Review

This Review is in a thematic series on Sticky Notes on Blood Platelets, which includes the following articles:


Thiol Isomerases in Thrombus Formation

Platelet Immunoreceptor Tyrosine-Based Activation Motif (ITAM) Signaling and Vascular Integrity
Platelet Lipidomics: Modern Day Perspective on Lipid Discovery and Characterization in Platelets
What Can Proteomics Tell Us About Platelets?
Assessment of Platelet Function and the Arguable Need for More Antiplatelet Drugs
Genomics of Megakaryocytes and Platelets

Garret FitzGerald, Guest Editor

Thiol Isomerases in Thrombus Formation

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Abstract: Protein disulfide isomerase (PDI), ERp5, and ERp57, among perhaps other thiol isomerases, are important for the initiation of thrombus formation. Using the laser injury thrombosis model in mice to induce in vivo arterial thrombus formation, it was shown that thrombus formation is associated with PDI secretion by platelets, that inhibition of PDI blocked platelet thrombus formation and fibrin generation, and that endothelial cell activation leads to PDI secretion. Similar results using this and other thrombosis models in mice have demonstrated the importance of ERp5 and ERp57 in the initiation of thrombus formation. The integrins, α_{IIb}β_{3} and α_{V}β_{3}, play a key role in this process and interact directly with PDI, ERp5, and ERp57. The mechanism by which thiol isomerases participate in thrombus generation is being evaluated using trapping mutant forms to identify substrates of thiol isomerases that participate in the network pathways linking thiol isomerases, platelet receptor activation, and fibrin generation. PDI as an antithrombotic target is being explored using isoquercetin and quercetin 3-rutinoside, inhibitors of PDI identified by high throughput screening. Regulation of thiol isomerase expression, analysis of the storage, and secretion of thiol isomerases and determination of the electron transfer pathway are key issues to understanding this newly discovered mechanism of regulation of the initiation of thrombus formation. (Circ Res. 2014;114:1162-1173.)

Key Words: antiplatelet agents ▪ antithrombotic agents ▪ blood platelets ▪ platelet aggregation inhibitor ▪ thrombosis

Protein disulfide isomerase (PDI) has long been identified as a critical functional component of the biosynthetic pathway in the synthesis of proteins. With the classic observation that denatured ribonuclease had the capacity to refold into its native structure, a, b, b′, and a′. The a and a′ domains contain the characteristic active site motif, CXXC, where X can be any one of a number of amino acids and C is cysteine. These domains are enzymatically active.

Although PDI, whose structure is remarkably preserved across species, contains a KDEL retention sequence to keep it in the endoplasmic reticulum (ER) bound to a KDEL receptor protein, PDI also resides in extracellular locations.

How they get secreted is another matter that is currently unknown.
Platelet-Mediated Thrombosis In Vitro Versus In Vivo

The molecular and cellular basis of hemostasis and thrombosis have been mainly studied by the purification of components, including proteins and cells, followed by their evaluation of their action in chemically defined systems. The blood coagulation cascade has been analyzed in vitro by initiating this process with exogenous tissue factor—to turn on the extrinsic agglutination cascade has been analyzed in vitro by initiating this process with exogenous tissue factor.8,12 It would seem that an electron transport pathway exists to convert the inactive components for thrombus formation into active components, and this requirement is only operational in in vivo systems during the initiation phase of thrombus formation.15 Perhaps the most unanticipated discovery was the requirement for extracellular thiol isomerases in the initiation of thrombus formation.8 It would seem that an electron transport pathway exists to convert the inactive components for thrombus formation into active components, and this requirement is only operational in vivo systems during the initiation phase of thrombus formation. This can be seen as a regulatory system, to maintain these components temporally and spatially separate, so that pathological thrombus formation does not occur. Thrombus formation is initiated through vascular injury—and thiol isomerases, secreted from the injured cells, are the critical initiating signals.

Vascular Thiol Isomerases

PDI and other thiol isomerases are secreted on cell activation into extracellular locations outside of the ER. Platelets and endothelial cells are among the cells that secrete PDI and other thiol isomerases.5,7,11 PDI, ERp5, ERp57, ERp44, ERp29, ERp72, and TMX3 are stored and released from platelets.8,9,11,18 (Figure 1). Similarly, PDI, ERp5, ERp57, and ERp46 are released from endothelial cells on cell activation.9,12,13 PDI, ERp5, and ERp57 have been implicated in the initiation of thrombus formation in vivo.8,11–13,19 PDI, ERp5, and ERp57 have been implicated in the initiation of thrombus formation in vivo.8,11–13,19 PDI, ERp5, and ERp57 have been implicated in the initiation of thrombus formation in vivo.8,11–13,19 PDI, ERp5, and ERp57 have been implicated in the initiation of thrombus formation in vivo.8,11–13,19 PDI, ERp5, and ERp57 have been implicated in the initiation of thrombus formation in vivo.8,11–13,19

One of the most striking discoveries is that PDI is expressed in the lung and the heart. In fact, it has been hypothesized that PDI may participate in the conversion of encrypted tissue factor to its active form in a favorable oxidative environment.22 However, to date, we nor others have been able to prove (or disprove) this concept experimentally, and it remains controversial.21

Three thiol isomerases have been shown in in vivo studies to be important for thrombus formation (Figure 2). It is possible, even likely, that other thiol isomerases secreted during vascular injury also play a role in thrombus formation, but there are currently no experimental data to support or refute this concept. Their functional activity takes place extracellularly after secretion from platelets and endothelium. Those thiol isomerases documented to participate in vivo in thrombus formation include PDI, ERp57, and ERp5.8,9,11,19,20

Each of the thiol isomerases is known by multiple names, thus confusing the neophyte being introduced to this field. In this review, we will refer to the vascular thiol isomerases by their common, trivial names. However, the formal nomenclature is also indicated (Figure 1).

