Simultaneous Exposure of Sites in von Willebrand Factor for Glycoprotein Ib Binding and ADAMTS13 Cleavage Studies With Ristocetin

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Objective—Platelet-bound von Willebrand factor (VWF) was recently demonstrated to be a better substrate for ADAMTS13, suggesting that 1 conformational change exposes both the glycoprotein Ibα binding site in the A1 domain and the ADAMTS13 cleavage site in the A2 domain. Because ristocetin induces VWF to bind glycoprotein Ibα in the absence of shear stress, we evaluated whether it could also enhance ADAMTS13 proteolysis of VWF.

Methods and Results—We used several VWF sources: plasma, purified plasma VWF, recombinant VWF fragments encompassing A1A2A3, A1A2, and 2 A2 domains, 1 containing a ristocetin-binding site (Asp1459–His1472) and the other lacking it. Ristocetin accelerated ADAMTS13 cleavage of multimeric VWF and of each of the recombinant VWF fragments except for the A2 domain lacking the ristocetin-binding site. We also examined the effect of ristocetin on the conformation of the A2 domain by assessing its effect on the susceptibility of Met1606 at the ADAMTS13 cleavage site to be oxidized by hypochlorous acid. Ristocetin markedly enhanced oxidation of Met1606 and Met1521 of the A2 domain.

Conclusion—These data indicate that exposure of the sites for glycoprotein Ibα and ADAMTS13 are coupled, explaining why platelet-bound VWF is a better ADAMTS13 substrate and why enhanced proteolysis is often observed in type 2B von Willebrand disease. (Arterioscler Thromb Vasc Biol. 2012;32:2625-2630.)

Key Words: von Willebrand factor ■ ADAMTS13 ■ ristocetin ■ platelet adhesion ■ oxidation

Ristocetin, a peptide antibiotic from the soil bacterium Nocardia lurida, has been used for decades as a tool to diagnose deficiency or dysfunction of von Willebrand factor (VWF) in von Willebrand disease.1–3 Ristocetin is able to assess the functional state of VWF because it induces the interaction of VWF with the platelet glycoprotein (GP) Ib-IX-V complex in the absence of shear stress or VWF immobilization, conditions normally required in vivo for this interaction.4 Presumably, ristocetin is able to do this by inducing an allosteric change in VWF that exposes the binding site for glycoprotein Ibα (GPIbα).5,6 Ristocetin is 1 of the 2 widely used modulators of the VWF–GPIbα interaction (the other being botrocetin), and the one that induces an interaction that most closely mimics shear-induced platelet adhesion and aggregation.5,7 Two binding sites for ristocetin have been identified within VWF flanking the platelet-binding A1 domain: Cys1237–Pro1251 and Glu1463–Asp14728–10 (Figure 1A). Both sites are proline rich and brought into proximity by a disulfide bond between Cys1272 and Cys1458. An Asp/His1472 polymorphism in the second ristocetin-binding site has been shown to affect platelet aggregation at low concentrations of ristocetin.11

Previous studies12,13 found that GPIbα-bound VWF fragments or platelet-bound VWF multimers were better substrates for the plasma metalloprotease ADAMTS13, raising the possibility that exposure of the GPIbα binding site on VWF could be coupled to the exposure of the ADAMTS13 cleavage site. Another possibility to explain accelerated proteolysis of platelet-bound VWF multimers would be that the tensile force experienced by a VWF multimer with multiple bound platelets in a shear field would be sufficient to stretch VWF and expose the ADAMTS13 cleavage site, as force-induced exposure of this site has been demonstrated in several studies.14–16 Here, we evaluated whether ristocetin alone could enhance ADAMTS13 cleavage of VWF in the absence of shear force, using both multimeric plasma VWF and recombinant monomeric VWF fragments.

