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Forum Review

The Role of Thiols and Disulfides in Platelet Function

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ABSTRACT

Disulfide bonds formed in newly synthesized proteins in the endoplasmic reticulum of cells are important for protein structure and stability. Recent research, however, emphasizes a role for thiol–disulfide reactions with disulfide bond rearrangement as a dynamic process in cell and protein function, and in platelet function in particular. Protein disulfide isomerase was found on the platelet surface where it appears to play an important role in the platelet responses of aggregation and secretion, as well as activation of the platelet fibrinogen receptor, the αIIbβ3 integrin. Additionally, sulfhydryl groups in αIIbβ3 have been implicated in the activation of this integrin. Physiologic concentrations of reduced glutathione generate sulfhydryls in αIIbβ3 and potentiate sulfhydryl-dependent reactions in αIIbβ3. Sulfhydryl labeling in αIIbβ3 is inhibited by phenylarsine oxide, a reagent that binds to vicinal thiols. As vicinal thiols are in equilibrium with disulfide bonds, they provide redox-sensitive sites in αIIbβ3 able to respond to external or cytoplasmic reducing equivalents. Furthermore, protein disulfide isomerase and sulfhydryls are now implicated in platelet adhesion by a second platelet integrin, the α2β1 collagen receptor. Most recently, extracellular sulfhydryls in the P2Y12 ADP receptor were found to be required for platelet activation by this receptor. We here provide an overview of this field with a focus on recent developments, and conclude with a working model. *Antioxid. Redox Signal.* 6, 736–746.

INTRODUCTION

Platelets are small subcellular fragments found in blood that are involved in the processes of hemostasis, atherosclerosis, and wound healing. In hemostasis, platelet plug formation represents the primary response to vascular injury, with the coagulation cascade and fibrin formation comprising the secondary response. Normal primary hemostasis requires three critical events: platelet adhesion, granule content release, and platelet aggregation. Within seconds of vascular injury, platelets adhere to collagen fibrils in the vascular subendothelium. This initial interaction is mediated through the collagen receptor, α2β1, a member of the integrin family of receptors, and by a second platelet receptor, glycoprotein Ib/IX, which binds to exposed subendothelial collagen fibrils through the adhesive protein von Willebrand factor. Adherent platelets then release granule contents, including ADP, and generate other mediators of platelet activation/aggregation, such as thromboxane A_2. Collagen, thrombin, and ADP are all important physiologic agonists in the activation of platelets. Similar to other cells, platelet activation is controlled by changes in the level of cyclic nucleotides, calcium influx, hydrolysis of membrane phospholipids, and phosphorylation of intracellular proteins. Platelet activation leads to activation of the αIIbβ3 integrin, (also known as glycoprotein IIb/IIIa). Upon activation, αIIbβ3 binds fibrinogen, causing adhesion of adjacent platelets resulting in the formation of the primary hemostatic plug.

The αIIbβ3 platelet integrin is part of the integrin family of receptors that are heterodimeric transmembrane receptor complexes, each with an α and β subunit. Integrins function in numerous physiological processes, such as hemostasis, immune responses, and angiogenesis (73). They function in cell adhesion and signaling by interacting with the extracellular matrix or other cellular receptors. Integrins exist in different activation states that have different affinities for ligands (54, 73). Activation of integrins controls cell adhesion, and integrin activation is reported to control metastasis in breast

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cancer (27). αIIbβ3 has provided the prototype example of integrin modulation as it goes through a transition from a low-affinity/avidity state to a state where it effectively binds soluble ligands, such as fibrinogen.

One general model for activation of the αIIbβ3 integrin is as follows (54, 73): (a) Platelet stimulation leads to changes in αIIbβ3 by inside-out signaling. This signaling is a response to intracellular signaling events and involves the propagation of conformational changes from the cytoplasmic domains of integrins to the extracellular ligand binding site; (b) this results in fibrinogen binding followed by (c) clustering of αIIbβ3; (d) the αIIbβ3-fibrinogen association provides the physical link for aggregation; and (e) further conformational changes in αIIbβ3 occur leading to outside-in signaling and additional platelet responses.