Protein Disulfide Isomerase

PDI, the prototype of these thiol isomerases, has a molecular weight of 57 000 and includes 508 amino acids. Encoded by the P4HB gene, it is composed of 4 thioredoxin-like domains a-b-b'-a', where a and a' are catalytically active units with the CGHC motif in the active site and preceded by a signal sequence. The C-terminal segment contains the KDEL sequence, a motif that binds to the KDEL receptor4 and recycles the protein within the ER and cell membranes, specifically peripheral membranes. Within the cell, this enzyme is primarily involved in the formation and rearrangement of disulfide bonds. The crystal structure of human PDI in both the reduced and the oxidized forms shows that the 4 thioredoxin domains are arranged as a U, with 2 active sites in domains a and a' facing each other.24 In contrast to the closed conformation of reduced PDI, oxidized PDI exists in an open state with more exposed areas and a larger cleft available for substrate binding.

ERp57

ERp57 has a molecular weight of 57 000 and includes 505 amino acids. It is encoded by the gene PDIA3. With significant structural homology to PDI, it too is composed of 4 thioredoxin-like domains a-b-b'-a', where a and a' are active.
thiol isomerases with the CGHC motif in the active site and preceeded by a signal sequence. The active thioredoxin domains in ERp57 show 50% sequence similarity to parallel domains in PDI. The C-terminal segment contains the QEDL motif. Within the cell, this enzyme is primarily involved in the formation and rearrangement of disulfide bonds. ERp57 interacts with ER lectins calnexin and calreticulin and has been implicated in glycoprotein folding.25

ERp5
ERp5, encoded by the gene PDIA6, has a molecular weight of 48,000 and includes 440 amino acids. It is composed of 3 thioredoxin-like domains a-a′-b, where a and a′ are active thiol isomerases with the CGHC motif in the active site and are preceeded by a signal sequence. The C-terminal segment contains the KDEL sequence that binds to the KDEL receptor. This enzyme is broadly expressed in cells from a spectrum of tissues. Within the cell, this enzyme is primarily involved in the formation and rearrangement of disulfide bonds.

Storage and Secretion of Thiol Isomerases in Platelets and Endothelial Cells
The observation that antibodies to thiol isomerases block thrombus formation indicates that extracellular thiol isomerases participate in thrombus formation (Figure 2B). The subcellular localization of thiol isomerases and, in particular, thiol isomerase localization to the plasma membrane is an essential feature of their participation in blood coagulation. Thiol isomerases typically localize to the ER. PDI, for example, is highly enriched in ER, with an estimated concentration of 200 μmol/L.26 Such enrichment of thiol isomerases is achieved by the ER retention machinery. The KDEL ER retention sequence at its C terminus is recognized by a member of the KDEL receptor family located in the Golgi.27 The receptor mediates the recycling of the protein back to the ER. ERp57 and ERp72 contain QDEL and KEEL ER retention sequences, respectively. Despite this mechanism for ER retention, localization of thiol isomerases to the Golgi apparatus, secretory granules, and on plasma membrane after secretion is observed in many cell types,28 and extracellular thiol isomerases mediate numerous biological functions in addition to thrombus formation.29–34

How do extracellular thiol isomerases escape the ER retrieval mechanism? One possibility is that non-ER thiol isomerases are either splice variants that lack the ER retention sequence or proteolytic products from which the ER retention sequence has been removed. Yet secreted thiol isomerases retain their ER retention sequence. In hepatocytes and exocrine pancreatic cells, the KDEL sequence is identified in PDI localized to the extracellular surface of the plasma membrane.35,36 Saturation of the ER retention machinery has been proposed as a mechanism by which thiol isomerases escape retrieval to the ER.37 Another possibility is that thiol isomerases escape ER retention by complex formation with other proteins that prevent the interaction of thiol isomerases with KDEL family receptors.38 Both facultative translocation in which PDI is partitioned between cytosolic and ER compartments39 and retrotranslocation40 have been proposed.41 More recently, a KDEL receptor-dependent pathway that traffics PDI from the Golgi to the plasma membrane has been identified in endothelial cells.42 This pathway is dependent on KDEL receptor-mediated activation of src kinases43,44 and is blocked by knockdown of the KDEL receptor or inhibition by brefeldin A, an inhibitor of ER–Golgi trafficking.45,42 This pathway could provide a mechanism for thiol isomerases transport to either the cell surface or the secretory granules.