Materials and Methods

Materials

The following materials were used in this study: ristocetin (American Biochemical and Pharmaceutical Ltd, Marlton, NJ); H2O2 (VWR International, West Chester, PA); methionine (MP Biomedicals, Inc, Solon, OH); biotin, doxycycline, and vancomycin (Sigma-Aldrich, St. Louis, MO); hypochlorous acid (Ancillary Science, Inc, St. Louis, MO); ADAMTS13 (Recombinant ADAMTS13, purity > 95%, Air judicata, Inc, San Diego, CA); heparin (Innovative Reagents, Inc, Los Angeles, CA); 1, 235-Iodoiodoacetate (Amersham Biosciences, Piscataway, NJ); N-ethylmaleimide (NEM, Sigma-Aldrich); and the VWF-containing plasma products and recombinant VWF fragments were from the Plasma Products Institute (North Providence, RI).

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Inc, St. Louis, MO); sequencing-grade trypsin (Promega, Madison, WI); trifluoroacetic acid and formic acid (EMD, Merck KGaA, Darmstadt, Germany); rabbit anti-human VWF polyclonal antibody (Dako North America, Inc, Carpinteria, CA); monoclonal ADAMTS13 antibody (A10) and control antibody (Abcam, Cambridge, MA); myeloperoxidase (Athens Research Technologies, Athens, Georgia); FreeStyle 293 serum-free medium and puromycin (Invitrogen Corporation, Carlsbad, CA); HEK 293 Tet-On cells (Clontech, Mountain View, CA); streptavidin–HRP (Thermal Scientific, Rockford, IL); Ultra-10K filter and Immobilon Western HRP substrate (Millipore, Billerica, MA); gradient polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA); citrated pooled normal plasma (Precision BioLogic, Nova Scotia, Canada); Seakem gold agarose (Cambrex Bio Science Rockland Inc., ME); Sephadex G25 (GE Healthcare, Piscataway, NJ); Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA); and synthetic peptides (GenScript, Piscataway, NJ). Plasma VWF was purified from cryoprecipitate as described previously.17

Recombinant Proteins
Recombinant human ADAMTS13 and VWF fragments were expressed as fusion proteins with the pNBioSec vector in HEK293 Tet-On cells, as previously described.17 In this expression system, the secreted recombinant proteins contained 2 tags: an N-terminal biotin tag and a C-terminal protein C epitope tag. Serum-free cell-culture medium containing the secreted recombinant proteins was concentrated 10-fold by centrifugation over an Ultra-10K filter and desalted over Sephadex G25 into 10 mmol/L HEPES and 2 mmol/L CaCl₂, pH 7.4. The recombinant proteins were stable and were used without further purification. The concentration of recombinant proteins was determined in Western blots in comparison with albumin which was biotinylated to 4 biotins per molecule. Figure 1B shows the VWF sequences encompassed in the recombinant fragments and synthetic peptides.

ADAMTS13 Cleavage Assays
We examined ADAMTS13 cleavage of VWF in several ways: (1) cleavage of purified VWF with recombinant ADAMTS13; (2) cleavage of VWF in plasma by endogenous plasma ADAMTS13; and (3) cleavage of recombinant VWF fragments by recombinant ADAMTS13. In each case, the reaction mixture contained 1.5 mol/L urea. Each assay is a modification of the ADAMTS13 cleavage assay published by Nishio et al.12

Cleavage of Purified VWF With Recombinant ADAMTS13
Purified VWF and recombinant ADAMTS13 were incubated at 37°C in buffer (10 mmol/L HEPES and 6.5 mmol/L BaCl₂, pH 7.4), in the presence of urea or ristocetin. The reactions were stopped at the indicated times with EDTA (final concentration 10 mmol/L). The concentrations of ADAMTS13 and VWF were 0.4 µg/mL (2 nmol/L, molecular weight 190,000) and 2 µg/mL (8 nmol/L of VWF monomer, molecular weight: 250,000), respectively. The molar ratio of recombinant ADAMTS13 to VWF monomer in our assay was 1:4, which is higher than that in plasma (1:8), assuming ADAMTS13 is 5 nmol/L and VWF monomer is 40 nmol/L in plasma. One µg/mL of recombinant ADAMTS13 is equivalent to 1 U/mL of ADAMTS13 in plasma.

