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Advances in Vascular Thiol Isomerase Function

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Abstract

PURPOSE OF REVIEW—This review will provide an overview of several recent advances in the field of vascular thiol isomerase function.

RECENT FINDINGS—The initial observation that protein disulfide isomerase (PDI) functions in thrombus formation occurred approximately a decade ago. At the time, there was little understanding regarding how PDI or other vascular thiol isomerases contribute to thrombosis. While this problem is far from solved, the past few years have seen substantial progress in several areas that will be reviewed in this article. (1) The relationship between PDI structure and its function has been investigated and applied to identify domains of PDI that are critical for thrombus formation. (2) The mechanisms that direct thiol isomerase storage and release from platelets and endothelium have been studied. (3) New techniques including kinetic-based trapping have identified substrates that vascular thiol isomerases modify during thrombus formation. (4) Novel inhibitors of thiol isomerases have been developed that are useful both as tools to interrogate PDI function and as potential therapeutics. (5) Human studies have been conducted to measure circulating PDI in disease states and evaluate the effect of oral administration of a PDI inhibitor on *ex vivo* thrombin generation.

SUMMARY—Current findings indicate that thiol isomerase-mediated disulfide bond modification in receptors and plasma proteins is an important layer of control of thrombosis and vascular function more generally.

Keywords

thiol isomerase; thrombus formation; protein disulfide isomerase

Introduction

Thiol isomerases are a large family of oxidoreductases that function in disulfide bond shuffling and proper folding of nascent proteins as they are produced in the endoplasmic reticulum (ER) of eukaryotic cells. Release of vascular thiol isomerases from platelets and endothelium into the extracellular environment contributes to several pathological processes

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Conflicts of interest

The author has no conflict of interest to report.

including thrombus formation [1–8]. PDI was the first thiol isomerase to be associated with thrombus formation [1,2]. ERp5 and ERp57 were subsequently found to also participate [3–5]. Several antagonists to PDI have been identified and one of these, isoquercetin, is currently being used in a phase II/III trial [9]. Yet despite this rapid progress from initial *in vivo* observation to clinical trial, surprisingly little is known about how PDI or other thiol isomerases function during thrombus formation. We know that thiol isomerases must be released into the extracellular environment in order to function in thrombosis, but the mechanisms mediating their release from platelets and endothelium are poorly understood. Similarly, we have not identified the relevant substrates on which thiol isomerases act to promote thrombus formation. Understanding how to measure thiol isomerase activity in humans and how to assess the effects of thiol isomerase-targeted therapies will be important in directing pharmacological interventions that are currently being studied. Although these issues are just beginning to be addressed, recent work in the field has begun to unravel this entirely new layer of control of thrombus formation.

PDI structure-function

PDI is the best studied of the vascular thiol isomerases. The domain structure of PDI is **a-b-b'-x-a'** [7] (Fig. 1). The thiol redoxin-like **a** and **a'** domains mediate disulfide bond shuffling. In contrast, the **b** and **b'** domains mediate substrate binding. A hydrophobic patch on the **b'** domain is particularly important for binding both protein substrates and small molecules [10,11]. The **x**-linker is a 19-amino acid peptide that connects the **b'** and **a'** regions [12]. PDI is a highly flexible molecule that exhibits a particularly high degree of flexibility in the **b'-x-a'** region. In its reduced form, PDI can assume a very compact structure, enabling interaction of the N-terminal and C-terminal active site motifs [13,14]. In contrast, oxidized PDI demonstrates a more extended conformation, as evidenced by high resolution evaluation of crystal structure [15] and small angle x-ray scattering [16]. This substantial flexibility enables PDI to interact with a wide range of substrates.

PDI appears to undergo dynamic changes during substrate binding. Bekendam et al. demonstrated an allosteric switch mechanism, whereby binding of peptides or proteins to the hydrophobic binding site on the **b'** domain of PDI displaces the **x**-linker [17]. This displacement results in a conformational change in PDI, causing the structure to become more compact (Fig. 1). Differential cysteine alkylation coupled with mass spectroscopy showed an increase in active site cysteine thiolates and an increase in the reductase activity of PDI in both the **a** and **a'** domains [17]. Thus, activation of the allosteric switch appears to enhance reductase activity. PDI antagonists that targeted the hydrophobic patch also elicited the allosteric switch mechanism and were found to be potent inhibitors of PDI. This mechanism may represent a means by which substrate binding enhances thiol isomerase activity.