It has long been known that the poorly membrane-permeant sulfhydryl blocking reagent *p*-chloromercuribenzene sulfonate (pCMBS) inhibits platelet aggregation (1) and that reduction of disulfide levels in the fibrinogen receptor by reducing agents induces platelet aggregation (56). However, the role of thiols and disulfides in platelet function remained an underdeveloped area of platelet research. The report of protein disulfide isomerase (PDI) activity being secreted by activated platelets (12) and the localization of a functionally active PDI to the external surface of the platelet suggested functions for PDI not previously thought of (13, 24). PDI was found to mediate platelet aggregation and secretion, as well as activation of the αIIbβ3 integrin (22). A number of recent reports further implicate thiol groups or the rearrangement of disulfide bonds as part of a process that couples platelet stimulation to various platelet responses, including aggregation and secretion (10, 23, 26, 49–51, 67, 70, 83).

ROLE OF EXTRACELLULAR THIOLS AND DISULFIDES IN PLATELET FUNCTION

Platelet surface thiols and disulfides

Early reports demonstrated a role for platelet surface sulfhydryl groups in platelet responses and identified several classes of sulfhydryls on the platelet surface. The membrane-impermeant sulfhydryl reactive reagent pCMBS inhibits platelet aggregation induced by ADP (1, 58), collagen, and thrombin (36). By measuring the kinetics of binding of *p*-chloro[^{203}Hg]mercuribenzoate, at least two classes of thiols were found on purified platelet membranes (3). Spin-labeled probes detected four major classes of sulfhydryl groups on intact platelets (69) based on mobility. Using the membrane impermeant 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 3.1×10^{-18} mol of SH were found per platelet (36), whereas pCMBS reacted with 10×10^{-18} mol of SH per platelet (1). Using radioactive sulfhydryl agents, several groups identified four to nine labeled proteins on the platelet surface (4, 64, 68). However, the proteins and their reactive sulfhydryls that are critical to these platelet responses were until recently unknown.

Both subunits of the platelet fibrinogen receptor, αIIb and β3, contain disulfide bonds. αIIb contains 18 cysteine residues, and β3 contain 56 cysteine residues. Thirty-one of these are in a cysteine-rich region containing four repeats in the extracellular portion of the molecule. Cysteine residues of integrins are highly conserved, underscoring their importance. All of the cysteines in αIIbβ3 have generally been assumed to be disulfide-bonded, but recent results show that some exist as free thiols. The idea that the cysteines in αIIbβ3 are entirely in the disulfide form is based partly on work noting an "apparent lack of free thiol group" in the purified β3 (20) and αIIb (11) subunits using the reagent DTNB. Now at least three groups have labeled sulfhydryls in both αIIb and β3 (23, 26, 68, 83). There are several possible explanations for the difference in results, including the use of labeling reagents inherently more sensitive than DTNB, and the fact that SH groups in some sulfhydryl-containing proteins are inaccessible to DTNB for steric reasons. Additionally, sulfhydryl groups are subject to oxidation during purification procedures (78), and maximal labeling of αIIbβ3 on intact platelets is found with conditions that disrupt or dissociate the αIIbβ3 subunits (23).

Functional role for disulfides in platelets

Cleavage of disulfide bonds appears to be involved in conversion of αIIbβ3 to a fibrinogen-binding conformation. Concentrations of the reducing agent dithiothreitol (DTT) above 1 m*M* caused slow progressive platelet aggregation after a lag period of 1–4 min, provided that fibrinogen was present (56, 58). Unlike ADP-induced aggregation, DTT-induced aggregation was not inhibited by prostaglandin E1 (56, 87), suggesting that reduction of disulfide bonds triggers a later step in the platelet response, specifically, the development of the ability to bind fibrinogen. DTT activates purified αIIbβ3 to a fibrinogen binding state by reduction of disulfide bonds within the integrin's cysteine-rich repeats (84). This leads to global conformational changes in both αIIb and β3 and exposure of fibrinogen binding sites.

