-dispensed into each well at a final concentration of 50 nM immediately prior to the start of the assay. Control experiments with equal volumes of MGO dilutions were included to define if MGO carry-over in the ATIII samples had any effect on the inactivation rates.

Purified factor Xa kinetic assay

Assays were performed as described above on a Synergy2 reader with an excitation wavelength of 390 nm and an emission wavelength of 460 nm. Bachem Xa substrate was warmed to room temperature and added to wells at a final concentration of 210 μ M in PBS. Native and MGO-incubated ATIII was added to wells at a final concentration of 250 nM. Factor Xa was diluted and auto-dispensed into each well at a final concentration of 50 nM immediately prior to the start of the assay.

Thrombin Generation Assay

Assays in human plasma were performed as a modified version of the protocol described by Chandler et al. [18]. Briefly, 80 μ L of plasma was added to wells followed by the addition of 12.5 μ L of PBS and 420 μ M thrombin substrate (Z-Gly-Gly-Arg-AMC), which generates a fluorescent product upon thrombin-catalyzed hydrolysis. Finally, calcium chloride was injected into the wells at a final concentration of 14 mM for a final volume of 115 μ L per well. The plate was shaken for 3 seconds and change in fluorescence was monitored every 30 seconds for 2 hours on the Synergy 2 plate reader at an excitation wavelength of 390 nm and emission wavelength of 460 nm. All data was then adjusted for substrate depletion and fluorescent inner filter effect using the third order polynomial described by Chandler et al. [18]. Change in slope of the corrected data was then graphed vs time, and peak height, time to peak height, lag time, and ETP were calculated.

Purified ATIII was added back to ATIII-deficient plasma (ATD) at multiple concentrations to determine the final molarity that matched the ATIII activity in whole plasma as measured by decreased thrombin generation. ATIII added back at 1.3 μ M restored activity in ATD to whole plasma levels and was used for all MGO-treated runs. Purified human ATIII was incubated at 37 °C for 48 hours with various concentrations of MGO, all in the physiological range for diabetics, and added back to ATD plasma.

LC-Coupled Tandem Mass Spectrometry

ATII and MGO-treated ATIII (1.5 μ g each) were diluted with 100 mM ammonium bicarbonate, treated with 1 μ L of 50 mM TCEP for 30 min, followed by 1 μ L of 100 mM iodoacetamide to carbamidomethylate Cys residues. ATIII was then digested with 80 ng of

endoproteinase AspN at 37 °C overnight, followed by digestion with 200 ng of trypsin overnight at 37 °C. ATIII digestions were then acidified to 0.1% formic acid. For analysis of each protein digest by LC-coupled tandem mass spectrometry (LC-MS/MS), peptides were loaded onto a capillary reverse-phase analytical column (360 µm o.d. × 100 µm i.d.) using an Eksigent NanoLC Ultra HPLC and autosampler. The analytical column was packed with 20 cm of C18 reversed-phase material (Jupiter, 3 µm beads, 300 Å, Phenomenex), directly into a laser-pulled emitter tip. Peptides were gradient-eluted over a 90-minute gradient at a flow rate of 500 nL/min. The mobile phase solvents consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Gradient-eluted peptides were mass analyzed on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific), equipped with a nanoelectrospray ionization source. The instrument was operated using a data-dependent method with dynamic exclusion enabled. Full-scan spectra were acquired with the Orbitrap (resolution 60,000), and the top 16 most abundant ions in each MS scan were selected for fragmentation by collision-induced dissociation (CID) in the LTQ Velos ion trap in a data-dependent fashion, with dynamic exclusion enabled. For identification of ATIII peptides, tandem mass spectra were searched with Sequest (Thermo Fisher Scientific) against a human subset database created from the UniprotKB protein database (www.uniprot.org). Variable modifications of + 57.0214 on Cys (carbamidomethylation), + 15.9949 on Met (oxidation), and + 54.010565 and + 72.021129 on Arg (corresponding to MGO adducts MG-HI and MG-DH, respectively) were included for database searching. Search results were assembled using Scaffold 3.0 (Proteome Software), and spectra acquired of ATIII peptides of interest were inspected using Xcalibur 2.2 Qual Browser software (Thermo Scientific). Tandem mass spectra were examined by manual interrogation to validate MG-derived adducts on R393. Accurate mass measurements acquired in the Orbitrap were used to generate extracted ion chromatograms (XICs) for R393 modified peptides of ATIII. Using a 10 ppm tolerance, the theoretical monoisotopic m/z values of the observed $[M + 3H]^+$ precursor ions were used for generating XICs.