Cleavage of VWF in Plasma by Endogenous Plasma ADAMTS13
Cleavage of VWF by ADAMTS13 in citrated pooled normal plasma was examined by incubating 10-fold diluted plasma in buffer (10 mmol/L HEPES and 6.5 mmol/L BaCl₂, pH 7.4) containing either urea or ristocetin at 37°C for various times. Those samples incubated overnight were either left untreated, or treated with 10 mmol/L EDTA or a monoclonal ADAMTS13 antibody or control antibody (final concentration 20 µg/mL). At the end of the incubations (at the indicated times or after overnight incubation), EDTA was added to each sample to prevent further VWF cleavage by ADAMTS13.

The VWF multimer pattern was evaluated by electrophoresis on 1.5% agarose gels, followed by Western blotting and detection with a horseradish peroxidase–conjugated polyclonal VWF antibody as previously described.19

Cleavage of Recombinant VWF Fragments by Recombinant ADAMTS13
Cleavage of recombinant VWF fragments (1 µg/mL) by recombinant ADAMTS13 (0.15 µg/mL) was carried out at 37°C for 5 hours in the presence of either 1.5 mol/L urea or 1.5 mg/mL ristocetin in plasma.
10 mmol/L HEPES and 2 mmol/L CaCl₂, pH 7.4. The reactions were stopped using EDTA. Cleavage products were reduced with 0.36 mol/L β-mercaptoethanol in SDS sample buffer and analyzed by electrophoresis on 4% to 15% gradient polyacrylamide gels. Cleavage products were transferred to nitrocellulose membranes, blocked with 1% albumin in Tris-buffered saline with 0.05% Tween-20, probed with horseradish peroxidase–conjugated streptavidin, and visualized with the chemiluminescent Immobilon Western HRP substrate. Cleavage products were quantified by densitometry, and the extent of cleavage was expressed as fractional cleavage based on the ratio of band density of the cleavage product to the total of the substrate plus cleavage product.

**Results**

**Ristocetin Enhances ADAMTS13 Cleavage of Multimeric VWF in the Absence of Shear Stress**

One possible reason that A2 proteolysis is enhanced by the binding of GPIbα to the A1 domain is that exposure of the GPIbα and ADAMTS13 sites on VWF is allosterically coupled. Because ristocetin exposes the GPIbα-binding site, we evaluated its capacity to enhance ADAMTS13-mediated proteolysis of VWF in the absence of applied shear stress (Figure 2). At both 0.75 and 1.0 mg/mL, ristocetin accelerated the ability of recombinant ADAMTS13 to cleave purified multimeric plasma VWF (Figure 2A). Similarly, ristocetin (1.0 mg/mL) added to diluted plasma enhanced the ability of endogenous ADAMTS13 to cleave VWF in a manner similar to the effect of 1.5 mol/L urea (Figure 2B), a denaturing agent used in clinical tests to partially unfold VWF and potentiate its proteolysis. Consistent with the responsible protease being ADAMTS13, proteolysis was inhibited by either a monoclonal antibody against ADAMTS13 or metal ion chelation with EDTA.

**Ristocetin Enhances ADAMTS13 Cleavage of a Recombinant VWF Fragment Containing the A1A2A3 Region**

VWF is known to have only 1 cleavage site for ADAMTS13, the Tyr1605–Met1606 bond within the A2 domain. We, therefore, assessed whether ristocetin enhances cleavage of smaller monomeric recombinant fragments of VWF containing this site. Ristocetin induced ADAMTS13 to cleave a VWF fragment encompassing the tandemly repeated A domains (A1A2A3), yielding a cleaved product of a size identical to that produced in the presence of urea (Figure 3A). The lowest ristocetin concentration that produced maximum proteolysis was ≈0.8 mg/mL (Figure 3B). The ristocetin effect was not mimicked by a related antibiotic, vancomycin. Similarly, ristocetin also induced cleavage of an A1A2A3 fragment with the Asp1472 polymorphic variant in the ristocetin binding site (Figure 3C).

**Detection of Methionine Oxidation in VWF by Mass Spectrometry**

Multimeric VWF was incubated with an HOCl-generating system containing 25 nmol/L myeloperoxidase plus H₂O₂ at various concentrations in PBS (pH 7.4) at 37ºC for 1 hour in the presence or absence of 1.0 mg/mL ristocetin. The oxidation reaction was stopped by the addition of excess free methionine. Oxidized VWF was digested with trypsin, and the peptides were analyzed by nano liquid chromatography–electrospray ionization–tandem mass spectrometry as described previously.