Figure 2. Protein disulfide isomerase (PDI) is released during and is required for thrombus formation. Intravital microscopy images of thrombus formation in a live mouse. A, PDI antigen (green) is secreted from platelets and endothelium during thrombus formation. B, Blocking anti-PDI antibody inhibits platelet thrombus formation (red) and fibrin (green) generation. Left, 0 μg/g mouse; right, 3.0 μg/g mouse. Time of images: 60 seconds after injury.
The observation that thiol isomerases localize both to secretory granules and to the plasma membrane indicates that they can partition to either regulated or constitutive secretory pathways. However, the mechanisms that underlie the partitioning are not well understood. To appreciate the implications of subcellular localization of thiol isomerases for thrombus formation, one must consider subcellular localization in vascular cells, including platelets and endothelial cells.

Platelet thiol isomerases are stored in intracellular compartments and are released on activation. Chen et al. demonstrated PDI in the supernatants of activated platelet >20 years ago. Many other reports confirmed that PDI is released from activated platelets. Whether PDI localizes to the extracellular surface of the plasma membrane of resting platelets has been more difficult to assess because it is difficult to rule out activation during processing as a source of PDI. The observation that PDI is released in an activation-dependent manner raises the question of what granule type PDI is stored in before release. The major secretory granules in platelets are α-granules (50–80 per platelet), dense granules (3–6 per platelet), and lysosomes (0–3 per platelet). PDI showed a normal distribution in platelets from subjects with gray platelet syndrome, which lack α-granules, and in platelets from subjects with Hermansky Pudlak Syndrome, which lack dense granules. These results indicated that PDI is not stored primarily in dense or α-granules. In contrast to nucleated cells that store the majority of their PDI in the ER, platelets do not have a mature ER. They do have a dense tubular system, which is considered a remnant of the ER. However, the dense tubular system is not considered a secreted compartment. Nonetheless, we recently identified PDI in platelet T-granules, a newly defined electron dense tubular system-related granular compartment that contains TLR9 (toll-like receptor 9) and PDI (Figure 3). T-granules are released in an activation-dependent manner. How PDI is released from T-granules remains an area of active investigation. Whether T-granules represent the storage site of other platelet thiol isomerases also remains to be determined.

Endothelial cell thiol isomerases have been identified in several subcellular compartments, and the distribution of PDI is best established. Both microscopy and density gradient centrifugation show that endothelial PDI is concentrated in the ER. Several experiments, however, indicate that PDI also resides on the surface of endothelial cells. Immunolocalization of PDI on nonpermeabilized endothelial cells demonstrated its cell surface localization. Endothelial PDI also colocalizes with plasma membrane markers when analyzed by density gradient centrifugation. PDI has been identified by mass spectrometry of a highly purified endothelial cell plasma membranes preparation. In addition, surface labeling of endothelial cells with sulfosuccinimidobiotin followed by streptavidin precipitation demonstrated endothelial cell surface PDI. Functional assays also indicate the presence of PDI on the extracellular surface of endothelial cells. Endothelial cell surface PDI participates in transnitrosation of proteins and in modifying cell surface α,β. Additional PDI is released from endothelial cells on stimulation by agonists, such as thrombin, PMA (phorbol 12-myristate 13-acetate), or calcium ionophore, suggesting that it is stored in secretory granules.

Colocalization studies showed that PDI does not localize to Weibel–Palade bodies, but is found in chemokine-containing, small secretory granules colocalized with GRO-α and monocyte chemotactic protein-1. The mechanism by which these small secretory granules are exocytosed on stimulation is not well defined.

Real-time monitoring of thrombus formation in live mice using intravital microscopy demonstrates that thiol isomerases are rapidly released after vascular injury. Laser-induced injury of cremaster muscle arterioles results in rapid accumulation of PDI at the site of injury. The appearance of PDI precedes the accumulation of platelets. Studies in which platelet accumulation is inhibited proved that the PDI that is rapidly released after vascular injury is derived from endothelium although secretion from platelets contributes as they accumulate over time. Once released at the site of injury, PDI is captured by endothelial α,β, and platelet α,β. ERp57 also accumulates at sites of vascular injury and is required for thrombus formation. Because thiol isomerase release is essential for its function in thrombus formation, it is possible that defects in granule secretion could result in decreased thiol isomerase release and impaired thrombus formation. It is also possible that pharmacological blockade of PDI release could impair thrombus formation.

Critical and Unanticipated Role for Thiol Isomerases in Thrombus Formation In Vivo

PDI: the Prototypic Thiol Isomerase of the PDI Family

Although PDI has been known to be secreted from platelets and to be present on the platelet membrane surface for several decades, and inhibitors of thiol isomerases were known to
alter platelet aggregation and fibrinogen interaction with the platelet integrin \( \alpha_{IIb}\beta_3 \). In vitro, the biological importance of these earlier observations was not appreciated until the demonstration in in vivo mouse models of thrombosis that inhibition of PDI inhibited thrombus formation. In one study, thrombus formation in the cremaster arterioles was monitored by intravital microscopy, and PDI antigen secretion into the blood after laser-induced vascular injury was visualized. This PDI became associated with the growing thrombus. Inhibition of PDI with a blocking antibody completely inhibited both platelet thrombus formation and fibrin generation. In the other study, thrombus formation was initiated with a ligation injury. Fibrin, visualized by intravital microscopy, was significantly attenuated by infusion of an inhibitory antibody to PDI. Both studies, using different vascular injury models, allowed the conclusion that fibrin generation is dependent on the presence of PDI. After vascular injury, PDI is first secreted from the endothelium and then secreted from the bound platelets.