Results

MGO incubation concentration-dependently decreases ATIII activity in a purified PBS system

Anti-Thrombin activity—We first set out to validate a thrombin activity assay based on cleavage of the fluorogenic substrate Z-Gly-Gly-Arg-AMC. Kinetic activity assays were performed with a substrate concentration of 420 µM and a thrombin concentration of 50 nM in PBS buffer to optimize the signal while preserving a linear response of fluorescent product formation. Under our experimental conditions a linear response over 20 minutes was obtained, which allowed comparison of initial rates of product generation in assays containing ATIII or MGO-modified ATIII. We then measured ATIII inhibitory activity in coupled assays with 50 nM thrombin and the fluorogenic substrate as competitor. Increasing ATIII concentrations caused a decrease in limiting fluorescence signal due to the increasing rates of thrombin inactivation. For the remaining kinetic experiments in PBS, 250 nM ATIII was selected, which represents a 5:1 ATIII:thrombin molar ratio. Control assays using fluorogenic substrate alone in PBS, and assays containing maximum MGO carry-over

concentrations in PBS of 13 μM in the final reaction mixtures in the absence of ATIII were identical, indicating that these low concentrations did not affect thrombin activity in the assay.

Assays were performed with 250 nM ATIII that had been incubated for 48 hours with increasing concentrations of MGO, at molar MGO: ATIII ratios of 2:1, 10:1, 20:1 and 54:1. Two sets of experiments were performed, one in the absence of heparin, and one in the presence of 250 nM heparin with an average molecular weight of 4500 Da. Schematics of these assays are shown in Fig. 1. The results are shown in Fig. 2. Incubation with MGO impaired ATIII's ability to inhibit thrombin in a concentration-dependent manner (Fig. 2A,B). The peak fluorescence increased, and the inactivation rate constants decreased (**2A**), and the initial rate of change (**2B**) in fluorescence after 20 seconds increased with increasing concentrations of MGO, reflecting less thrombin inactivation with impaired ATIII.

Heparin in the presence of ATIII acts as a scaffold, increasing its inhibitory capacity through steric alignment of inhibitor and enzyme on a template, and by invoking a conformational change in ATIII. Whereas high MW heparin (> MW 6,000) mainly acts by forming a template, low MW heparin causes a conformational change in ATIII that renders it more reactive to factor Xa, while exhibiting modest but measurable catalytic effects on the thrombin reaction. As expected, thrombin was inactivated more rapidly by ATIII in the presence of heparin (Fig. 2C,D). Importantly, the trends in the effect of MGO on ATIII held true in the presence of heparin. Incubation with increasing concentrations of MGO again concentration-dependently diminished the inhibition of thrombin by ATIII as measured by peak fluorescence and inactivation rate constants, (**2C**) and initial rate of change in fluorescence (**2D**).

Anti-Xa activity—To confirm that our results were a global inhibition of ATIII activity rather than specific to its activity toward thrombin, we performed kinetic assays with active factor Xa and MGO-treated ATIII. Assays performed in PBS solution with purified factor Xa, its specific fluorogenic substrate Bachem I-1100, and ATIII recapitulated the results observed in thrombin activity assays. Optimization of fluorogenic substrate concentration indicated a good signal at 210 μM with 50 nM factor Xa, which was used in further experiments. As expected, increasing concentrations of ATIII with constant concentrations of factor Xa (50 nM) and substrate (210 μM) inhibited the ability of factor Xa to cleave its substrate as indicated by decreasing peak fluorescence and initial rate of change in fluorescence, and increasing inactivation rate constants. When assays were performed with constant concentrations of factor Xa (50 nM), fluorogenic substrate (210 μM) and ATIII (250 nM), preincubation of ATIII with increasing molar ratios of MGO showed a concentration-dependent decrease of ATIII's ability to inhibit factor Xa substrate cleavage in the absence and presence of 250 nM heparin (Fig. 3A, B).