To assess whether one catalytic domain is more important for thrombosis than the other, Zhou et al. generated transgenic mice with mutations in the catalytic cysteines of the C-terminus active site motif of PDI [18]. Interestingly, mice with defective N-terminus PDI catalytic motifs were not viable. Activation-induced aggregation was impaired in platelets derived from mice lacking catalytic cysteines in the C-terminus of PDI. The aggregation

defect was reversed by incubation with a recombinant C-terminal active site only PDI mutant that possessed a functional C-terminal active site motif, but lacked catalytic cysteines in the N-terminus [18]. The PDI C-terminus defective mice showed decreased platelet accumulation and fibrin deposition in an intravital model of thrombosis in which thrombus formation is directly imaged following laser-induced vascular injury. The thrombosis defects were reversed by infusion of the recombinant C-terminal active site only PDI mutant [18]. These studies provide compelling *in vivo* evidence that the C-terminal active site motif mediates PDI activity during thrombus formation and raise the possibility of targeting this site for inhibition of thrombus formation.

Thiol isomerase trafficking and release

In nucleated cells, thiol isomerases are highly concentrated in the endoplasmic reticulum where they serve an essential function in folding nascent proteins and are present in extremely high concentrations. For example, PDI is estimated to achieve a concentration of 100–200 μM in the ER [19,20]. Vascular thiol isomerases contain ER retention sequences (e.g., PDI, ERp5 - KDEL; ERp57 - QEDL) that facilitate the concentration of thiol isomerases in ER [7]. Only a fraction of this high concentration of thiol isomerases need be released to achieve thrombus formation. A functionally significant portion of the PDI that is released from platelets and endothelium is retained on the surface through interactions membrane receptors such as $\alpha_{\text{IIb}}\beta_3$ and $\alpha_V\beta_3$, respectively [21]. Yet how vascular thiol isomerases are stored and released is poorly understood.

In platelets, PDI localizes to the dense tubular system, which is considered by many to be a remnant ER [22]. Subsequent studies showed that PDI containing granules are distinct from classical secretory granules, such as α -granules and dense granules [23]. Rather electron microscopy showed PDI in membrane structures, termed T-granules, in the periphery of the platelet. PDI-containing granules were normal in platelets from patients with defective α -granules or dense granules [23]. Whether T-granules represent *bona fide* granules or cross-sections through the dense tubular system remains to be determined. A recent study by Crescente et al. tracked PDI during its trafficking through megakaryocytes and into platelets [24]. They showed that PDI and ERp57 co-localize with markers of the endoplasmic reticulum such as calnexin and SERCA3 and not to granular compartments [24]. They also observed PDI and ERp57 in proplatelets, localized to dense tubular system membranes that are delivered to mature platelets where they localize within the platelet near the inner plasma membrane surface.

How the cargo of these thiol isomerase-containing membrane structures is released remains an important, but unsolved problem. The fundamental question of whether these membrane structures fuse in a coordinated way with plasma membrane to release their cargo (as observed with classical platelet granules [25–29]) is unknown. Nonetheless, recent studies show that, like secretion of other platelet cargo, release of PDI is dependent on feedback via release of ADP from dense granules, as evidenced by the fact that PDI release is impaired in Hermansky-Pudlak platelets, which lack normal dense granules [30]. Despite our limited knowledge of the molecular mechanisms of PDI release, it is clear that these mechanisms differ from those of classical granules such as dense granules and α -granules. PDI does not

localize to either α -granules or dense granules and the studies of Crescente et al. show that secretion is independent of Munc13-4, a chaperone protein that is critical for the secretion of α -granules and dense granules [31]. Nonetheless, PDI release is sensitive to pharmacological inhibition of actin depolymerization (e.g., by latrunculin A), similar to classical granule release [24,25,32], implicating actin in the process of PDI secretion [31]. Thus, while PDI release shares some characteristics of granule exocytosis observed in classical platelet granules, there are significant mechanistic differences and the PDI release mechanism remains an active area of investigation.