Both the endothelium and the platelets contribute to the generation of extracellular PDI. The kinetics of appearance of PDI during thrombus formation indicate that, before the deposition of platelets, PDI lines the area of vascular injury. This strongly implicates the endothelium as the initial source of PDI. If platelets are blocked from participating in thrombus formation using eptifibatide, and thus the platelet contribution of PDI eliminated, PDI accumulates on the injured vessel wall because of secretion from the endothelium. Genetically modified mice lacking platelet PDI initially demonstrated platelet adhesion and fibrin formation, consistent with the importance of the endothelium contribution of extracellular PDI.

Does platelet-derived PDI differ in function from endothelium-derived PDI? Jasuja et al demonstrated that, in the absence of platelets and thus the absence of platelet-derived PDI, fibrin generation is normal, despite the absence of platelet accumulation. In a parallel series of experiments with the exception that platelets were present, Kim et al showed that in genetically modified mice lacking platelet PDI, platelet PDI is critical for thrombus growth but is not required for initial platelet adhesion. This is likely supported by endothelial PDI. Platelet-derived PDI is specifically not required for P-selectin expression, calcium mobilization, certain phosphorylation reactions, \( \beta_3 \)-talin interaction, and platelet spreading, as might be expected a priori.

PDI binds to the active conformer of the \( \beta_3 \) integrin subunit. The prominent platelet integrin \( \alpha_{IIb}\beta_3 \) is located solely on platelets. On activation and a conformational transition, this integrin binds to fibrinogen. \( \alpha_{IIb}\beta_3 \) is located on the endothelium although there is modest expression on platelets as well. This integrin is a vitronectin receptor although other protein ligands also interact with it. PDI binds to the \( \beta_3 \) subunit with a Kd of \( \pm 1 \) to 2 \( \mu \)M. The fact that the binding constant is about the same for both \( \alpha_{IIb}\beta_3 \) and the \( \beta_3 \) subunit suggests that PDI interacts predominantly with the \( \beta_3 \) subunit. The interaction between PDI and the \( \beta_3 \) subunit requires the active conformer of \( \beta_3 \)—a form stabilized in the presence of Mn

Earlier in vitro studies have pointed to the role of thiol isomerases and their inhibitors in the activation or inhibition of integrin function. For example, \( \alpha_{IIb}\beta_3 \) exposes free sulphydryl groups during platelet activation. The addition of anti-PDI antibodies to platelets blocks specific platelet functions attributed to both \( \alpha_{IIb}\beta_3 \) and \( \alpha_{IIb}\beta_1 \). If these integrins were substrates of PDI, \( \alpha_{IIb}\beta_3 \) would have to have free sulphydryls. Yet, the crystal structure of human \( \alpha_{IIb}\beta_3 \) does not show any free sulphydryls; all 56 cysteines form 23 disulfide bonds in \( \beta_3 \). Nonetheless, mutation of specific cysteine residues can convert \( \alpha_{IIb}\beta_3 \) into its active conformer that binds to fibrinogen. Furthermore, \( \alpha_{IIb}\beta_3 \) with 9 CXXC motifs, has endogenous thiol isomerase activity. These Cys residues are engaged in disulfide bonds, and thiol isomerase activity requires 2 free sulphydryls in the CXXC motif. The key question is whether some of these disulfide bonds break spontaneously, for brief intervals, as the protein breathes, and crystallography only captures the major oxidized structure. The mechanism of \( \alpha_{IIb}\beta_3 \) activation, be it involve thiol isomerases or the cytoplasmic tails of the integrin subunits and inside-out signaling, remains elusive.

PDI is essential for platelet thrombus formation and fibrin generation in vivo. In its absence, neither platelets aggregate nor fibrin forms in the injured vascular bed. An unexplained observation has been that \( \beta_3 \) null mice demonstrated no fibrin generation in vivo, which was unanticipated given that wild-type mice treated with eptifibatide that blocks platelet accumulation and Par4 null mice with platelets that cannot be activated by thrombin, generate normal levels of fibrin despite the absence of platelet thrombi. All 3 of these mice—\( \beta_3 \) null, Par4 null, and wild-type mice treated with eptifibatide—fail to generate a platelet thrombus in vivo. A major difference is that only \( \beta_3 \) null mice lack \( \alpha_{IIb}\beta_3 \) in the endothelium. Perhaps \( \beta_3 \), in either \( \alpha_{IIb}\beta_3 \) or \( \alpha_{IIb}\beta_1 \), is a critical intermediate in the initiation pathway to fibrin formation? This poses a potential important role for PDI in fibrin formation.