**Minimum Sequence Required for Ristocetin to Expose the ADAMTS13-Cleavage Site in the A2 Domain**

Ristocetin is known to bind 2 sequences in VWF, Cys1237–Pro1251 at the N terminus of the A1 domain and Glu1463–Asp1472, located between the A1 and A2 domains. We, therefore, examined whether one of these known sites was required for ristocetin to expose the ADAMTS13 cleavage site in the A2 domain. We compared ADAMTS13 cleavage of 2 recombinant proteins in the presence of ristocetin, an A1A2 fragment and an extended A2 fragment, both of which contain only the ristocetin-binding site located between the A1 and A2 domains but not the N-terminal ristocetin site. ADAMTS13 was able to cleave both fragments, indicating that the sequence Asp1459–His1472 was required for the ristocetin effect (Figure 4A). In support of this interpretation, ADAMTS13 was unable to cleave the A2 domain lacking the Asp1459–His1472 sequence in the
presence of ristocetin but cleaved it normally in the presence of urea (Figure 5A). Addition of a synthetic peptide containing the ristocetin-binding sequence (Asp1459-His1472) to A2 domain lacking the Asp1459–His1472 sequence did not enhance its cleavage (Figure 5B), indicating that the ristocetin-binding site must be physically connected to the A2 domain to mediate the ristocetin enhancement of cleavage. Addition of the synthetic ristocetin-binding peptide to extended A2 fragment competed for ristocetin binding and reduced ristocetin-induced cleavage of extended A2 fragment (Figure 5B). A scrambled peptide was unable to compete and inhibit cleavage, consistent with the notion that the interaction of ristocetin with the Asp1459-His1472 region is sequence specific.

Ristocetin enhanced ADAMTS13 cleavage more efficiently in the A1A2 than in the extended A2 fragment, but the A1 domain did not improve cleavage efficiency if it was not in the same polypeptide fragment as the A2 domain (Figure 4A). This phenomenon is unique to the A1 domain because the presence or absence of the A3 domain had no significant effect on the ADAMTS13 cleavage of the A2 domain (Figure 4B).

**Figure 3.** Ristocetin, but not vancomycin, enhances ADAMTS13 cleavage of A1A2A3 in a concentration-dependent manner. A, A1A2A3(His1472) was incubated with ADAMTS13 in the presence of either urea or ristocetin with or without EDTA at 37°C for 5 hours. Cleavage products were detected by SDS-PAGE and Western blotting. B, A1A2A3(His1472) was incubated with ADAMTS13 at 37°C for 5 hours in the presence of increasing concentrations of ristocetin or vancomycin. C, A1A2A3(Asp1472) was incubated with ADAMTS13 at 37°C for 5 hours in the presence of increasing concentrations of ristocetin.

**Figure 4.** Effect of the A1 or A3 domain on ADAMTS13 cleavage of A2 in the presence of ristocetin. A, Either A1A2 or extended A2 was incubated with ADAMTS13 in the presence of either urea or ristocetin at 37°C for 5 hours. On the right, isolated A1 with or without a biotin tag was added to aliquots of the reaction mixture. B, A1A2A3 or A1A2 was incubated with ADAMTS13 in the presence of either urea or ristocetin with or without EDTA at 37°C for 5 hours. Cleavage products were detected by SDS-PAGE and Western blotting. exA2 indicates extended A2 fragment.

