



## FORUM REVIEW ARTICLE

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# Extracellular Thiol Isomerases and Their Role in Thrombus Formation

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### Abstract

**Significance:** The mammalian endoplasmic reticulum (ER) houses a large family of twenty thioredoxin-like proteins of which protein disulfide isomerase (PDI) is the archetypal member. Although the PDI family is best known for its role in oxidative protein folding of secretory proteins in the ER, these thioredoxin-like proteins fulfill ever-expanding roles, both within the secretory pathway and beyond. **Recent Advances:** Secreted PDI family proteins have now been shown to serve a critical role in platelet thrombus formation and fibrin generation. Utilizing intravital microscopy to visualize thrombus formation in mice, we have demonstrated the presence of extracellular PDI antigen during thrombus formation following injury of the vascular wall. Inhibition of PDI abrogates thrombus formation *in vivo* (16, 26, 46, 55). These observations have been extended to other PDI family members, including ERp57 (39, 116, 118, 123) and ERp5 (77). The vascular thiol isomerases are those PDI family members secreted from platelets and/or endothelium (40): PDI, ERp57, ERp5, ERp72, ERp44, ERp29, and TMX3. We focus here on PDI (16, 46, 55), ERp57 (39, 116, 118, 123), and ERp5 (77), which have been implicated in thrombus formation *in vivo*. **Critical Issues:** It would appear that a system of thiol isomerase redox catalysts has been hijacked from the ER to regulate thrombus formation in the vasculature. **Future Directions:** How this redox system is trafficked to and regulated at the cell surface, the identity of extracellular substrates, why so many thiol isomerases are required, and which thiol isomerase functions are necessary are critical unanswered questions in understanding the role of thiol isomerases in thrombus formation. *Antioxid. Redox Signal.* 24, 1–15.

### Thrombus Formation

**T**HROMBUS FORMATION is a host defense mechanism to minimize the loss of blood following a breach in the high-pressure circulatory system (30). Under normal physiologic conditions, thrombus formation preserves the normal blood pressure. Under pathologic conditions, thrombi fail to form, leading to bleeding, or are formed inappropriately, leading to blockage of arteries and veins and ischemia of distal tissue. We and others have demonstrated the importance of vascular thiol isomerases during thrombus formation following injury of the vascular wall, including protein disulfide isomerase (PDI) (16, 26, 46, 55), ERp57 (39, 116, 118, 123), and ERp5 (77). These enzymes are required for platelet thrombus formation and fibrin generation.

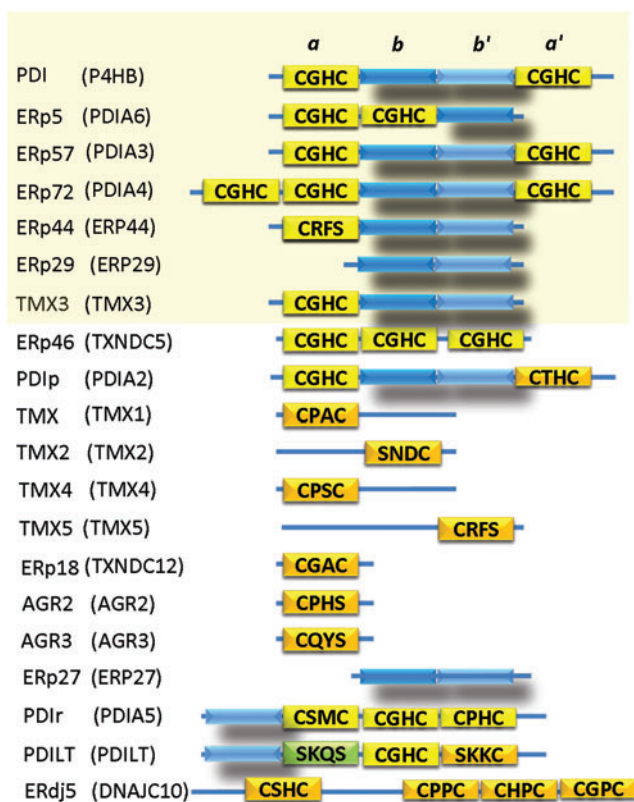
### Overview of Thiol Isomerases

There are 20 known members of the thiol isomerase family (Fig. 1), PDI being the best known since the pioneering work of Anfinsen and colleagues (19, 34). PDI family proteins share at least one thioredoxin-like fold, a molecular scaffold consisting of a four-stranded antiparallel  $\beta$  sheet surrounded by three  $\alpha$  helices (82). Eleven of these proteins contain at least one and as many as four active site Cysteine-X-X-Cysteine (CXXC) motifs that can alternate between the disulfide and dithiol states to transfer electrons in redox processes. Eight of these thioredoxin domain-containing proteins are redox inactive, either lacking any functional CXXC motif (ERp27, ERp29, *etc.*) or possessing a substitution of a single CXXC motif cysteine coupled with serine

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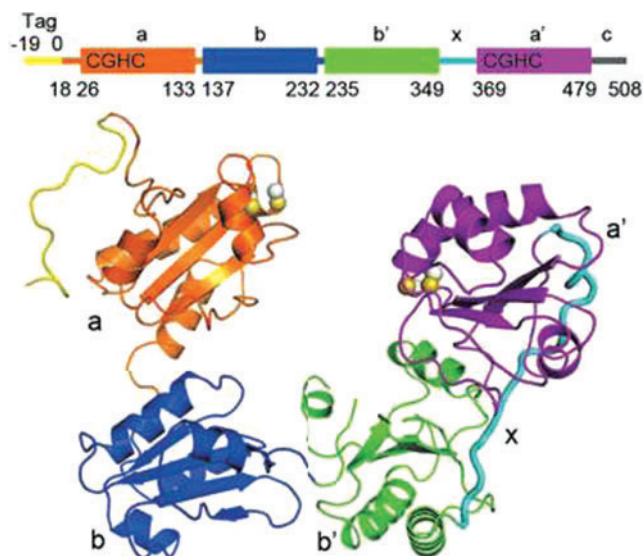
**FIG. 1. PDI family of thiol isomerases.** PDI is the archetypal member of the thiol isomerase family. It contains four domains (a, b, b', a'), including catalytically active thioredoxin-like domains containing the CGHC motif (*yellow*). Catalytically inactive thioredoxin-like domains are shown in *blue* and *light blue*. The vascular thiol isomerases are displayed with a pale *yellow* background. Family members known to be involved with thrombus formation include PDI, ERp57, and ERp5. Adapted from (26). To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

(ERp44, TMX2, *etc.*). Others contain additional domains such as the J domain (ERdj5) or a single transmembrane segment (TMX, TMX4, *etc.*). While many of these proteins have been purified and studied *in vitro*, the unique function and endogenous substrates of most PDI family proteins remain uncertain (4, 37).

### Protein Disulfide Isomerase

PDI is the best characterized and most abundant thioredoxin-like protein in the ER. A polypeptide with a molecular weight of 57,000 encoded by the *P4HB* gene consists of four thioredoxin domains designated a-b-b'-a', with the a and a' domains, including catalytic active sites containing a Cysteine–Glycine–Histidine–Cysteine (CGHC) motif (34, 58). An intervening “x” segment is C-terminal to the b' domain and a short “c” segment is C-terminal to the a' domain. These four independent domains are folded into a twisted U-shaped conformation to create the putative substrate binding groove within its cleft (113) (Fig. 2).