In endothelial cells, PDI localizes to secondary granules that are distinct from Weibel-Palade bodies [33]. The membrane trafficking and mechanism of PDI release from endothelial cells are just beginning to be dissected. Sharda et al. showed that externalization of PDI from endothelium is dependent on HPS6 [30]. Both brefeldin A and, paradoxically, knockdown of the KDEL receptor block PDI externalization in endothelial cells infected with Dengue virus, suggesting a non-canonical KDEL-dependent secretory pathway involving transport from ER to Golgi [34]. However, Araujo et al. describe an additional or alternative Golgi bypass pathway that is resistant to inhibitors of Golgi trafficking [35]. Furthermore, they show that cytoskeletal disruption enhances PDI externalization in endothelial cells. Additional studies are needed to define the pathways whereby thiol isomerases are trafficked to endothelial storage granules and determine the mechanisms of activation-dependent release.

Substrates of vascular thiol isomerases

Among the fundamental questions regarding the role of thiol isomerases in thrombus formation is what are the relevant substrates subject to thiol isomerase-mediated disulfide bond rearrangements. Several substrates that could be modified by extracellular PDI during thrombus formation have been identified. PDI interacts with integrins on vascular cells including $\alpha_{IIb}\beta_3$ on platelets, $\alpha_V\beta_3$ on endothelium, and $\alpha_M\beta_2$ on neutrophils [7,21]. Mouse platelets genetically engineered to lack PDI have impaired $\alpha_{IIb}\beta_3$ activation, implying a role for PDI activity in $\alpha_{IIb}\beta_3$ activation [36]. Yet whether and how PDI functions in platelet $\alpha_{IIb}\beta_3$ activation during thrombus formation remains debated. PDI has been proposed to facilitate the ability of tissue factor to generate factor Xa, although this supposition and the precise mechanism remain controversial [37,38]. Thrombospondin, von Willebrand factor, and GPIIb α are reported to be PDI substrates [7]. What remains unknown is whether thiol isomerase-mediated modifications of these proteins contribute to thrombus formation.

A novel strategy to address this problem has been the application of kinetic-based trapping techniques to identify thiol isomerase substrates. This technique has thus far been applied to identification of PDI substrates (Fig. 2). The strategy uses a mutant PDI that is modified such that the second cysteine in its CGHC motif is mutated to alanine (CGHA). The CGHA mutant forms an intramolecular disulfide bond that cannot be resolved, leaving PDI covalently bound to its substrate (Fig. 2). The PDI-substrate complex can then be isolated and the trapped substrate identified by mass spectroscopy. Bowley et al. used this technique to identify a number of putative substrates (Fig. 2) [39], including vitronectin, complement factor 3, complement factor 5, C4b-binding protein, α_2 -macroglobulin, protein S, histidine-

rich glycoprotein, thrombospondin 1, prothrombin, and CD5 antigen-like protein [39]. They selected vitronectin for further characterization, identifying two disulfides (Cys137-Cys161 and Cys274-Cys453) that are cleaved by PDI and demonstrating that reduction of vitronectin by PDI enables binding to $\beta 3$ integrins. Using intravital microscopy, they further showed that vitronectin accumulates into thrombi and that inhibition of PDI blocks vitronectin accumulation [39]. Mice lacking vitronectin demonstrated defective thrombus formation.

While kinetic-based trapping using CGHA mutants is a powerful, unbiased approach for substrate identification, an inherent limitation is that it only identifies substrates that are *reduced* by PDI. To circumvent this limitation, Stopa et al. engineered trapping mutants with intervening sequence variants, CGPC and CGRC, which are capable of capturing substrates that are *either reduced or oxidized* by PDI [40]. Among the putative substrates captured by these variants was factor V [41]. Inhibition of PDI impaired factor Va generation and thrombin generation in a platelet-dependent thrombin generation assay. These results indicate a role for PDI in activation of factor Va, possibly by releasing factor V from its binding protein multimerin thereby enabling proteolytic cleavage [41].

Thiol isomerase inhibitors

The fact that the thiol isomerases PDI, ERp5 and ERp57 have been shown to be important for thrombus formation has rendered them targets for antithrombotic therapy. Flavonoid quercetins such as quercetin-3-rutinoside (rutin) and isoquercetin inhibit PDI and are potently antithrombotic in animal models of thrombosis [42]. Lin et al. showed that quercetin-3-rutinoside binds PDI at the hydrophobic binding site on **b'** domain of the receptor (Fig. 3), but blocks reductase activity at the active site motif [16]. These authors demonstrated that infusion of isolated **b'x** domain reversed quercetin-3-rutinoside-mediated inhibition of PDI. Thus, **b'x** could be used clinically as a reversible agent for quercetin-3-rutinoside in the setting of bleeding [16].