Kim et al proposed that PDI is essential for thrombus formation but not for maintaining hemostasis. This conclusion was based on the observation that the tail bleeding times in mice lacking PDI within platelets were normal, whereas mice in which all extracellular PDI was inhibited using a blocking antibody had prolonged bleeding times. However, this conclusion requires an assumption that hemostasis, the host defense system for maintaining the integrity of a high-pressure circulatory system, can be predicted from the bleeding time. Several arguments question this conclusion. First, the bleeding time in humans has essentially been abandoned as a clinical predictor of bleeding risk. Second, even some mice known to have a severe bleeding tendency, such as those lacking factor VIII, can express a normal tail bleeding time. We too have demonstrated that an inhibitor of PDI, isoqueretin, administered to mice does not increase the tail bleeding time. However, we do not conclude based on this observation that hemostasis is preserved. Unfortunately, there are limited experimental approaches to the evaluation of hemostasis in laboratory animals.

**ERp57: a Protein Whose Domain Architecture Parallels PDI**

ERp57 has been shown by 3 independent groups to be important for thrombus formation in vivo. Antibodies to ERp57 inhibited platelet aggregation, fibrinogen binding to \( \alpha_{IIb}\beta_3 \), dense granule secretion, and P-selectin expression in vitro in...
one study but not in the other. An inhibitory anti-ERp57 antibody prolonged the bleeding time in mice and inhibited thrombosis in the ferric chloride–induced injury model applied to the carotid artery and the laser-induced injury model applied to the cremaster muscle. Given the marked sequence and structural similarity between ERp57 and PDI, the problem of antibody cross-reactivity poses a significant concern in assigning particular functions to these thiol isomerases. Although a polyclonal antibody to ERp57 inhibited some degree of PDI activity, Wu et al generated a monoclonal antibody to ERp57 that seems to be highly specific for ERp57. They also demonstrated that a widely used monoclonal antibody RL90 directed at PDI shows significant cross-reactivity to ERp57. Using translation blocking antisense vivo-morpholinos targeted to ERp57 (vivo-ERp57) that reduced ERp57 expression in mouse tissues, Jasuja et al extended these studies to demonstrate that ERp57 is released from both platelets and endothelial cells. Laser-induced platelet thrombus formation and fibrin accumulation were significantly decreased in vivo-ERp57–treated mice. Platelet thrombus formation was absent after laser injury in mice with a platelet-specific deletion of ERp57 but fibrin generation is normal. Thus, ERp57 secreted from endothelial cells at sites of laser injury does not rescue platelet thrombus formation in mice lacking platelet ERp57.

Anti-ERp57 antibodies inhibit the activation of α₂β₃. Similarly, catalytically inactive ERp57 inhibited platelet aggregation and prolonged the tail bleeding time in mice. A mouse model lacking megakaryocyte/platelet ERp57 similarly had prolonged tail bleeding times and prolonged times to occlusion using the ferric chloride–induced carotid injury model applied to the cremaster muscle. Given the marked sequence and structural similarity between ERp57 and PDI, the problem of antibody cross-reactivity poses a significant concern in assigning particular functions to these thiol isomerases. Although a polyclonal antibody to ERp57 inhibited some degree of PDI activity, Wu et al generated a monoclonal antibody to ERp57 that seems to be highly specific for ERp57. They also demonstrated that a widely used monoclonal antibody RL90 directed at PDI shows significant cross-reactivity to ERp57. Using translation blocking antisense vivo-morpholinos targeted to ERp57 (vivo-ERp57) that reduced ERp57 expression in mouse tissues, Jasuja et al extended these studies to demonstrate that ERp57 is released from both platelets and endothelial cells. Laser-induced platelet thrombus formation and fibrin accumulation were significantly decreased in vivo-ERp57–treated mice. Platelet thrombus formation was absent after laser injury in mice with a platelet-specific deletion of ERp57 but fibrin generation is normal. Thus, ERp57 secreted from endothelial cells at sites of laser injury does not rescue platelet thrombus formation in mice lacking platelet ERp57.

Potential Substrates of Thiol Isomerases During Thrombus Formation

A method for identifying substrates of extracellular vascular thiol isomerases based on mechanism-based kinetic trapping of extracellular PDI substrates has been developed. The basic approach, used for determining the electron transport pathway in the ER and on cell surfaces, was modified to study mixed disulfides involving thiol isomerases and their covalently bound substrates that originate in plasma and platelet releasate. The reduced function of thiol isomerases is dictated by the state of the CXXC motif of the active site. For almost all of the thiol isomerases identified to date that are associated with the platelet, this motif is CGHC in all of the catalytically active domains of these enzymes. It is thought that the redox potential of these thioredoxin-like domains is defined by the XX within the CXXC motif. This would argue that the redox potentials are similar in these 3 enzymes, whereas sequences of the b and b’ domains are different and likely responsible for the potential variety of substrate specificities. Second, PDI, ERp57, and ERp5 all bind to α₂β₃. Direct binding studies of PDI to α₂β₃ and β₃ indicate a Kd of 1 to 2 μM, direct binding studies of ERp5 to α₂β₃ and β₃ indicate a Kd of about 7 μM. In both cases, blocking antibodies to the thiol isomerase leads to inhibition of platelet aggregation and blockage of activation of α₂β₃. In the case of ERp5, ERp57 also seems to interact with β₃, although direct binding experiments have not been performed. Third, α₂β₃ seems to be a central actor in the pathway to both platelet activation and fibrin generation. However, the mechanism by which these 3 isomerases interact with active α₂β₃, either directly or indirectly, remains to be determined.