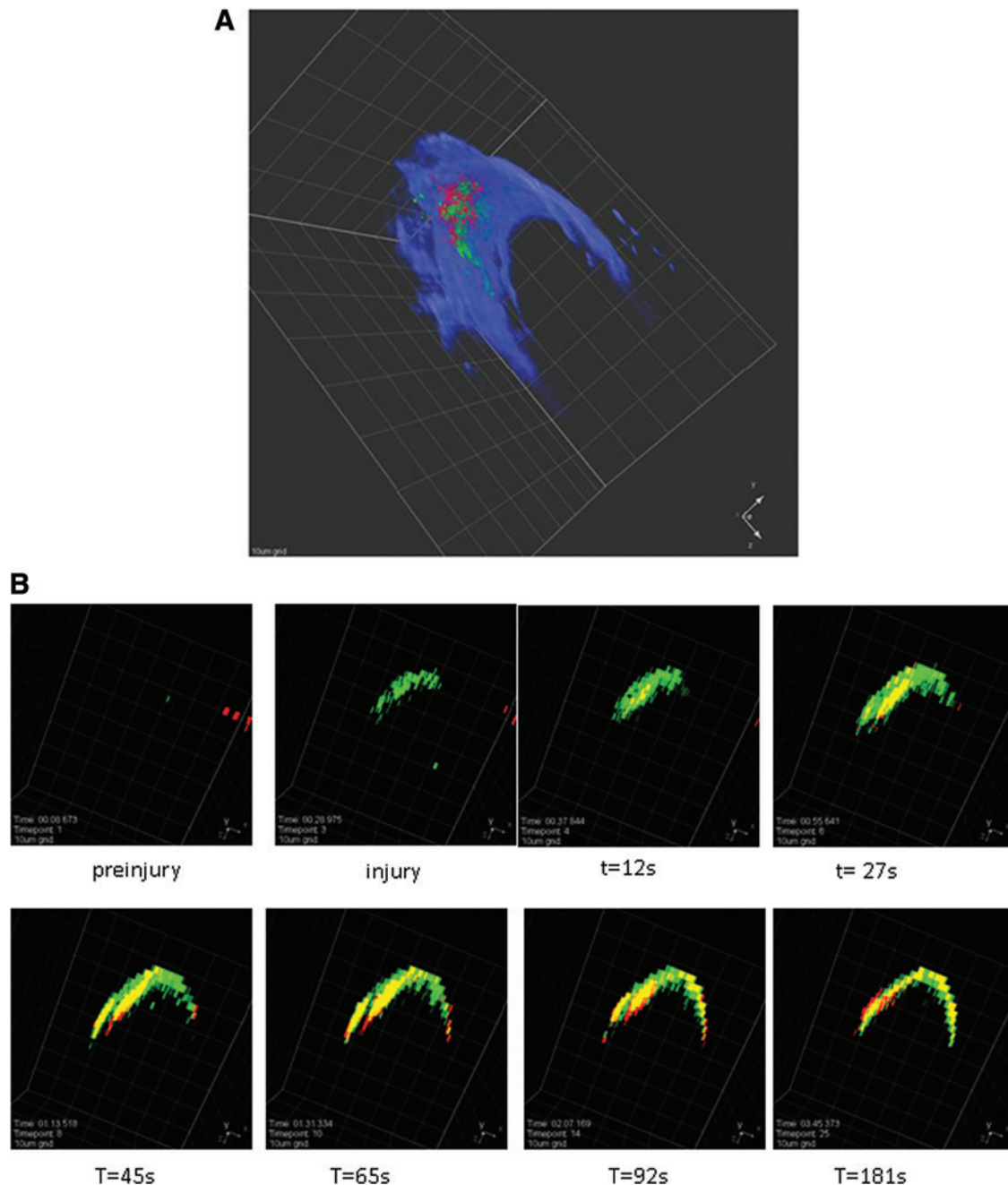
PDI was originally identified as the microsomal disulfide interchange activity capable of accelerating the oxidative folding of ribonuclease A, a protein that contains four



**FIG. 2. X-ray crystal structure of reduced human PDI.** PDI is composed of four thioredoxin-like domains: a, b, b', and a'. The CXXC active site motifs are found in a and a'. The b' domain is thought to be important for substrate specificity. This U-shaped protein appears flexible, and the a and a' domains can interact simultaneously with the substrate protein. The four domains have the characteristic thioredoxin-like fold, homologous to thioredoxin. From (113). To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

disulfide bonds (34, 108). A number of specific oxidative folding substrates have been described for PDI in the ER, including procollagen (27), thyroglobulin (20), and  $\gamma$ -gliadin (9). Beyond its role in disulfide oxidation, reduction, and isomerization reactions, PDI also acts as a non-catalytic chaperone in complex with a variety of enzymes to facilitate, for example, the hydroxylation of proline (78) and membrane transport of triglycerides (117). The entrance of PDI into the ER is specified by a cleavable N-terminal signal sequence containing the C-terminal KDEL ER retrieval motif (107). The a and a' domain CXXC motifs of PDI appear to be predominantly reduced *in vivo* (4), although the redox state of these dithiols is dynamically regulated by sulfhydryl oxidases such as Ero1 (28, 106) to drive oxidative protein folding.

PDI is abundant in the ER. However, it is also located in small secretory granules, including the GRO- $\alpha$  granule of the endothelial cell and the T-granule of the platelet (45, 105). Following laser injury in a live mouse, PDI appears rapidly first on the surface of the endothelium and subsequently on the surface of bound platelets (16, 45). The binding of secreted PDI to the developing thrombus involves the interaction of PDI with the  $\beta_3$  integrins,  $\alpha v\beta_3$  on the endothelium, and  $\alpha IIb\beta_3$  on bound platelets (17). Tissue factor and PDI are colocalized on the endothelium surface following cell activation by the laser injury (Fig. 3A). The expression of PDI on the luminal wall of an arteriole immediately follows laser injury and is rapidly followed by the expression of tissue factor (Fig. 3B). The endothelial surface is also shown. These events occur following calcium mobilization (45). Similarly, PDI has been known for some



**FIG. 3. Three-dimensional image reconstruction from confocal stacks taken during *in vivo* laser injury-induced thrombus formation in the mouse.** (A) This arteriole demonstrates localization of PDI (green) and tissue factor (red) with endothelium (blue). Endothelium is marked with fluorescently tagged anti-CD146 antibody, while PDI and tissue factor are designated with corresponding fluorescently tagged antibodies. (B) Sequence of confocal images of tissue factor antigen (red) and PDI antigen (green) following laser-induced injury of the vessel wall of a mouse cremaster arteriole. Merge between tissue factor and PDI is visualized as yellow. Initial injury monitored for 120 s. Images are 3D reconstructions from confocal stacks using maximum intensity projection mode. The image coordinate arrows for x, y, z planes are provided for orientation. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

time to be secreted rapidly from platelets *in vitro* upon stimulation by platelet agonists (14).

#### *Oxidoreductase activity of PDI*

The CGHC motif active sites of PDI enable it to alternate between the reduced dithiol and disulfide-bonded states and,

thus, participate in biochemical oxidation and reduction reactions. While PDI does directly oxidize disulfide bridge formation in a number of proteins, it is itself unable to generate a disulfide *de novo*. The oxidized form of glutathione (GSSG) is able to oxidize the PDI active site (43, 53), but the reaction is stoichiometric and GSSG would also need to be recycled. As such, PDI is dependent upon a true sulphydryl