High throughput screening has identified several potent and selective inhibitors of PDI (Table 1). Two of these compounds were found to act at the hydrophobic binding pocket of the **b'** domain and were therefore termed bepristats [17]. Unlike flavonoid quercetins, however, whose binding to **b'** blocks reductase activity at the active site motifs, bepristats paradoxically enhanced reductase activity. Further evaluation showed that the bepristats displaced the **x**-linker, activating the allosteric switch mechanism and enhancing the free dithiol state of the catalytic cysteines. Bepristats selectively inhibit PDI and not ERp5, ERp57, or thioredoxin and block platelet aggregation in a reversible manner. These compounds were also antithrombotic in a murine model of laser-induced thrombus formation. The distinguishing feature of bepristats compared with previously described PDI inhibitors is their selectivity as assessed, for example, by an analysis of off-target effects using the PubChem database.

Several potent PDI inhibitors act at the catalytic cysteines on PDI. These include LOC14 [43], PACMA-31 [44], and CCF642 [45] (Fig. 3). RB-11-ca reportedly interacts selectively with Cys53 of the **a** domain [46]. Peptides represent an alternative to small molecules for targeting catalytic cysteines. Sousa et al. used a peptide (VEFYAPWCGHCK) derived from

the C-terminus of PDI as an inhibitor that targets the C-terminal catalytic motif of PDI [47]. This peptide, termed CxxC, blocks platelet aggregation and $\alpha_{IIb}\beta_3$ activation. Reagents targeting catalytic cysteines tend to be irreversible inhibitors.

Human studies of PDI levels and PDI inhibitors

Studies involving either measurements of PDI released from platelets or associated with microparticles have recently been published. A study by Voightleander et al. showed increased PDI release from platelets of patients with hemophilia A compared to matched controls [48]. This same study showed a positive correlation between age and increased activation-induced PDI release from platelets regardless of whether the subject has hemophilia or not [48]. PDI associated with circulating microparticles was found to be increased in the setting of metabolic syndrome [49] or diabetes [50]. Although plasma levels of circulating free PDI are low, investigators are currently developing methods for evaluating these levels with the objective of measuring circulating free PDI levels in disease states.

In the first example of a PDI-targeted therapy given to humans, Stopa et al. evaluated the effect of orally administered isoquercetin on platelet-dependent thrombin generation in healthy human subjects [41]. This thrombin generation assay was selected to monitor the antithrombotic effect of isoquercetin based on a previous study that demonstrated that platelet-dependent thrombin generation is dependent on PDI [51]. Oral administration of 1 g isoquercetin resulted in isoquercetin levels in plasma sufficient to block recombinant PDI when tested in an *ex vivo* assay [41]. Higher plasma isoquercetin levels were associated with increased PDI inhibition [41]. Ingestion of isoquercetin also inhibited platelet-dependent thrombin generation. This inhibition could be overcome by addition of recombinant PDI to the assay, indicating that isoquercetin was acting via inhibition of PDI. The inhibitory effect of PDI inhibition by isoquercetin was even more marked in patients with antiphospholipid syndrome [41]. To evaluate the mechanism by which inhibition of PDI blocks thrombin generation, the authors evaluated the effect of isoquercetin on factor V activation. They showed that PDI inhibition blocks generation of factor Va in platelet-rich plasma. These results support a model whereby ingestion of isoquercetin causes inhibition of PDI, impairing the conversion of factor V to Va and thereby limiting thrombin generation. The utility of this strategy in blocking thrombosis in the setting of cancer thrombosis is currently being evaluated [9].