Activation of αIIbβ3 can also be effected by disruption of certain disulfide bonds in the β3 subunit, including the long-range disulfide bond Cys5–435 (76), the disulfide bond involving Cys560 in the third cysteine-rich repeat (70), or that involving Cys598 of the fourth repeat of the cysteine-rich region (14). Activation can also be effected by a noncysteine point mutation in the extracellular cysteine-rich repeat region of β3 (42) and substitutions in amino acids neighboring certain cysteines in the cysteine-rich region of the β subunits of integrins (86). This indicates that the cysteine-rich regions cooperate to restrain the integrin in the inactive conformation. Using [^{14}C]iodoacetamide labeling of the purified αIIbβ3 receptor, labeling was reported in 2.6 and 4.4 free cysteines of the nonactivated and activated forms of the receptor, respectively, suggesting that reduction of a disulfide bond is associated with αIIbβ3 activation (83). Together these results point to the involvement of disulfide bond cleavage in activation of αIIbβ3.

A number of recent advances made in structural studies of integrins are consistent with the concept that disulfide rearrangement is involved in the process of integrin activation. In the presence of calcium, the crystal structure of the extracellular segment of the αvβ3 molecules shows a compact v-shaped structure (82). One leg of the v-shaped form ends in

the large globule corresponding to the integrin headpiece, whereas the other leg corresponds to the tailpiece. In one model, upon activation the bent conformation extends in a "switchblade"-like fashion to a very different, completely extended conformation, with a globule head and two long tails (77). The possibility has been raised that inside-out activation of integrins is primarily triggered through protein movements in the β subunit (5). This is based on the findings that the vast majority of activating antibodies for integrins map to the β subunit (39) and that three of the β-domains in αvβ3 that are linked to activation are disordered and presumably flexible. Furthermore, the cysteine-rich region of the β subunit was found to be the fulcrum for integrin rearrangement upon activation (7). Together these findings suggest instability of disulfide bonds in these regions and the possibility of disulfide rearrangement in activation of integrins.

Platelet PDI

Traditionally, PDI has been known as an endoplasmic reticulum protein, but it has recently been identified on cell surfaces and shown to be functionally active (41, 59, 71). Our group demonstrated that PDI is on the platelet surface (13, 24). We found that PDI mediated platelet aggregation and secretion, as well as activation of the αIIbβ3 integrin to a full fibrinogen binding conformation (22). A role for PDI in adhesion of platelets by β_1 and β_3 integrins has also been reported, including adhesion by the α2β1 collagen receptor (49, 51). A physical and functional relationship of PDI to the adhesion receptor glycoprotein Ib on the platelet surface has also been reported (10), although sulfhydryl groups were not found to be involved in ristocetin-induced platelet aggregation mediated by this platelet receptor (50).

PDI is an enzyme that can catalyze three different reactions, isomerization of disulfide bonds including thiol–disulfide exchange, reduction, and oxidation (33, 55, 72) (Fig. 1). The oxidation of sulfhydryls to disulfides occurs primarily in the endoplasmic reticulum, whereas PDI on the cell surface is thought to primarily catalyze reduction or isomerization of disulfide bonds (65). Human PDI has a single subunit of 491 amino acids and has two regions of internal homology, each of which contains an active site with the sequence of Cys-Gly-His-Cys (Fig. 2). The active site Cys

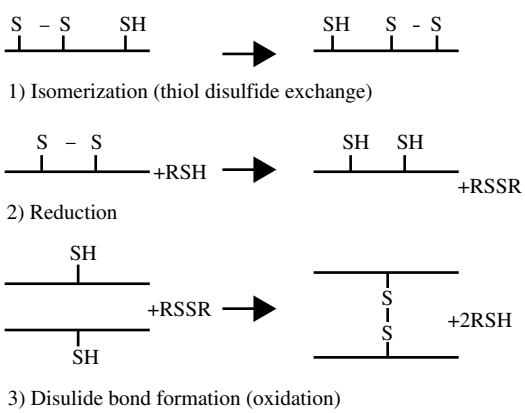

FIG. 1. Reactions catalyzed by PDI.