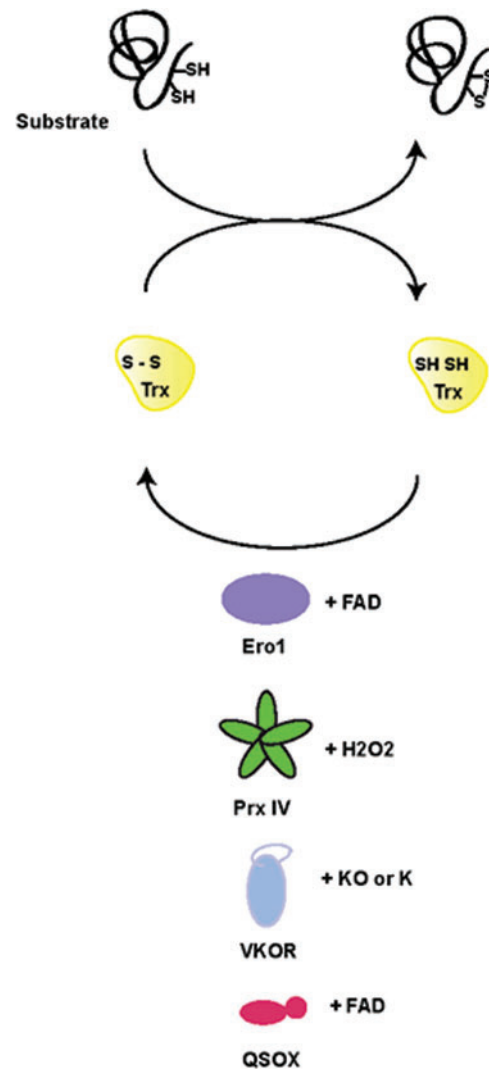
oxidase and must itself be oxidized before it can catalyze oxidative protein folding. This is accomplished by cooperating with a sulfhydryl oxidase or a small molecule oxidant such as glutathione or molecular oxygen. At least four families of sulfhydryl oxidases reside within the eukaryotic secretory pathway. These include the ER oxidase 1 proteins (Ero1- $\alpha$  and - $\beta$ ), the quiescin-sulfhydryl oxidases (QSOX1 and 2), the vitamin K epoxide reductase paralogs (VKOR and the VKOR-like protein), and peroxiredoxin-4.

A general molecular logic unites these pathways (Fig. 4). A sulfhydryl oxidase couples the oxidation of a CXXC motif in PDI to the reduction of a small molecule cofactor; this cofactor may be a flavin adenine dinucleotide (FAD) molecule for the Ero1 and QSOX proteins or a quinone such as vitamin K in the case of the VKORs. The reaction proceeds through a mixed disulfide intermediate between the sulfhydryl oxidase and a catalytic thioredoxin domain of the PDI family protein that can be trapped *in vivo* (3, 72, 93, 104). This describes a linear electron transfer pathway supporting disulfide formation, with the enzyme first oxidizing an active site CXXC motif in PDI, which subsequently donates its disulfide to a nascent secretory protein. Some thiol isomerases preferentially pair with specific sulfhydryl oxidases, such as peroxiredoxin IV with ERp5 (92) and VKOR with TMX (94). In an additional layer of complexity, some PDI family thiol isomerases may also participate in redox cascades with one another as a bridge between sulfhydryl oxidase and the ultimately oxidized thioredoxin-like protein (74).

#### Chaperone activity of PDI

Chaperones share the ability to recognize and bind non-native proteins under biophysical conditions that can promote proper folding, as occurs in the ER (8). PDI has molecular chaperone activity (115). The complete deletion of PDI in yeast is lethal, yet cells with mutant PDI that possess only inactivated catalytic sites (AXXA) are viable (62). Consistent with these observations, no viable strains of PDI knockout mice have been described (37). These findings suggest that the essential function of PDI in cell growth and survival may lie not in its catalytic activity but rather in its role as a chaperone protein. The structural determinants of PDI chaperone activity remain unclear. A mutant PDI with the 51 C-terminal amino acid residues deleted (abb'xa') lost its chaperone activity but retained its isomerase and oxidoreductase activities (18). While this result seems to indicate that the C-terminus of PDI may be necessary for its chaperone activity, the abb'xa' mutant may also result in a loss of substrate binding by the b' domain due to destabilization of the a' domain (37). If true, this would, in turn, suggest that the main effect being observed is not the importance for chaperone activity of the c-extension but rather the critical substrate binding *via* the b' domain. Hybrid PDI mutants that contained various combinations of PDI domains with or without thioredoxin (101) indicated that full-length PDI is required for chaperone activity but not catalytic function. The role of the b' domain in PDI's chaperone activity appears critical.

We have recently demonstrated that a specific inhibitor of PDI reductase activity identified from a high-throughput screen, quercetin-3-rutinoside, binds directly to the PDI b' domain (65). Quercetin-3-rutinoside binds to the b'x domain



**FIG. 4. Catalytic disulfide bond formation requires enzymatic reoxidation of thiol isomerases.** A thioredoxin-like thiol isomerase (Trx, yellow) donates its disulfide to a reduced protein substrate with concomitant reduction of the CXXC motif cysteine residues in Trx. In order for a thiol isomerase to assist oxidation of another folding protein, the active site CXXC motif must first be reoxidized. A family of sulfhydryl oxidases exists within the mammalian secretory pathway that couple oxidation of Trx-like thiol isomerases to the reduction of small molecule cofactors (FAD, vitamin K) or chemical oxidants ( $H_2O_2$ ). These include the endoplasmic reticulum oxidase 1 (Ero1-a and Ero1-b), quiescin-sulfhydryl oxidases (QSOX1 and 2), the vitamin K epoxide reductase paralogs (VKOR and VKOR-like protein), and peroxiredoxin 4 (Prx4). To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

and to the bb' domains and inhibits PDI reductase activity. Reversal of quercetin-3-rutinoside inhibition of thrombus formation in a mouse model is observed with infusion of the PDI b'x domain (65).

A role for the chaperone function of PDI in thrombosis has been suggested. Versteeg used S-methyl methanethiosulfonate (MMTS) to inactivate the catalytic sites of PDI (109).

PDI modified covalently with MMTS showed a significant reduction in oxidoreductase activity, but its chaperone activity was unaffected. However, both unmodified and MMTS-treated PDI significantly increased the rate of tissue factor-dependent factor Xa generation in a chromogenic assay (99, 109). Furthermore, the wasp venom-derived peptide mastoparan, which binds in the b' hydrophobic pocket of PDI necessary for chaperone activity, inhibited the ability of bovine PDI to enhance factor Xa generation. PDI treated with N-ethylmaleimide enhanced factor Xa generation in a manner similar to unmodified PDI (86).

#### *PDI and thrombus formation*

Of uncertain physiologic relevance at the time, PDI was identified on the surface of resting platelets (22) and secreted from activated platelets (13, 14). The supernatant of activated platelets contained a bacitracin-sensitive activity capable of refolding disulfide-reduced RNase to restore its function (14). Membrane impermeable thiol modifiers, anti-PDI Fab fragments, and the nonselective thiol isomerase inhibitor bacitracin impaired platelet aggregation *in vitro* and also prevented stable binding (40) of fibrinogen to its platelet surface receptor  $\alpha_{IIb}\beta_3$  (23). Disulfide exchange is important to the adhesion of platelets to fibrinogen, fibronectin, and collagen (33, 59, 60). In the case of collagen, PDI exerts this effect by acting on integrin  $\alpha_b\beta_1$  rather than GPVI (60, 61).

Thrombospondin-1 was the first cell surface substrate of PDI to be identified (41), with the resulting disulfide arrangement of TSP-1 exposing a cryptic RGD motif to facilitate enhanced integrin receptor binding (42, 100). PDI was also demonstrated to catalyze the formation of thrombin–antithrombin–vitronectin and thrombin–antithrombin–thrombospondin-1 complexes *in vitro* (24, 73). These studies received little traction in the platelet biology field, given that numerous exogenous compounds are known to interfere with the function of hemostatic proteins and detailed mechanistic studies were lacking to understand the physiologic relevance of these findings.