ERp5

Like PDI and ERp57, ERp5 is secreted from platelets on cell activation. Inhibition of ERp5 function with an anti-ERp5 antibody prevented fibrinogen binding to activated platelets and platelet aggregation in vitro. The fibrinogen receptor α₂β₃ is a potential substrate of ERp5 as the enzyme coimmunoprecipitates with the β₃ chain of the integrin. We investigated whether ERp5 is released at the site of thrombus formation in vivo and whether inhibition of the reductase activity of ERp5 influences platelet thrombus formation and fibrin generation in a laser-induced mouse model of thrombosis. Anti-ERp5 antibody inhibited ERp5-dependent platelet and endothelial cell disulfide reductase activity in vitro and laser-induced thrombus formation in vivo, with a significant decrease in the deposition of platelets and in fibrin accumulation. ERp5 binds to wild-type α₂β₃ but does not bind to a mutant α₂β₃, which is unable to bind fibrinogen. ERp5 binds to β₃ with a Kd of 6.9 μM. These results provide evidence for a novel role of ERp5 in thrombus formation.

These studies have established several common features of PDI, ERp57, and ERp5. First, the active site motif is characterized by CGHC in all of the catalytically active domains of these enzymes. It is thought that the redox potential of these thioredoxin-like domains is defined by the XX within the CXXC motif. This would argue that the redox potentials are similar in these 3 enzymes, whereas sequences of the b and b’ domains are different and likely responsible for the potential variety of substrate specificities. Second, PDI, ERp57, and ERp5 all bind to α₂β₃. Direct binding studies of PDI to α₂β₃ and β₃ indicate a Kd of 1 to 2 μM, direct binding studies of ERp5 to α₂β₃ and β₃ indicate a Kd of about 7 μM. In both cases, blocking antibodies to the thiol isomerase leads to inhibition of platelet aggregation and blockage of activation of α₂β₃. In the case of ERp5, ERp57 also seems to interact with β₃, although direct binding experiments have not been performed. Third, α₂β₃ seems to be a central actor in the pathway to both platelet activation and fibrin generation. However, the mechanism by which these 3 isomerases interact with active α₂β₃, either directly or indirectly, remains to be determined.

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The PDI cDNAs of the wild-type; a PDI with a modified active site leading to covalent interaction with a substrate (trapping mutant) with alanine substitutions of the C-terminal Cys
at the α or α’ domains (C56A,C400A); and an inactive control mutant containing alanine substitutions at all active site Cys residues in both the α- and α’ domains (C53,56,397,400A) were cloned and expressed with an N-terminal FLAG-epitope and a C-terminal streptavidin-binding peptide-epitope. To identify PDI substrates in platelet-rich plasma, wild-type PDI-CGHC, the trapping mutant PDI-CGHA, and the inactive PDI-AGHA, all containing both the FLAG- and streptavidin-binding peptide-tag, were reacted with components of platelet-rich plasma. PDI and any PDI–substrate complexes is immunoprecipitated using streptavidin-agarose beads that bind the streptavidin-binding peptide-tag on the PDI, then eluted using biotin. Our results indicate that the trapping mutant is reactive with multiple protein substrates in both plasma/platelet releasate and platelet lysate. Protein bands resolved by SDS-PAGE are candidate PDI substrates subsequently identified by mass spectrometry and confirmed by Western blot using an antibody specific for that identified PDI substrate. To date, thrombospondin-1 has been identified as PDI substrates on platelet membranes.

To identify PDI substrates undergoing reduction, oxidation or isomerization, the GH residues of the PDI active site, CGHC, was mutated. Some of these mutations resulted in active PDI variants with altered kinetics of enzyme–substrate interaction, resulting in stable reaction intermediates with enzyme covalently linked to the substrate. Two GH variants trapped proteins in platelet releasate. Because enzymatically active PDI is required for thrombus formation, only those substrate proteins that bound to the enzymatically active variant PDI and not to an enzymatically inactive PDI were analyzed further. Of the PDI-associated proteins identified in releasate, only multimerin-1 and factor V were covalently bound to PDI through a disulfide bond. Multimerin-1 and platelet factor V are reported to be in a disulfide-linked complex in platelet α-granules. These data suggest a role for PDI in the early stages of thrombus formation, where PDI is required for platelet factor V release from multimerin-1, allowing formation of the prothrombinase complex, and generation of necessary levels of thrombin for initial platelet-dependent thrombin generation.

Control of Nitric Oxide and Reactive Oxygen Species by Thiol Isomerases

In addition to their oxidoreductive activities, thiol isomerases can react with nitric oxide (NO) and reactive oxygen species (ROS). The abilities of thiol isomerases to transfer NO to a target protein, remove NO from a protein, and transfer NO from the extracellular environment into the cytosol have all been documented. Thiol isomerases have also been shown to bind to NADPH oxidase and facilitate ROS production in vascular cells. NO and ROS serve critical roles in the thrombus formation, and the ability of thiol isomerases to control NO and ROS is an important consideration in understanding the role of thiol isomerases in thrombus formation.