Conclusion

Thiol isomerases have many functions and it is currently not certain which of these promote thrombus formation. Modulation of functional disulfide bonds is perhaps their best-studied function and could represent a previously unappreciated layer of control of thrombus formation. Thiol isomerase-mediated modification of disulfides in coagulation proteins and platelet receptors may be required to 'unlock' proteins to enable their participation in thrombosis. Such disulfide bond modifications could result in exposure of binding sites, enzyme activation, receptor activation, and/or release of proteins from their binding partners [7]. These modifications by thiol isomerases do not elicit coagulation or platelet activation themselves (e.g., addition of PDI to plasma does not cause it to clot nor does incubation of

platelets with PDI cause them to activate), yet are essential for the initiation of thrombus formation *in vivo*. A potential explanation for this apparent paradox is that allosteric disulfides in vascular proteins form an embedded network of clamps that serve an antithrombotic function in the quiescent vasculature. Following injury, thiol isomerases released from vascular cells are required to deactivate this antithrombotic disulfide bond network and enable thrombi to form. Vascular thiol isomerases also act as redox sensors, responding to redox active molecules in the vasculature and connecting changes in redox environment with modification of disulfide bonds. This redox function may also contribute their vascular function. While the study of vascular thiol isomerases remains in its infancy, the importance of the endeavor is plainly evidenced by the efficacy of thiol isomerase inhibitors in preventing thrombosis in multiple animal models and by the initial studies testing PDI antagonists in humans.

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Bullet Points

- The active site motif of the **a'** domain and hydrophobic pocket of the **b'** domain are important for PDI function in thrombus formation.
- In platelets, PDI is stored in the dense tubular system and released via mechanisms distinct from classical platelet granules.
- In endothelial cells, PDI is trafficked via unconventional pathways and stored in secondary granules.
- Kinetic-based trapping strategies have identified many new putative PDI substrates.
- Novel inhibitors of PDI primarily target the active site motif of the **a'** domain or the hydrophobic pocket of the **b'** domain.

Figure 1. The allosteric switch mechanism

In its unligated state, the **x-linker** interacts with the hydrophobic binding pocket on the **b'** domain (capped conformation). In this conformation, the active site motifs on the **a** and **a'** domains are prone to disulfide bonding. The binding of a substrate to the hydrophobic pocket results in displacement of the **x-linker**. This displacement causes a conformational change resulting in a more compact structure and a propensity towards a free dithiol state for the catalytic cysteines in the active site motif.

Figure 2. Kinetic mechanism-based trapping of thiol isomerase substrates

A. Reduced thiol isomerase cleaves disulfide bonds in substrates by nucleophilic attack in which the active site sulfur ion nucleophile of the thiol isomerase attacks the adjacent sulfur atoms of the disulfide bond in the substrate. This nucleophilic substitution results in a transient mixed disulfide. The mixed disulfide then decomposes with a disulfide bond forming in the thiol isomerase. **B.** A trapping mutant with a CGHA active site motif has a free thiol that is capable of nucleophilic attack of a disulfide bond on a substrate. However, the mixed disulfide bond that forms fails to resolve into a disulfide on the thiol isomerase since it lacks a second cysteine. Instead, the mixed disulfide is stable, enabling isolation of the complex and identification of the substrate.

Figure 3. Mechanism of action of PDI antagonists

Several PDI inhibitors have been identified over the past few years. Evaluation of their mechanism of action indicates that most act either at the catalytic cysteines within the active site motifs or at the hydrophobic binding pocket on the **b'** domain. In general, compounds that act at the active site motif tend to be irreversible inhibitors while those that act at the hydrophobic binding pocket tend to be reversible inhibitors.

Table I

Inhibitors of PDI.

	Reversibility	IC ₅₀ (μ M)	Site of action	ref
Bepristats	Reversible	0.3–0.6	b' domain	[17]
Flavanoid Quercetins (Quercetin-3-rutinoside, isoquercetin, etc.)	Reversible	6	b' domain	[42]
Juniferdin	Reversible	0.16–3	Not specified	[52]
RB-11-ca*	Irreversible	30–50	Shows selectivity for Cys53 of the a domain.	[46]
PACMA-31*	Irreversible	10	Catalytic motif of a and a' domains	[44]
P1 (phenyl vinyl sulfonate)*	Irreversible	1.7	Catalytic motif of a and a' domains	[53]
LOC14	Reversible	0.062	Binds adjacent to active site and forces PDI into oxidized conformation.	[43]
CCF642	Irreversible	0.2–0.8	Catalytic motif of a and a' domains	[45]
CxxC peptide	Irreversible	NA	Catalytic motif of a and a' domains	[47]