The rate constants of thrombin and factor Xa inactivation by control and MGO-treated ATIII were calculated, and their ratios were plotted vs. the MGO concentrations that were used in the 48-hour ATIII incubations. As shown in Fig. 4, ATIII modification equally affected thrombin inactivation in the absence and presence of low MW heparin, and factor Xa inactivation in the presence of low MW heparin. The chosen concentration of low MW

heparin was sufficient to cause discernible rate acceleration in the thrombin and factor Xa assays.

ATIII inactivation by MGO was quantified using second-order rate constants, as shown in Table 1. Activity of ATIII against thrombin is significantly attenuated by MGO in both the presence and absence of low molecular weight heparin. Notably, anti-Xa activity is attenuated significantly more in the presence of low molecular weight heparin than in its absence (not shown).

MGO incubation concentration-dependently decreases ATIII activity in human plasma

We designed the following ex vivo experiments to test whether ATIII activity was also attenuated in plasma-based assays containing a full complement of coagulation factors. ATIII incubated with either PBS or the indicated molar ratios of MGO was added back to ATIII-deficient (ATD) plasma. Optimization assays (data not shown) revealed that control (PBS-incubated) ATIII showed added back to ATD plasma at 1.3 μ M inhibited thrombin generation at a level similar to native ATIII in whole plasma.

ATD plasma supplemented with MGO-treated ATIII displayed increasing thrombin generation in a direct relationship to the concentration of MGO in incubation with ATIII (Fig. 5A). ATIII incubated with 6.5 μ M MGO (5:1 MG:ATIII molar ratio), added back to deficient plasma permitted thrombin activity equal to that of completely ATIII-deficient plasma.

In plasma with 1.3 μ M heparin added, thrombin activity in full plasma was significantly decreased, as expected. Again, we observed a concentration-dependent attenuation of ATIII activity directly related to the concentration of MGO in incubation with ATIII before add-back into deficient plasma (Fig. 5B). Thrombin activity equal to ATD plasma was reached in assays using ATIII incubated with a 10:1 molar ratio of MGO:ATIII, compared to 5:1 in the absence of heparin.

Incubation with MGO leads to covalent adduction of a functionally critical arginine residue on ATIII

As incubation with MGO led to global inhibition of ATIII serpin activity, we next chose to investigate the mechanism of inhibition. The active site R393 of ATIII is the proteolytic target of both thrombin and factor Xa, and as such we chose to investigate whether or not this site was covalently adducted, and thus its affinity for thrombin and Xa altered, by MGO during incubation. In the current study, we used LC coupled-tandem mass spectrometry to identify sites of modification following MGO treatment of ATIII. After 48-hr incubation with MGO, ATIII was proteolytically digested to generate peptides for mass spectrometric analysis. Due to the presence of Arg and Lys residues in particular regions of the ATIII primary amino acid sequence, ATIII digestion with trypsin, which cleaves C-terminal to Arg and Lys residues, was preferred for generating peptides that would perform well during LC-MS/MS. However, since ATIII is a trypsin inhibitor, endoprotease AspN which is unaffected by ATIII activity was used initially for proteolysis of ATIII.

After treatment with AspN, ATIII was subsequently digested with trypsin, and ATIII peptides were gradient-eluted using reverse phase LC and mass analyzed on an LTQ-Orbitrap Velos mass spectrometer. Following data acquisition, the resulting tandem mass spectra were searched via SEQUEST against a human database, and assigned spectra to ATIII peptides of interest were manually examined and validated. Among those peptides detected, ATIII peptides containing R393 were identified and are of particular interest. In addition to detecting the peptide unmodified, two different MGO-modified forms of peptide AFLEVNEEGSEAAASTAVVIAGRSLNPNR (371-399) were identified in LC-MS/MS analysis of the 1:20 MGO-treated ATIII sample. Relative to the mass of the unmodified peptide, the adducted ATIII peptides have mass shifts of 54.01 Da and 72.02 Da, corresponding to the methylglyoxal-derived adducts hydroimidazolone (MG-HI) and dihydroxyimidazolidine (MG-DH), respectively. The tandem mass spectrum of the MG-HI adducted ATIII peptide is shown in Fig. 6. A prominent series of b- and y-type product ions were observed and are annotated accordingly in the spectrum. Product ions in the y ion series beginning with the y8 ion displayed mass shifts consistent with MG-HI adduction, confirming modification of R393. The calculated mass errors of the unmodified and MGO-adducted peptides were each within 5 ppm of theoretical values, adding high confidence in the identification of the MGO-modified peptide forms.