**Ristocetin Unfolds the A2 Domain and Enhances Oxidation of Met1606 at the ADAMTS13 Cleavage Site**

Met1606 at the ADAMTS13 cleavage site is buried within the A2 domain.21 Our data indicate that ristocetin can induce or expedite A2 unfolding, a process that may or may not be facilitated by ADAMTS13. We previously showed that the chaotropic agent urea and applied shear force are both able to increase oxidation of Met1606 by HOCl, which provides an independent measure of exposure of the ADAMTS13 cleavage site, without the necessity of having ADAMTS13 in the mix.17,22 We, therefore, assessed the effect of ristocetin on HOCl-induced oxidation of Met1606. Multimeric VWF was incubated with an HOCl-generating system, myeloperoxidase plus H2O2 at various concentrations, in the presence of ristocetin or buffer for 1 hour. The treated VWF was then digested with trypsin, and methionine oxidation (methionine sulfoxide) in the A1 and A2 domains was examined by mass spectrometry as described previously.17 Two methionines buried in the native A2 structure, Met1521 and Met1606, were more heavily oxidized in the presence of ristocetin.
than in buffer (Figure 6A), showing that ristocetin indeed changed the conformation of the A2 domain and exposed the 2 buried methionine residues to HOCl. Ristocetin also changed the conformation of the A1 domain, but the extent of methionine oxidation in A1 was much less than in the A2 domain (Figure 6B).

**Discussion**

Ristocetin is known to induce GPIbα–VWF-dependent platelet aggregation by exposing the cryptic A1 domain in plasma VWF, faithfully mimicking the interaction induced by shear stress. In this study, we discovered that ristocetin also changes the conformation of the A2 domain, making the Tyr1605–Met1606 peptide bond available for ADAMTS13 cleavage or oxidation by HOCl. This effect required 1 of the ristocetin-binding sites (Asp1459–His1472) located between the A1 and A2 domains and was enhanced by the physically connected A1 domain. Further, the enhancing effect was observed in both dimorphic forms of VWF in which the dimorphism is in the ristocetin-binding site (Asp/His1472).

These findings have several implications. First, they clearly indicate that exposure of the sites in VWF for binding GPIbα and cleavage by ADAMTS13 within a single VWF subunit are coupled, explaining why elevated shear stress enhances both platelet binding and VWF proteolysis and partially explaining why VWF or VWF fragments are cleaved more rapidly when bound to GPIbα. Enhanced cleavage of VWF multimers associated with GPIbα binding undoubtedly also involves an increase in tensile stress on the multimers, facilitating force-induced unfolding of A2, and exposure of the Tyr1605–Met1606 bond. The effect of ristocetin also provides a plausible explanation for why endothelial ultra-large VWF can be cleaved in the absence of shear stress.

This form of VWF can also form spontaneous, high-strength bonds with GPIbα in the absence of shear stress or modulator. Together, the 2 observations indicate that in ultra-large VWF at least a portion of the monomers making up the multimer have both the GPIbα and ADAMTS13 sites exposed. Simultaneous exposure of the GPIbα and ADAMTS13 sites is also consistent with the frequently observed increased VWF proteolysis associated with the gain-of-function phenotype of type 2B von Willebrand disease. In this form of the disease, loss of high-molecular-weight multimers is usually ascribed to increased clearance resulting from enhanced platelet binding, but increased proteolysis has also been described.
Second, the data also shed light on a potential mechanism of modulating the exposure of the ADAMTS13 cleavage site. Previous studies have suggested that shear stress is the only means by which the ADAMTS13 cleavage site can be exposed in circulating VWF. A recent study estimated that 11 pN of force is needed to unfold a single A2 domain and that under a shear stress of 100 dyn/cm², a VWF multimer must contain at least 200 monomers to experience this amount of force in an A2 domain near the center of the polymer. A VWF polymer of this size has never been shown to exist in human blood. The effect of ristocetin found in our studies, requiring a short modulator sequence (Asp1459–His1472) but no applied force, indicates that exposure of the ADAMTS13 cleavage site may be modulated biochemically and mechanistically, suggesting that both of these factors may be involved in VWF proteolysis in vivo.

Finally, our findings also suggest that ristocetin might be a more specific reagent than chaotropic agents, such as urea, to evaluate the activity of ADAMTS13 for cleaving multimeric VWF in vitro. This is particularly true when exosite interactions of ADAMTS13 with VWF are being studied or when the goal is to investigate the ability of endogenous ADAMTS13 to cleave endogenous VWF. For example, oxidation of VWF at Met1606 will render it uncleavable but no applied force, indicates that exposure of the ADAMTS13 cleavage site may be modulated biochemically and mechanistically, suggesting that both of these factors may be involved in VWF proteolysis in vivo.

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Disclosures
None.

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