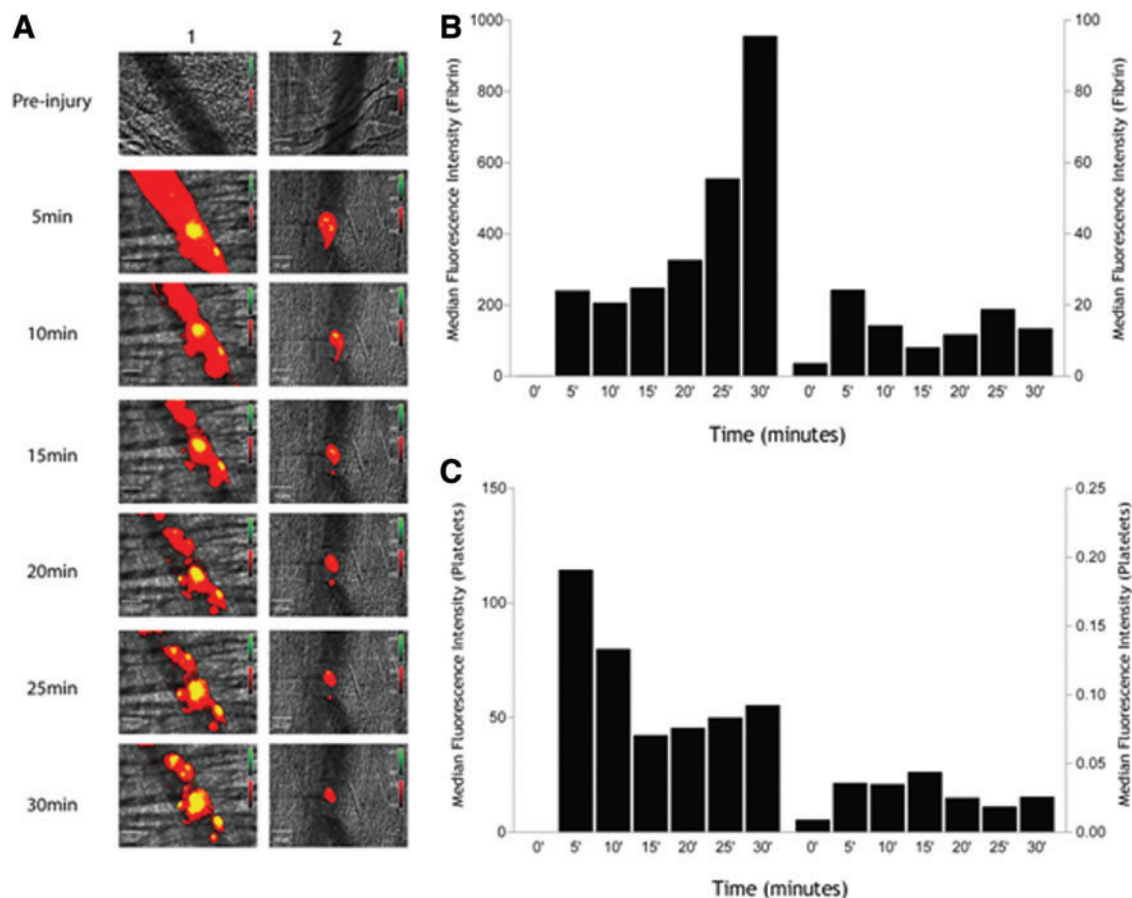
The relevance of these observations was realized by *in vivo* studies demonstrating that inhibition of PDI prevents thrombus formation in mice (16, 87). Given that redox reactions require study in the native environment, the development of a high-speed digital intravital microscopy system for the study of thrombus formation in live mice proved essential for what was otherwise an unanticipated discovery (25); the release of PDI following vascular injury is required to support not only platelet aggregation but also fibrin generation. PDI antigen is rapidly detected at sites of vascular injury even before the local accumulation of platelets (16, 45). Using laser injury directed at the vessel wall, PDI antigen first appears along the vessel wall and is then followed by the accumulation of platelets. The amount of PDI generated increased with increasing platelet thrombus size. Concomitantly, fibrin appears within and around the platelet thrombus. This indicates that endothelial-derived PDI is secreted from the injured vasculature and that platelet accumulation and platelet activation lead to the release of a second wave of PDI (16). Inhibition of PDI with the blocking antibody RL90 or the small molecule, quercetin-3-rutinoside, identified by high-throughput screening, inhibited both platelet thrombus formation and fibrin generation (16, 46).

The role of PDI in thrombus formation was also studied in a thrombosis model in which vessel injury was initiated with ferric chloride (Fig. 5). PDI antigen, not detected in the intact arteriole of the mesentery, accumulated at the site of injury. PDI antigen expression in the injured arteriole peaked at approximately 5 min and then slowly decreased. Thus, PDI antigen is expressed extracellularly during FeCl<sub>3</sub>-initiated thrombus formation as well as following laser-induced thrombus formation.

To prove that PDI specifically plays a role in FeCl<sub>3</sub>-induced platelet thrombus formation, the effect of an inhibitory anti-PDI antibody RL90 [known to cross-react with ERp57 (118)] on platelet deposition after FeCl<sub>3</sub>-induced laser injury was studied (Fig. 5). Pretreatment of mice with this antibody delayed the appearance of the initial platelet thrombus; no platelet accumulation was observed in about half of the mice treated at higher antibody doses and only about one third of injured vessels occluded within 30 min. Markedly reduced fibrin deposition was detected following administration of this antibody. These results indicate that PDI, or possibly ERp57 as well, is important for both platelet accumulation and fibrin deposition during thrombus formation following FeCl<sub>3</sub>-induced vessel wall injury. Quercetin-3-rutinoside, which inhibits PDI but not ERp57 activity, also blocks platelet accumulation and fibrin formation following FeCl<sub>3</sub> exposure, confirming a role for PDI (46). This extracellular PDI release triggered by cell activation is further supported by the observations that PDI rapidly adheres to sites of tissue injury even in severely thrombocytopenic mice (87), animals treated with the  $\alpha_{IIb}\beta_3$  inhibitor eptifibatid (45), and mice genetically deficient in platelet-derived PDI (55).

PDI appears to control the initiation of thrombus formation, but the mechanism by which it does so is currently unknown. Specifically, the targets of vascular PDI enzymatic activity have not been identified although some preliminary results are available. PDI has been proposed to modulate the initiation of tissue factor activity, thus initiating the blood coagulation cascade and ultimately thrombin generation and fibrin formation (15). Tissue factor exists in an encrypted inactive form, but the molecular basis of this inactivity has been elusive. Encrypted tissue factor can be detected as a tissue factor antigen on cell surfaces that binds to factor VIIa but its procoagulant activity is diminished or absent (6, 71).

Competing hypotheses include the dimerization of tissue factor (7), the absence of negatively charged phospholipid or the presence of neutral or anticoagulant phospholipid (85), sequestration of tissue factor in cholesterol-rich lipid rafts (21, 97), and the reduction–oxidation of the Cys 186–Cys 209 allosteric disulfide bond that corresponds functionally to a molecular switch from inactive to active (15). It has been suggested that the oxidation of tissue factor to generate a disulfide bond between these two residues is the key event in converting the encrypted tissue factor into its active conformation, with PDI playing a crucial role in this process *via* its oxidoreductase function (15, 80). Cys209 in tissue factor has been reported to be constitutively S-glutathionylated (88) or S-nitrosylated (2). This modification was hypothesized to prevent formation of the proposed Cys186–Cys209 disulfide. PDI has been suggested to deencrypt tissue factor to yield active tissue factor (2), whereas the inhibition of PDI has been suggested to increase tissue factor activity *via* expression of phosphatidylserine (79). PDI also regulates a critical



**FIG. 5. Anti-PDI monoclonal antibody inhibits thrombus formation and fibrin generation after  $\text{FeCl}_3$  injury.** Inhibitory anti-PDI or an isotype-matched IgG2a was infused into a wild-type mouse 10 min before  $\text{FeCl}_3$  injury. Fibrin was imaged using a fibrin-specific antibody conjugated to Alexa 488, and platelets were imaged using anti-CD42 antibodies conjugated to DyLight649. (A) The time course of appearance of fluorescence associated with fibrin and platelets as a function of time. Isotype-matched IgG2a (column 1) and inhibitory monoclonal anti-PDI antibody (column 2). (B, C) Median maximum integrated fibrin (B) and platelet (C) fluorescence infused with the IgG2a (left panels) and infused with anti-PDI antibody (right panels) versus time after arteriolar injury. This antibody has since been shown to cross react with ERp57 (118). To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

P2X7 receptor-dependent signaling pathway that is associated with the deencryption of tissue factor (31). The role of PDI in tissue factor activation remains unsettled.