The same catalytic cysteines that mediate the oxidoreductive activities of PDI can be subjected to S-nitrosylation at physiological NO levels. S-nitrosylation of recombinant PDI occurs at both of its 2 catalytic domains. PDI mutants in which cysteines were replaced with serines in either the N-terminal CXXC motif or the C-terminal CXXC motif each demonstrated 50% of the S-nitrosylation of wild-type PDI when incubated with an NO donor. S-nitrosylation did not occur when both CXXC motifs were mutated. Thus, S-nitrosylation of PDI occurred only at the active site cysteines. Functional studies showed that S-nitrosylation of recombinant PDI inhibits both its chaperone activity and its isomerase activity. S-nitrosylation of PDI has physiological consequences. Increased levels of S-nitrosylation were identified in brain slices of patients with Parkinson and Alzheimer disease, and overexpression of wild-type PDI protects neurons from death is several models of neurodegeneration.

PDI and NO also interact in the vasculature. Proteomics studies have identified S-nitrosylated PDI in endothelium and shown increased PDI S-nitrosylation with statin use, shear stress, or hypoxia. The inhibition of PDI by NO could contribute to vascular quiescence. PDI may further facilitate the ability of NO to maintain vascular quiescence by transferring NO from the extracellular environment into cytosol. The original studies demonstrating that PDI regulates the transfer of NO into cytosol were performed in human erythroleukemia cells. Knockdown of PDI by antisense mRNA resulted in a substantial decrease in NO-mediated cGMP generation after S-nitrosothiol exposure. Subsequent studies in endothelial cells demonstrated that cell surface PDI transferred NO from S-nitroso-albumin, the major NO carrier in plasma, to endothelial cell cytosol. Knockdown of cell surface PDI in endothelial cells inhibited transfer of NO from extracellular S-nitroso-albumin to cytosolic proteins, such as metallothionein.

PDI can also transfer NO into platelets. Incubation of platelets with an NO donor results in increased fluorescence of an NO-sensitive intracellular probe. Inhibition of cell surface PDI using a PDI-specific antibody blocked the increased fluorescence. Once introduced into the platelet cytosol, NO can interact with soluble guanylyl cyclase. Soluble guanylyl cyclase is a heme-containing protein and NO binds avidly to the porphyrin ring, activating the enzyme. When accumulation of NO into platelet cytosol was blocked by inhibition of PDI, cGMP synthesis was inhibited. NO serves an essential role in maintaining vascular quiescence. Although platelets possess NO synthase, transfer of NO from endothelial cells is considered the major source of NO in the vasculature. PDI-mediated transfer of NO produced by the endothelium and transferred into platelet cytosol could help regulate platelet activation.

Although platelet and endothelial cell PDI facilitate maintenance of quiescence in the resting blood vessels, several changes occur with cell activation that could reverse the role that PDI has in controlling vascular NO and ROS. NO levels decrease as a result of endothelial dysfunction and may decrease acutely during thrombus formation. Furthermore, reduced PDI that is secreted from platelets and endothelial cells during thrombus formation could remove NO from S-nitrosylated proteins. Reduced PDI is capable of removing NO from S-nitrosoglutathione and target proteins, including S-nitroso-albumin and S-nitroso-PDI.

Another mechanism by which PDI could contribute to thrombus formation is by facilitating the generation of ROS. PDI physically associates with NAPDH oxidase, a
Thiol Isomerases as Drug Targets

Arterial thrombosis, the underlying cause of myocardial infarction, stroke, and peripheral vascular disease, is the most common cause of morbidity and mortality in the United States. The fact that current anti-thrombotic therapies are insufficient is evidenced by the high rate of recurrent thrombosis, despite convention treatment. Alternative anti-thrombotic strategies based on new knowledge of in vivo thrombus formation are required to improve anti-thrombotic therapy. Thiol isomerases represent an important class of anti-thrombotic targets. Preclinical studies performed using different murine models of thrombus formation consistently indicate that PDI functions in thrombus formation. These murine models demonstrate that inhibition of thiol isomerases blocks both platelet accumulation and fibrin formation at sites of vascular injury. ERp57 has also been shown to participate in thrombus formation and fibrin formation at sites of vascular injury. These preclinical studies demonstrate the efficacy of thiol isomerase inhibition in the setting of thrombosis.

Yet can thiol isomerases be inhibited without significant toxicity given their role in protein folding? The most compelling studies to address this question are a series of experiments performed using quercetin-3-rutinoside. Quercetin-3-rutinoside was identified as an inhibitor of PDI while screening a library of bioactive compounds with known activities. Structure function studies showed that not only did quercetin-3-rutinoside inhibit PDI function but also all other quercetin flavonoids tested that possessed a 3-O-glycosidic linkage at the 3′ position of the C ring blocked PDI activity (Figure 4). Quercetin flavonoids that lacked this sugar failed to inhibit PDI. This same glycosidic linkage that endows a quercetin flavonoid with the ability to inhibit PDI likely impairs its cell permeability.

Quercetin-3-rutinoside blocked thrombus formation in mice after laser-induced injury or ferric chloride-induced damage of cremaster arterioles. In contrast, a quercetin flavonoid that lacked the 3-O-glycosidic linkage, diosmetin, neither inhibited PDI activity nor blocked thrombus formation in vivo. Furthermore, infusion of recombinant PDI reversed quercetin-3-rutinoside inhibition, demonstrating that PDI was indeed the relevant target of its antithrombotic activity. Quercetin-3-rutinoside demonstrated antithrombotic activity regardless of whether it was administered intravenously or by oral gavage.