The abundance of MGO modifications at the ATIII active site depended directly on the concentration of MGO in incubation, as shown in Fig. 7. Extracted ion chromatogram data from our LC-MS-MS experiments was used to quantify the absolute amount of adducted peptide, following MGO treatment. The intensities of ATIII peptides modified by MG-HI and MG-DH reveal a concentration-responsive relationship.

Discussion

We present a mechanistic investigation into the clinical observation of hypercoagulability in diabetic patients. In this study, we tested the hypothesis that the decrease in ATIII activity known to occur after exposure to MGO is due to covalent adduction of the protein at functionally critical residues. Our initial *in vitro* assays included purified proteins in a PBS based kinetic assay, which recapitulated past work in the field based on MGO-mediated attenuation of ATIII's inhibition of both thrombin and factor Xa [14,16]. We expanded the concept of MGO-mediated ATIII inactivation to include a thrombin generation assay in human plasma both in the presence and absence of heparin. In addition, we have confirmed two specific reactions leading to covalent adduction of the ATIII active site, arginine 393, by MGO using mass spectrometry.

Thrombin generation assays measure the capacity of whole plasma to activate thrombin, an essential coagulation protease [18]. Their output, endogenous thrombin potential (ETP) correlates with a procoagulant phenotype. Our data in ATIII-deficient plasma represent concentration dependent attenuation of ATIII's inhibitory activity on thrombin in the presence of a full complement of coagulation proteins. Our plasma-based system expands on past work showing decreased ATIII inhibitory activity in purified systems containing only one other coagulation protein. Significantly, the absolute and relative concentrations of MGO and ATIII in these studies match those observed *in vivo*. Strengths of this study

include the fact that ATIII activity was significantly attenuated after incubation with MGO concentrations at or below those observed in diabetics [8]. Relative ATIII activities after incubation in the diabetic range of MGO concentration were at or below those previously shown to be associated with clinical thrombotic complications [10]. Limitations of our model include the fact that ATIII was incubated as a purified protein in PBS, meaning that the remaining pro-coagulant and anti-coagulant proteins present in plasma were not exposed to MGO prior to supplementation with ATIII. Further studies, possibly including the development of a monoclonal antibody specific for MGO-modified ATIII, are necessary to confirm *in vivo* adduction of ATIII in diabetic patients.

To our knowledge, specific residues of ATIII that are adducted by MGO have not previously been identified. We believe our mass spectro-metric findings are particularly relevant, as they confirm adduction of the active site of ATIII. We thus propose that MGO impairs ATIII's inhibitory action through direct interference with the interactions between the active site Arg-393 and its targets on both thrombin and factor Xa. Notably, the effects on anti-thrombin activity are significant in both the presence and absence of low molecular weight heparin, whereas effects on anti-Xa activity are significantly more pronounced in the presence of low molecular weight heparin. Thrombin and factor Xa cleave the active site loop of ATIII, resulting in their entrapment and permanent inactivation through a suicide inhibitory mechanism [20]. MGO's impairment of ATIII activity against both thrombin and factor Xa supports our observation that the active site of ATIII, rather than a substrate-specific site has been inhibited. In addition, loss of activity in ATIII may be due to adducted arginine residues in the heparin-binding domain. Steric inhibition of the ATIII-heparin interaction could hinder the enhancement of ATIII activity in a mechanism independent from active site adduction. However, since ATIII inhibition was observed both in the presence and absence of heparin, further investigation into the heparin-related mechanism is needed. Our judicious choice of low concentrations of low MW heparin allowed selective investigation of Arg-393 adduction as a cause of inhibitory activity loss. Future experiments of thrombin inactivation accelerated by high MW heparins spanning the complete heparin-binding site of ATIII may determine whether critical Arg or Lys residues in this heparin-binding site are also susceptible to adduction.

Prior work has shown a decrease in secretion and thus circulating levels and activity of ATIII in mice with streptozotocin-induced diabetes [7]. However, this contradicts human studies illustrating normal circulating antigen levels of ATIII with significantly decreased activity [5]. The mechanism proposed in the current study, active site adduction and inactivation of soluble ATIII, is more illustrative of pathophysiology observed in human subjects with diabetes. In addition, the extent of glyoxalation of circulating ATIII could provide a useful clinical marker for the severity of diabetes-induced hypercoagulability.