Using mechanism-based kinetic trapping with active site variants of PDI, we have identified mixed disulfide intermediates between PDI and a number of novel substrates potentially critical for thrombus formation: vitronectin, complement factor 3, complement factor 5, C4b-binding protein,  $\alpha$ 2-macroglobulin, protein S, histidine-rich glycoprotein, thrombospondin 1, prothrombin, and CD5 antigen-like protein (Bowley, Furie and Furie; unpublished).

### ERp57

ERp57 is a single chain protein of 505 amino acids with a molecular weight of 57,000. It is expressed in eukaryotic cells from yeast to man and is widely distributed among mammalian tissues (70). Its domain structure is a-b-b'-a', and its domains share a high degree of similarity with those of PDI. Both enzymes have four thioredoxin-like domains with the a and a' domains each characterized by a catalytic active site motif CGHC. A monoclonal antibody, RL90, that was de-

veloped against PDI cross reacts with and inhibits the function of ERp57 (118), underscoring the degree of homology between the active domains of these enzymes. In contrast, the b' domain of ERp57, which is responsible for substrate binding, is distinct from that of PDI. This observation suggests that these two enzymes interact with different substrates (38, 56, 66, 90). That ERp57 is functionally distinct from PDI, despite their structural similarity evidenced by the fact that ERp57 cannot substitute for PDI in rescuing PDI deficiency in yeast (35). Moreover, ERp57 and PDI differ in their capacities for redox modulation by endogenous sulfhydryl oxidases such as Ero1 $\alpha$  (48, 57, 72). The gene for ERp57, *PDIA3*, is broadly expressed at low levels in various cell types (40), including the endothelium and megakaryocyte.

ERp57 is localized primarily to the ER of nucleated cells and contains an ER retrieval motif. A subpopulation of ERp57 escapes retention in the ER by a poorly understood mechanism and can be released into the extracellular environment, where it acts on extracellular substrates. As is the case with PDI, it is the extracellular subpopulation of ERp57 that participates in hemostasis and thrombosis. ERp57 is released from platelets upon platelet stimulation (40) and is released from the endothelium upon

endothelial stimulation. Its location within platelets has not been determined, but it is present in the membrane fraction of endothelial cells in addition to the ER (45).

The ERp57 b' domain interacts with the ER lectin chaperones calnexin and calreticulin, while the b' domain of PDI does not. Thus, intracellular substrates of ERp57 include glycoproteins that enter the calnexin cycle. Both PDI and ERp57 possess similar reduction potentials that are substantially more oxidizing than those of substrate thiols (37). Free thiol groups on the cell surface are important for normal cellular functions. Thiol isomerases on the cell membrane have multiple biological functions, including NO transport *via* transnitrosation (83, 122), redox control of exofacial protein thiols (49), and, in the case of ERp57, participation in the process of antigen presentation (67). The physiological importance of ERp57 is highlighted by the finding that the genetic knockout of ERp57 in mice leads to embryonic lethality (32).

Because ERp57 has many other well-described functions in the ER, including the processing of glycoprotein substrates of the calnexin/calreticulin cycle (76), genetic studies alone would be insufficient to distinguish between a direct extracellular role for the thiol isomerase rather than a bystander effect on another endogenous substrate of ERp57 such as various  $\beta 3$  integrins. Interpreting the genetic (114) and inhibitory antibody (39, 118) studies together, the data favor a nonredundant extracellular role for platelet and endothelial ERp57 in thrombus formation.

#### ERp57 in thrombus formation

ERp57 was first detected in platelets in profiling studies designed to identify thiol isomerases in mouse and human platelets and megakaryocytes (40). These experiments showed that stimulated platelets release ERp57 and ERp57-containing microparticles. A second approach used proteomics to identify proteins that are upregulated upon ligation of platelet GPVI showed that ERp57 is released from platelets upon GPVI activation (95). Polyclonal sheep anti-ERp57 antibodies inhibited platelet aggregation and adenosine triphosphate (ATP) secretion from washed platelets stimulated with CRP-XL, whereas PF4 and P-selectin expression was minimally altered (39). These results suggest the involvement of ERp57 in dense granule secretion, but not alpha granule secretion under the conditions used. Furthermore, these antibodies inhibited calcium mobilization, an early event in platelet activation, and the activation of  $\alpha \text{IIb}\beta 3$ , as monitored by fibrinogen binding. Using an *in vitro* thrombus formation model utilizing collagen glass fibers, anti-ERp57 antibodies inhibited platelet accumulation. ERp57 antigen accumulated on the developing thrombus in a mouse laser injury model of thrombus formation and blocking antibodies to ERp57 inhibited platelet thrombus formation (39).

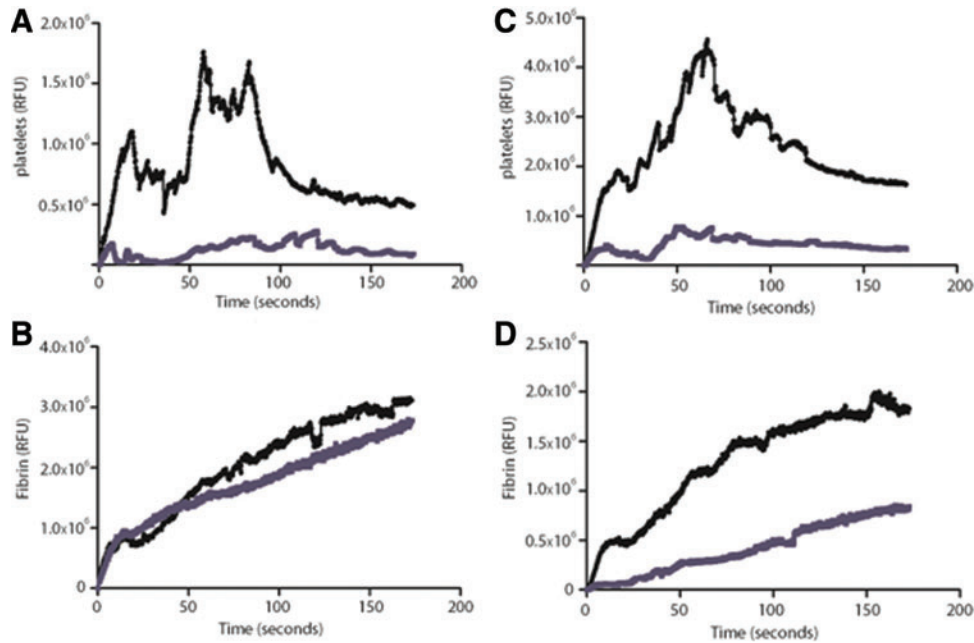
A monoclonal antibody to ERp57 inhibits platelet aggregation, whereas ERp57 enhances platelet aggregation and a mutant inactive form of ERp57 perturbs platelet aggregation (118). The activation of  $\alpha \text{IIb}\beta 3$  and the expression of P-selectin are inhibited by the anti-ERp57 monoclonal antibody under the conditions used. Genetically engineered mice lacking platelet-derived ERp57 have prolonged tail bleeding times and occlusion times in a mouse model of ferric chloride-induced carotid injury (116). In mesenteric arteri-

oles, incorporation of ERp57-null platelets into developing thrombi induced with ferric chloride was reduced compared to wild-type platelets and platelet incorporation was dependent upon the presence of  $\beta 3$  integrins. ERp57-null platelets revealed decreased platelet aggregation and decreased activation of  $\alpha \text{IIb}\beta 3$ . ERp57 bound to  $\beta 3$  in thrombin-activated platelets and not in platelets lacking  $\beta 3$ . These authors propose that ERp57 directly activates  $\alpha \text{IIb}\beta 3$  (116). These experiments were extended to the study of fibrin generation in platelets lacking ERp57 (123). Using the laser-induced thrombosis model, fibrin generation was reduced. In a mouse lacking ERp57 in the endothelium or upon addition of inhibitory antibodies to ERp57 into the mouse, fibrin generation was further reduced. These results parallel those observed for PDI (16, 45).