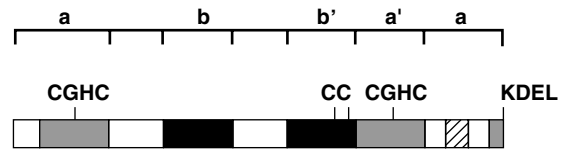

FIG. 2. Model of human PDI. Symbols: a and a′, internally homologous regions with sequence homology to thioredoxin; b and b′, internally homologous regions; c, highly acidic C-terminal region of the polypeptide. The active site sequences (CGHC), additional cysteine residues (C), and the C-terminal endoplasmic reticulum retention signal (KDEL) are also indicated.

residues are a disulfide in equilibrium with a dithiol, with the dithiol form catalyzing isomerization or reduction. In addition to the four cysteines at the active sites, there are two other cysteine residues. PDI contains a peptide/protein-binding site in the b′ domain (15, 46). An interaction of PDI with its substrate at this site appears to be required for catalytic activity (15, 29, 80). PDI also has a C-terminal tetrapeptide sequence, Lys-Asp-Glu-Leu (KDEL), which functions as an endoplasmic reticulum retention signal in cells.

The presence of a secreted platelet PDI was initially hypothesized on the basis of intramolecular and intermolecular thiol–disulfide exchange reactions occurring in the adhesive protein thrombospondin-1 in the supernatant solution of activated platelets (12, 75). We characterized PDI released from activated platelets (13). Platelet PDI has an apparent mass and isoelectric point similar to those of other PDIs, and the amino and sequence for the first 33 residues were identical to those predicted from chromosomal DNA (13). Using a rabbit antibody, we showed that PDI is on the external surface of platelets (13, 24). This PDI has activity against the soluble substrate ribonuclease A (RNase) that has been inactivated by formation of randomly mismatched disulfide bonds (scrambled RNase) (24). PDI can be released by microvesicle formation on platelet activation (13). It can also be released in a soluble form from the surface of resting platelets by raising the pH, beginning slightly above physiologic (25). This is consistent with the pH-dependent binding of PDI to its 26-kDa endoplasmic reticulum–Golgi receptor (79). Platelet PDI contains the C-terminal KDEL retention signal (25). Although we are unable to demonstrate a cytoplasmic pool of PDI in platelets (13, 24), others have found an approximately twofold increase of PDI on thrombin activated/aggregated platelets (10). This raises the possibility of an additional pool of PDI in platelets that may become exposed on the surface during the process of activation/aggregation.

The role of PDI in platelet responses

We initially investigated the role of PDI in platelet aggregation and secretion using (a) rabbit anti-PDI IgG specific for PDI, (b) a competing substrate (scrambled RNase), or (c) the PDI inhibitor, bacitracin (22). Strong inhibition of platelet responses to ADP or collagen by Fab fragments of the rabbit anti-PDI IgG was found at the same concentrations of Fab fragments that inhibited soluble PDI. Fab fragments prepared from normal rabbit IgG had no inhibitory effect. The role of

PDI in platelet aggregation was independently confirmed using a competitive substrate for PDI, scrambled RNase. Scrambled RNase has been shown to interact with PDI on the platelet surface (24) and to compete with peptides for the peptide-binding site of PDI, whereas native RNase competes only slightly (45). Scrambled RNase inhibited aggregation and the parallel secretion, whereas native RNase did not (22). Using flow cytometry and an antibody specific for activated αIIbβ3 (PAC-1), the rabbit anti-PDI Fab fragments, but not control Fab fragments, inhibited activation of αIIbβ3 (22).