The average biologic half-life of ATIII added to plasma is 60 hours [19]. Based on this, we posit that our incubations, with ATIII and MGO concentrations similar to diabetic physiology, effectively simulate the MGO exposure of circulating ATIII in diabetic patients. Our mass spectrometry results indicate that covalent adduction of the active site arginine 393 in ATIII underlies the declining inhibitory activity observed after incubation with MGO. Taken together, our functional assays and mass spectrometric results indicate that elevated

circulating MGO in diabetic plasma contributes to global and irreversible ATIII inactivation. Hypercoagulability in diabetic patients is multifactorial and also involves endothelial dysfunction [21]. However, modulation of activity of soluble anticoagulant proteins is a clinically tested method of preventing thrombosis, and our findings offer novel targets under this paradigm. Prior work has empirically identified nontoxic natural extracts that prevent loss of ATIII function during exposure to MGO in human plasma [14]. Our identification of the specific adduction reaction reveals a novel, targeted therapeutic opportunity for prevention of the diabetic chronic hypercoagulable state.

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Fig. 1. Schematic representation of fluorogenic assays determining MGO-induced ATIII inhibition

A) Thrombin cleaves its fluorogenic substrate, increasing fluorescence in a thrombin activity-dependent manner. B) ATIII inhibits thrombin substrate cleavage in an activity-dependent manner. C) MGO impairs ATIII inhibition of thrombin after pre-incubation in a concentration-dependent manner. Similar assays were run with factor Xa and its specific fluorogenic substrate, rather than thrombin.

Fig. 2. MGO concentration-dependently impairs thrombin inactivation by ATIII

Representative data from one of two identical experiments performed in triplicate. (T-TS assay in the absence of ATIII; 0 MGO: ATIII incubated in PBS; ATIII incubated with MGO at indicated molar ratios in PBS). (A) MGO impairs ATIII's inhibition of thrombin, causing elevated thrombin activity indicated by increased peak fluorescence and decreased inactivation rate. (B) Change of initial rate of fluorogenic substrate cleavage by thrombin in the presence of ATIII pre-incubated with indicated concentrations of MGO. Rate data show that higher MGO concentrations in pre-incubation produce higher initial reaction velocities, indicating decreased ATIII inhibition of thrombin. (C) MGO's effects on ATIII persist in the presence of a heparin scaffold. (D) Initial rate data for heparin-catalyzed reactions of incubated ATIII with thrombin again revealing increased reaction rates with increasing MGO concentrations during pre-incubation. * Indicates significantly higher thrombin activity than runs with ATIII incubated in the absence of MGO.

Fig. 3. MGO diminishes inhibition of factor Xa by ATIII in a purified system, resulting in a similar fluorogenic activity pattern as observed in thrombin activity assays

Representative data from one of two separate experiments with each treatment run in triplicate. Pre-incubation of ATIII with MGO at the indicated molar ratios impaired ATIII's ability to inhibit factor Xa in a concentration-dependent manner both in the absence (A) and in the presence (B) of 250 nM low molecular weight heparin. Impairment of ATIII activity was more pronounced in the presence of heparin, which significantly increased ATIII activity, as indicated by decreased Xa activity, in the absence of MGO in pre-incubation (0 MGO run).

Fig. 4. Ratio of the rate constants of protease inactivation by ATIII as a function of MGO in pre-incubation

Single exponential analysis of the time traces of inactivation gave first-order rate constants for thrombin and factor Xa inactivation by 250 nM native ATIII, or by 250 nM ATIII from a pre-incubated mixture of 25 μM ATIII with MGO at the indicated concentrations for 48 hours. The ratios of the rate constants for reactions with modified vs. native ATIII are shown as the ratio AT/AT_0 , with AT the remaining inhibitory active ATIII after pre-incubation, and AT_0 the reference ATIII inhibitory activity without MGO modification. Similar, significant decreases of inhibitory active ATIII as a function of increasing MGO concentration are shown for thrombin inactivation in the absence (●) and presence (○) of heparin, and factor Xa inactivation in the presence of heparin (◇).