We have also evaluated the role of platelet-derived and endothelium-derived ERp57 in thrombus formation in a mouse thrombosis model using laser injury of the cremaster arteriole vessel wall. Both the stimulated endothelium and activated platelets release ERp57 at the site of thrombus formation after laser injury. The role of ERp57 in promoting platelet activation was evaluated with knocked down ERp57 in mice using a targeted antisense Vivo-morpholino that acts upstream of the translation start site of ERp57 (Vivo-ERp57). Reduction in expression of ERp57 was induced by injecting mice with 12.5  $\mu\text{g}$  per day per mouse of Vivo-ERp57 or control Vivo-morpholino for 4 consecutive days. To determine the functional importance of ERp57 secreted from activated platelets and endothelium *in vivo*, we observed the kinetics of platelet and fibrin accumulation in Vivo-ERp57-treated mice. Platelet accumulation was monitored in the developing thrombus using DyLight 649-labeled anti-CD42 antibody, and fibrin deposition was monitored using an Alexa 488-labeled fibrin-specific antibody that is not reactive with fibrinogen.

Infusion of Vivo-ERp57 morpholino resulted in markedly reduced expression of ERp57 levels in platelets as well as the kidney and liver. Platelet aggregation was impaired in platelets from Vivo-ERp57-exposed mice. To determine the functional importance of ERp57 secreted from activated platelet and endothelium *in vivo*, the kinetics of platelet accumulation and fibrin formation was evaluated using intravital microscopy in Vivo-ERp57-treated animals. Both platelet thrombus formation and fibrin accumulation were significantly impaired in mice exposed to Vivo-ERp57 morpholino compared to control Vivo-morpholino (Fig. 6).

Our group has further explored the role of ERp57 in thrombus formation using the laser injury thrombosis model to determine how isolated deficiency of platelet ERp57 affects thrombus formation *in vivo*. We generated ERp57<sup>fl/fl</sup>/PF4Cre-positive mice that did not express ERp57 in their platelets, but expression of PDI, ERp5, and ERp72 was not affected. ERp57<sup>fl/fl</sup>/PF4Cre-positive mice had a prolonged bleeding time, and ERp57<sup>fl/fl</sup>/PF4Cre-positive platelets failed to aggregate in response to a PAR4 agonist. In response to laser-induced vessel wall injury, platelet thrombus size in ERp57<sup>fl/fl</sup>/PF4Cre-positive mice was reduced by 85% compared to ERp57<sup>fl/fl</sup>/PF4Cre-negative control animals (Fig. 6). However, fibrin deposition at sites of laser injury was comparable in the two experimental and control groups (Fig. 6D). A function blocking antibody directed against ERp57 also inhibited both platelet accumulation and fibrin formation following laser-induced injury. These results indicate that under



**FIG. 6. ERp57 deficiency in mice studied using Vivo-morpholino anti-sense short-term elimination and animals genetically deficient in platelet ERp57.** (A, B) Deficient platelet thrombus formation and normal fibrin generation in mice treated with Vivo-ERp57. Fibrin-specific mouse anti-human fibrin antibody 59D8 conjugated to Alexa 488 and anti-CD-42b antibody conjugated to DyLight 649 were infused into a mouse treated with a control Vivo-morpholino or an Vivo-ERp57 5 min before arteriolar injury, and thrombi were recorded. (A) Median integrated platelet fluorescent intensity or (B) median integrated fibrin fluorescent intensity presented *versus* time after vessel wall injury. *Black*, control morpholino-injected mice; *gray*, Vivo-ERp57-injected mice. (C, D) Platelet-independent fibrin generation in mice with ERp57 deficiency in platelets. Fibrin-specific mouse anti-human fibrin antibody 59D8 conjugated to Alexa 488 and anti-CD42b antibody conjugated to DyLight 649 were infused into ERp57<sup>fl/fl</sup>PF4 Cre-positive or ERp57<sup>fl/fl</sup>PF4 Cre-negative mice 5 min before arteriolar injury and thrombi were recorded. (C) Median integrated platelet fluorescent intensity or (D) median integrated fibrin fluorescent intensity presented *versus* time after vessel wall injury. *Black*, ERp57<sup>fl/fl</sup>PF4 Cre-negative mice; *gray*, ERp57<sup>fl/fl</sup>PF4 Cre-positive mice. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

the conditions used, ERp57 is important for platelet thrombus generation but not fibrin generation. This contrasts with earlier results of others (123). Our experiments show that antibody-mediated inhibition of ERp57 or reduction of ERp57 expression with Vivo-ERp-57 results in reduction in fibrin deposition in wild-type mice after laser-induced injury, but mice with conditional platelet ERp57 deficiency demonstrate normal fibrin generation. This suggests that ERp57 released from endothelium is critical for fibrin generation *in vivo*.

Infusion of specific inhibitory antibodies to ERp57 before laser-induced injury results in diminished platelet thrombus development and fibrin generation. In contrast, platelet-specific ERp57 knockout mice show reduced platelet thrombus formation but normal fibrin accumulation. Our results demonstrate that ERp57 derived from both platelets and the endothelium following vascular injury is important to the thrombotic response. In the laser-injury model, ERp57 secreted from endothelial cells is sufficient to maintain normal fibrin accumulation but cannot restore platelet aggregation in the absence of platelet-derived ERp57.

### ERp5

ERp5, encoded by the gene *PDIA6*, is a protein of 48,000 molecular weight containing two catalytic CGHC motifs in

its a and a' domains, as well as a noncatalytic thioredoxin-like fold in its b domain, which are linearly organized in a unique a-a'-b arrangement. This configuration enables catalysis of redox, isomerase, and chaperone activities, but *in vitro* assays of model substrates suggest ERp5 to be a less efficient thiol isomerase than PDI itself (54). Few distinct substrates of ERp5 have been identified in the ER.

Specific functions for ERp5 in the ER remain poorly characterized, although this thiol isomerase appears to undergo transcriptional upregulation by spliced XBP-1 as part of the unfolded protein response (63). Despite the presence of a classical C-terminal KDEL ER retrieval motif, many unique roles for ERp5 have been established beyond the cell surface, including thrombus formation (77) as well as in the shedding of tumor-associated ligands required for metastasis (52). For example, the extracellular catalytic activity of ERp5 acts on the tumor ligand, major histocompatibility complex class-I-related ligand MICA, and contributes to tumor immunoevasion (52).

### ERp5 in thrombus formation

This enzyme expresses oxidoreductase activity, as demonstrated by the insulin reductase activity and the renaturation of ribonuclease. Like PDI and ERp57, ERp5 is secreted from platelets upon cell activation (51). Inhibition



of ERp5 function with an anti-ERp5 antibody prevented fibrinogen binding to activated platelets and platelet aggregation *in vitro* (51). The fibrinogen receptor  $\alpha$ IIb $\beta$ 3 is a binding partner of ERp5 as the enzyme coimmunoprecipitates with the  $\beta$ 3 chain of the integrin (51). The involvement of ERp5 during thrombus formation was studied using the mouse laser injury model and a specific antibody raised against recombinant ERp5 (77). Anti-ERp5 antibody inhibited ERp5-dependent platelet and endothelial cell disulfide reductase activity *in vitro*. ERp5 antigen released at the thrombus site in a live mouse was detected after vascular injury. Anti-ERp5 at higher doses inhibited laser-induced thrombus formation *in vivo* by causing a major decrease in the deposition of platelets and fibrin accumulation. ERp5 binds to  $\beta$ 3 integrin with a Kd of 21  $\mu$ M, measured by surface plasmon resonance, and binds in the presence or absence of Mn<sup>++</sup>. The cysteine residues in the ERp5 active sites are not required for binding to  $\beta$ 3 integrin. These results provide evidence for a novel role of ERp5 in thrombus formation.