Quercetin flavonoids are abundant in commonly ingested fruits, vegetables, teas, and grains. Quercetin-3-rutinoside is also widely available as an over-the-counter nutritional supplement. These compounds have been designated Generally Recognized As Safe status by the Food and Drug Administration.

The evidence that PDI inhibition is both effective at blocking thrombus formation and safe raises the question of what antagonists should be used in the clinical setting. Many PDI inhibitors have been described. These include synthetic reagents that act at the catalytic cysteines, plant metabolites, antibiotics such as bacitracin, and hormones such as estrogens and thyroid hormones. However, only the plant metabolites and synthetic compounds have adequate efficacy and safety for use as antithrombotics.

The plant metabolites shown to inhibit PDI include juniferdin and quercetin flavonoids with 3-O-glycosidic linkages, such as quercetin-3-rutinoside and isoquercetin. Juniferdin...
was identified in a high throughput screen designed to identify inhibitors of PDI reductase activity using an insulin-based turbidimetric assay. The goal was to identify novel therapies targeting HIV-1, which requires PDI for entry into cells. Juniferdin itself demonstrated cytotoxicity in cultured cells. However, among several analogs that were tested, juniferdin epoxide retained PDI inhibition and did not demonstrate substantial cytotoxicity in cultured cells.

Many synthetic compounds that block PDI activity have been identified. The majority are sulfhydryl reagents that have long been used to study PDI in protein-based and cell-based assays but lack adequate potency and selectivity for clinical use. More recently, several more potent synthetic small molecules have been identified. 16F16 was discovered in a screen to identify compounds that suppress apoptosis. It is thought to bind active site cysteines and demonstrates cytotoxicity that may be related to off-target effects. RB-11-ca is interesting as a chemical probe because of its target selectivity: it covalently binds to Cys53 of PDI and not to other vicinal cysteines. A phenyl vinyl sulfonate-containing compound developed as an antineoplastic was found to inhibit PDI with a potency in the low micromolar range and was cytotoxic in several cancer cell lines. PACMA-31 is a cell-permeable propynoic acid carbamoyl methyl amide that was also cytotoxic to cancer cells. When tested in mice, PACMA-31 blocked ovarian tumor growth without causing toxicity to normal tissue. Oral dosing in these studies escalated to 200 mg/mL PACMA-31 and continued for 62 days without significantly effecting body weight or histology in major organs. The synthetic compounds mentioned above are all characterized by electrophilic moieties susceptible to covalent attack by the highly nucleophilic cysteine residues in the PDI active sites. As such, they are all irreversible inhibitors of PDI.

We have begun a trial in human subjects evaluating the pharmacokinetics and pharmacodynamics of isouqueretin. However, quercetin flavonoids are not without shortcomings as antithrombotics. They are poorly absorbed, and their absorption and metabolism is highly variable between individuals. Although they are selective for PDI among thiol isomerases and failed to inhibit ERp57, ERp5, ERp72, or thioredoxin reductase, they are highly hydrophobic and have alternative targets. More potent reversible PDI inhibitors with improved pharmacokinetics and selectivity may provide for a second generation of antithrombotic PDI inhibitors and such compounds are presently in development.

Conclusions
Platelet-mediated thrombus formation is a highly complex process influenced by multiple cell types, plasma factors, bioactive mediators, reactive oxygen species, and physical forces. Unlike in vitro models that typically focus on only few parameters, animal models of thrombus formation incorporate all these components. Although in vivo models have limitations, they provide information not easily ascertained by in vitro studies. The identification of thiol isomerases as important components of thrombus formation is a notable example of the use of studying thrombus formation in vivo. Although in vitro studies have provided inconsistent results with regard to the role of thiol isomerases in coagulation, in vivo studies have consistently demonstrated their importance in both platelet accumulation and fibrin generation after vascular injury.

The evidence that inhibition of thiol isomerases blocks thrombus formation is sufficiently strong that the PDI inhibitor isoquercetin has already entered clinical trials (NCT1722669). The number of other small molecule inhibitors of PDI has increased substantially in recent years. Many of these are far superior as biological probes with regard to potency and selectivity when compared with inhibitors that have been used for decades as thiol isomerases inhibitors, such as bacitracin and DTNB (5,5’-dithiobis-[2-nitrobenzoic acid]). However, whether these other recently reported thiol isomerase antagonists are viable drug candidates remains to be determined. Because thiol isomerase inhibition blocks both platelet accumulation and fibrin generation, thiol isomerase inhibitors could be used either in the setting of arterial thrombosis or venous thrombosis.

Improved understanding of the mechanism by which thiol isomerases participate in platelet recruitment and fibrin generation will help guide the selection of clinical settings in which to test this new class of antithrombotics. Yet little is known about how thiol isomerases mediate thrombosis or which thiol isomerases represent the best drug targets. The ability of these enzymes to interact with and modify multiple substrates suggests that they do not act on only a single component of the coagulation cascade or on a single platelet receptor but rather act as regulators that act on many substrates. The challenge will be to identify those thiol isomerase–substrate interactions that are critical for thrombus formation.

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References


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