Thrombosis Research 134 (2014) 1350–1357

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

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

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ARTICLE INFO

Article history:
Received 25 June 2014
Received in revised form 24 September 2014
Accepted 30 September 2014
Available online 5 October 2014

Keywords:
Antithrombin III
Hypercoagulability
Diabetes
Methylglyoxal

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 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.

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

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http://dx.doi.org/10.1016/j.thromres.2014.09.038
0049-3848/

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 microcentrifuge tube at 37 °C, 5% CO_2 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 μM 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 μM 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 μl of plasma was added to wells followed by the addition of 12.5 μl of PBS and 420 μM 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 μl 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 descried 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 μM 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 μg each) were diluted with 100 mM ammonium bicarbonate, treated with 1 μL of 50 mM TCEP for 30 min, followed by 1 μL 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