### Interaction of $\beta$ 3 Integrins and Thiol Isomerases

The  $\beta$ 3 integrins of the vasculature, including  $\alpha$ IIb $\beta$ 3 and  $\alpha$ V $\beta$ 3, have been established as binding partners to extracellular PDI both *in vitro* and *in vivo* (17) (Table 1), but there is no evidence that these integrins are redox substrates of PDI. Integrins are transmembrane receptors capable of binding extracellular protein ligands that activate the integrin to induce an intracellular signaling cascade (“outside-in signaling”).  $\beta$ 3 integrin pairs with  $\alpha$ IIb on the surface of platelets and  $\alpha$ v on the endothelium to create the fibrinogen receptor  $\alpha$ IIb $\beta$ 3 and vitronectin receptor  $\alpha$ V $\beta$ 3, respectively. Integrin activation is accompanied by a large conformational change between the bent and elongated conformations (98) that requires a new pattern of disulfide bond formation. In the case of  $\alpha$ IIb $\beta$ 3, all cysteines are disulfide bonded in the closed conformation (119), but modifiable sulfhydryl groups become exposed during platelet activation (69, 120). PDI binds to the active form of  $\beta$ 3 integrin with 1–2  $\mu$ M affinity (17), and small molecule or antibody inhibitors of PDI prevent activation of  $\alpha$ IIb $\beta$ 3 and hence fibrinogen binding (60, 61).

PDI, ERp5, or ERp57 influences  $\beta$ 3 integrin function, but the mechanism is unknown. Wu reported the ability of an inhibitory antibody to ERp57 to inhibit platelet aggregation and activation of  $\alpha$ IIb $\beta$ 3, whereas ERp57 potentiates platelet aggregation, and the active site-mutated ERp57 inhibits platelet activation and prolongs the tail bleeding time in mice (118). Exogenous PDI binds to platelet PDI-null platelets but not to  $\beta$ 3-null platelets (55). Using the monoclonal antibody, PAC-1, specific for the activated conformer of  $\beta$ 3, we ex-

amined the expression of this antigen in human platelets treated with PDI, ERp5, and ERp57, alone and in combination by flow cytometry. We did not observe expression of the active conformer upon incubation of PDI, ERp5, ERp57, or a mixture of PDI+ERp5+ERp57 (Fig. 7). Under the conditions studied,  $\beta$ 3 does not appear to be a substrate of these thiol isomerases. However, these thiol isomerases all bind to  $\beta$ 3. Therefore, it would appear that the pathway to  $\beta$ 3 activation involves these thiol isomerases, but they do not directly alter the  $\beta$ 3 conformation to express the structure associated with integrin activation.

### Future Directions

Despite a firm and growing body of evidence supporting a critical role for PDI family thiol isomerases in thrombus formation and fibrin generation, a number of major questions invite future investigation.

#### *Thiol isomerase substrates involved in thrombus formation*

Although thiol isomerases are important for thrombus formation, the mechanism by which they support thrombus formation remains to be elucidated. Specifically, the identity of the substrates of each thiol isomerase needs to be defined as do the changes in structure and function that each substrate undergoes *via* the enzymatic action of the thiol isomerases.

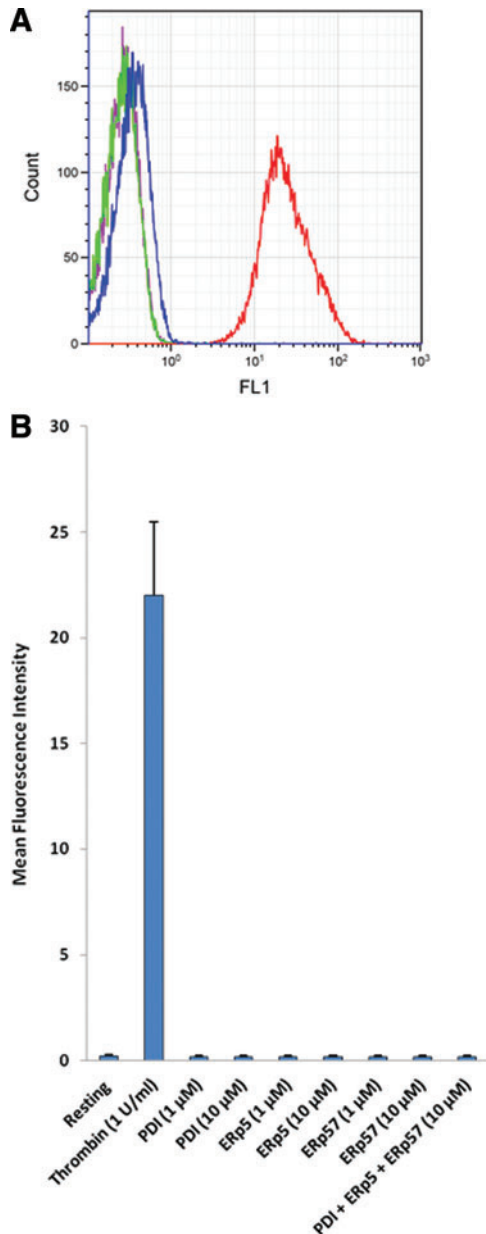
#### *Redox state*

Retaining one or more CXXC motifs, the PDI family thiol isomerases are able to catalyze a variety of chemical reactions, including reduction, oxidation, isomerization, and nitrosylation/denitrosylation of substrate thiols. The reduction potential and resulting redox state of various ER-resident PDI family proteins are not identical [for review, see Hatahet and Ruddock (37)]. However, the redox state of a thioredoxin-like protein dictates the types of reactions in which it may participate. While the active site of platelet surface PDI exists in both the dithiol and disulfide states, the fraction of PDI that is reduced increased substantially upon platelet activation (10). However, it is unclear whether PDI emerged in this state or had already undergone a redox process at the time of assay. The redox state of secreted thiol isomerases remains largely unknown. Similarly, as the key substrates of secreted thiol isomerases remain to be identified, the net vector of electron flow required to support hemostasis at sites of tissue injury must also be clarified.

A limitation of much recent work on PDI family proteins in thrombus formation has been the inability to determine the redox state of the released proteins, particularly *in vivo*. For example, many studies demonstrate rescue or creation of a thrombosis phenotype by infusion of purified wild-type or mutant PDI or ERp57 (46, 118, 123). Such a recombinant preparation could exist in a heterogeneous redox state, as PDI is often purified in the presence of a small molecule reducing agent that may not be completely eliminated, or alternatively, PDI may be variably oxidized by molecular oxygen during dialysis or freezer storage. Thus, unless the redox state of exogenous PDI is demonstrated before use or even at the site of action, the redox state could change following infusion. It remains unclear whether net oxidizing or reducing equivalents are introduced.