Fig. 5. MGO concentration-dependently decreases ATIII activity in plasma in the presence or absence of heparin

Thrombin activity was measured in ATIII-deficient plasma after addition of ATIII incubated with indicated concentrations of MGO. Fluorescence is shown as Endogenous Thrombin Potential (ETP), measured in arbitrary units derived from area under the curve of a Thrombin Generation Assay. Thrombin activity in whole plasma and ATIII-deficient plasma are indicated by “Plasma” and “Plasma (ATD)” dotted lines. (A) Increasing molarity of MGO in the incubation revealed concentration-dependent inactivation of ATIII. Samples with no significant difference in ETP when compared to ATD plasma were deemed to have maximum thrombin activity, first observed with a 5:1 MGO:ATIII molar ratio. (B) Low molecular weight heparin increased ATIII activity in whole plasma but not ATD plasma, as expected. MGO incubation again concentration-dependently decreased ATIII activity in the presence of heparin. Maximum thrombin activity was not reached until a 10: MGO:ATIII molar ratio was used.

Fig. 6. MGO covalently adducts the active site R393 of ATIII during incubation in PBS
 This figure shows the MS/MS spectrum of MG-HI-adducted ATIII peptide (residues 371-399). The $[M + 3H]^{+3}$ precursor ion with m/z 1009.8 was selected for fragmentation in the 1:20 molar ratio MGO-treated ATIII analysis. The observed singly and doubly protonated b- and y-type product ions are assigned to their corresponding m/z peaks in the tandem mass spectrum. The amino acid sequence is provided above the annotated spectrum, and the interresidue-placed brackets denote sites of amide bond fragmentation that occurred with collision-induced dissociation (CID). b-type product ions correspond to the resulting fragment ions that contain the N-terminus of the peptide, and y-type ions correspond to C-terminal peptide fragments. Asterisks are used to indicate product ions that are shifted in mass due to addition of the MG-HI adduct at R393.

Fig. 7. Relative abundance of ATIII peptide forms modified with MGO-derived adducts
 Extracted ion chromatograms (XICs) were generated using m/z values of 1009.5108 and 1015.5143, which correspond to the $[M + 3H]^{+3}$ precursor ions of both hydroimidazolone-modified (MG-HI) and dihydroxyimidazolidine-modified (MG-DH) adducted forms of ATIII peptide AFLEVNEEGSEAASTAVVIAGRSLNPNR (residues 371-399). The bar graph shows the signal intensities measured at the apex of the chromatographic peak for modified ATIII R393 containing peptides, and indicates that concentration of MGO in incubation with ATIII correlates to the amount of adduction at the active site arginine. The concentration response holds true for both types, MG-HI and MG-DH, of adduction observed on R393.

Table 1

Second-order rate constants were calculated for thrombin inactivation by antithrombin in the absence and presence of LMW heparin, and for factor Xa inactivation by antithrombin in the presence of LMW heparin, after incubation of antithrombin with increasing MGO concentrations. Second-order rate constants (k^*) were calculated by dividing the experimentally measured first-order rate constants from the competitive fluorogenic assays by the antithrombin concentration (250 nM). Correction factors ($1 + [S]_0/K_m$) were applied to the first-order rate constants to account for the competition of 420 μ M and 210 μ M fluorogenic substrate in the thrombin and fXa assays, respectively, with experimental K_m values of 200 μ M and 160 μ M in reaction buffer at 37 °C. Results are averages from assays in triplicate, and errors are given as 1SD.

MGO (μ M)	AT – IIa (no heparin) apparent k^* ($M^{-1} s^{-1}$)	AT – IIa (LMW heparin) apparent k^* ($M^{-1} s^{-1}$)	AT – fXa (LMW heparin) apparent k^* ($M^{-1} s^{-1}$)
0.00	10,800 $\pm$ 600	16,000 $\pm$ 1,000	122,000 $\pm$ 14,000
0.25	9,000 $\pm$ 300	13,000 $\pm$ 1,000	74,000 $\pm$ 9,000
0.50	6,700 $\pm$ 200	8,400 $\pm$ 1,000	83,000 $\pm$ 9,000
1.25	4,500 $\pm$ 90	7,800 $\pm$ 1,000	48,000 $\pm$ 5,000
2.50	2,900 $\pm$ 100	6,800 $\pm$ 300	37,000 $\pm$ 5,000
5.00	4,500 $\pm$ 200	4,300 $\pm$ 300	11,300 $\pm$ 1,000
13.50	4,500 $\pm$ 90	3,700 $\pm$ 300	9,400 $\pm$ 1,000