TABLE 1.  $\beta$ 3 INTEGRIN BINDING PROPERTIES OF THE VASCULAR THIOL ISOMERASES

	PDI	ERp57	ERp5
$\beta$ 3 binding	+	+	+
Binding to activated $\beta$ 3	+	+	+
Binding to unactivated $\beta$ 3	–	–	+
Inactive enzyme binding to $\beta$ 3	+	?	+
Binding constant	1 $\mu$ M	?	20 $\mu$ M



**FIG. 7. Thiol isomerases do not activate  $\beta_3$ .** Washed human platelets were incubated with recombinant human PDI (1 or 10  $\mu$ M), ERp5 (1 or 10  $\mu$ M), and ERp57 (1 or 10  $\mu$ M), either individually or in combination, in HEPES Tyrode buffer containing 5  $\mu$ M reduced glutathione (GSH), 1  $\mu$ M oxidized glutathione (GSSG), and 1 mM  $\text{CaCl}_2$  for 10 min at 37°C. Samples were then probed for activation of  $\beta_3$  integrin using conformation-specific antibody PAC-1 (FITC-labeled) using flow cytometry. (A) Histograms showing mean fluorescence intensity for 10  $\mu$ M PDI (green) and combination of PDI, ERp5, ERp57 (purple) each at 10  $\mu$ M compared to resting platelets (blue) and platelets stimulated with 1 U/ml  $\alpha$ -thrombin (red). (B) Mean fluorescence intensity of PAC-1 staining for various experimental conditions compared to resting platelets and platelets stimulated with 1 U/ml  $\alpha$ -thrombin. Bars represent mean  $\pm$  SD for three separate experiments. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

A major question surrounds whether the redox state of these thiol isomerases is actively regulated or modified, either immediately before secretion or beyond the plasma membrane. There is clear precedent for this within the cell, where enzymes such as thioredoxin reductase help maintain a reducing cytosol *via* reduction of thioredoxin (5), and thiol oxidases such as Ero1 create the oxidizing environment that drives disulfide bridge formation within the ER (28, 106). If any of the vascular thiol isomerases were to participate in a catalytic oxidative process (*e.g.*, a single thioredoxin was to participate in multiple sequential oxidation reactions with cell surface or plasma substrates), these thioredoxin-like proteins would need to cooperate with a sulfhydryl oxidase at the cell surface. Most of the topologically extracellular thiol oxidases, including the ER oxidase 1 proteins Ero1- $\alpha$  and - $\beta$ , the quiescin-sulfhydryl oxidases QSOX1 and 2, the vitamin K epoxide reductase paralogs VKORC1 and the VKORC1L1, and peroxiredoxin-4, are best characterized in the ER (11, 89, 124). However, Ero1- $\alpha$  may reside on the surface of activated platelets, where its association with PDI was proposed to support activation of  $\alpha$ IIB $\beta$ 3 (102).

By contrast, the QSOX enzymes have an established role in the late secretory pathway (12) and are present in a variety of extracellular fluids, including plasma (44). The single transmembrane domain of QSOX1a is proteolytically processed in the Golgi to enable its cellular egress *via* the secretory pathway (91). Although PDI itself has limited interaction with QSOX (84), a soluble secreted form of QSOX is a cell surface sulfhydryl oxidase that could provide oxidizing equivalents to support thrombus formation. In addition, peroxiredoxin IV is secreted, binds to human umbilical vein endothelial cells, and is a serum biomarker predicting cardiovascular disease, and all cause mortality (1, 75). Another enzyme that could contribute is the extracellular glutathione peroxidase 3 (GPx3), which metabolizes hydrogen peroxide to generate the oxidized disulfide-linked form of glutathione (GSSG) (103). Promoter mutations decreasing GPx3 expression lead to a hypercoagulable state characterized by platelet-dependent arterial thrombosis (50), and these mutations are associated with early-onset ischemic stroke (110). Generation of extracellular GSSG would indirectly bias the redox state of secreted thiol isomerases and, thus, regulate thrombosis.

#### Redundancy or specificity

Why are so many thioredoxin-like proteins required beyond the plasma membrane? Are there parallel and redundant pathways present to maximize redox capacity and ensure fidelity of extracellular redox reactions? Or, alternatively, are there separate and discriminatory pathways present to enable selective or regulated activation of specific substrates? As the redox potentials of the vascular thiol isomerases are not identical, one could even hypothesize that different thiol isomerases are secreted in distinct redox states, perhaps regulated by cooperation with specific thiol oxidases or reductases. ERp57 and PDI share a high degree of sequence homology, and the redox potentials for ERp57 (domain a,  $-0.167$ V; domain a'  $-0.156$ V) and PDI ( $-0.175$ V) are quite similar (29, 68). For these reasons, the question arises whether the roles of these two enzymes in thrombus formation are redundant or whether they have unique functions in this

process. Several lines of evidence suggest the latter. Function blocking anti-ERp57 antibodies further decrease thrombin-induced aggregation, ATP secretion, and  $\alpha_{IIb}\beta_3$  activation of PDI-null mouse platelets already impaired in these functions (55). A mechanism for the participation of both of these thiol isomerases in  $\alpha_{IIb}\beta_3$  activation is suggested by an elegant study of polyomavirus infection demonstrating that three thiol isomerases coordinately catalyze the unfolding of the C-terminal arm of VP1, the major coat protein of the virus, to facilitate infection (111). Alternatively or additionally, each of these thiol isomerases may interact with a unique set of substrates based on binding to their poorly conserved non-catalytic domains. The specialized role of ERp57 as a glycoprotein oxidoreductase suggests that it may be important in regulating platelet and endothelial cell surface glycoprotein function (47).

Mice specifically lacking platelet PDI suggested a defect in platelet aggregation *in vitro* as well as impaired platelet accumulation *in vivo* (55). This phenotype persisted despite upregulation of other thiol isomerases in PDI-null platelets in an apparent effort to compensate, suggesting these thioredoxin-like proteins are not redundant. One caveat is that it is difficult to distinguish impaired platelet activity due to global misfolding of platelet proteins dependent on ER PDI from a defect specific to the absence of secreted PDI. Although the defect can be rescued with exogenous PDI, it is unclear whether equimolar concentrations of other thiol isomerases would be sufficient.

#### Beyond the ER

Another unexplained question is how PDI and related thiol isomerases are secreted by platelets and endothelium? The vascular thiol isomerases all contain a classical C-terminal ER retrieval motif: KDEL in PDI and ERp5; QEDL in ERp57. These short peptide tags interact with a family of KDEL receptors in the early Golgi that should prevent escape to the cell surface and instead specify return of these cargos to the ER via COP-I-dependent retrograde transport (64). One could imagine a variety of mechanisms, including alternative splicing, regulated proteolysis, or posttranslational modification that would eliminate or mask the ER retrieval motif, but when examined, the KDEL sequence is preserved on the cell surface PDI (121). Other possibilities are that the retrieval motif receptors are actively downregulated or become saturated as previously described in states of ER stress (96), or that that the KDEL sequence or receptor becomes masked by interaction with chaperones or other regulatory molecules (36).

The cell biology of the endothelial cell and the platelet likely plays a critical role in PDI secretion. A KDEL receptor-dependent src kinase signaling pathway is activated by Golgi traffic (81) and has been implicated in cell surface expression of PDI in endothelial cells (112). Platelet PDI is stored in a newly described T-granule compartment that is secreted upon activation (105).

#### Conclusion

Thiol isomerases were originally described as intracellular actors that primarily functioned to assist in the process of protein folding. However, over the recent past, our understanding of these enzymes has expanded to include their role as extracellular regulators of thrombus formation *in vivo*.

While this function had not been anticipated, PDI and the other vascular thiol isomerases are now implicated at multiple steps in the thrombus formation, including their effects on platelet function and the initiation of coagulation. Although the specific mechanisms by which thiol isomerases perform this task remain to be clearly elucidated, the continued refinement of molecular probes and *in vivo* techniques promises to explain the role played by these regulators of thrombus formation.

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#### Abbreviations Used

ATP = adenosine triphosphate  
 AXXA = alanine-X-X-alanine  
 (where X is any amino acid)  
 CGHC = cysteine-glycine-histidine-cysteine  
 CXXC = cysteine-X-X-cysteine  
 (where X is any amino acid)  
 Cys = cysteine  
 ER = endoplasmic reticulum  
 FAD = flavin adenine dinucleotide  
 GPVI = glycoprotein VI  
 GPx3 = glutathione peroxidase 3  
 GSSG = glutathione (oxidized form)  
 KDEL = lysine-aspartate-glutamate-leucine  
 MMTS = S-methyl methanethiosulfonate  
 NO = nitric oxide  
 PDI = protein disulfide isomerase  
 PF4 = platelet factor 4