The inhibition of both ADP and collagen-induced aggregation by anti-PDI Fab fragments suggests that the PDI-catalyzed event is in the later phases of platelet activation. This is because it is unlikely that PDI would similarly affect different primary agonist receptors that differ markedly in structure.

More subtle aspects of platelet activation are seen when platelet aggregation is studied in citrated plasma. Here a primary aggregation response is distinguished from a stronger secondary aggregation response. When this biphasic aggregation was examined using epinephrine and ADP, the principle effect of bacitracin on platelets in citrated plasma was inhibition of the second or irreversible phase of aggregation and the accompanying secretion (22). There was little inhibition of the primary response, even with concentrations of bacitracin that provided strong inhibition of platelet surface PDI activity. This suggests that primary aggregation is largely independent of PDI. The blocking of transmission to second wave aggregation by bacitracin implies that this event requires a conformational change that is facilitated, if not caused, by PDI.

Role of sulfhydryls in platelet responses

The inhibition of platelet aggregation and secretion was studied next using covalent thiol reagents, maleimides, or mercuribenzoates (26). As expected, these sulfhydryl inhibitors were effective against stimulation by normal physiologic stimuli. Dose-dependent inhibition of collagen-induced aggregation by the membrane-impermeant sulfhydryl reagent pCMBS was found using concentrations of pCMBS well below those shown to be membrane-impermeant (1). These results were confirmed using an impermeant sulfhydryl reagent of the maleimide class 3-(*N*-maleimidylpropionyl) biocytin (MPB). Using ADP as the agonist, and citrated platelet-rich plasma, the second phase, or irreversible aggregation, is inhibited by pCMBS. First wave aggregation, on the other hand, persists even at high concentrations, suggesting that first wave responses are independent of both PDI and other potential sulfhydryl proteins.

The inhibition of platelet aggregation by impermeant thiol blocking reagents suggests that a thiol–disulfide exchange reaction occurs in platelet surface proteins during aggregation. There is evidence that a thiol–disulfide exchange reaction occurs in purified αIIbβ3 when it is activated by the reducing agent DTT (83). Treatment of the nonactive form of αIIbβ3 with DTT was shown to induce binding to an integrin-specific RGD ligand. However, incubation of the nonactive form of αIIbβ3 with a maleimide sulfhydryl reagent blocked the ability of DTT to activate the integrin. This implies that the mechanism of DTT-induced activation of αIIbβ3 is due not only to disulfide cleavage, but also to thiol–disulfide exchange. Additionally, using a reduced RNase assay, intrinsic thiol-isomerase activity was found in purified αIIbβ3 in the presence of EDTA (67). This provides evidence for thiol–disulfide reactivity in αIIbβ3.

To define better the site(s) of thiol–disulfide exchange on intact platelets, we tested the effect of PDI inhibitors and sulfhydryl reagents on stimulation by a peptide that directly activates αIIbβ3. The peptide LSARLAF (LSA) has been shown to be a stimulus of platelet aggregation and secretion (6, 16, 17). Evidence that this peptide directly activates αIIbβ3 is that antibodies to the putative binding site, the sequence 315–321 of αIIb, inhibit LSA activation of platelets (control antibodies do not), and that LSA does not induce release of platelet factor 4 from thrombasthenic platelets (missing αIIbβ3) as it does from normal platelets. When stimulation was initiated with this peptide, the PDI inhibitors were without effect (26). The lack of involvement of PDI, which is a sulfhydryl enzyme, in stimulation of LSA fortuitously allowed us to assess the role of sulfhydryl-dependent pathways in at least one kind of stimulation. In contrast with this lack of inhibition by PDI inhibitors, the sulfhydryl inhibitor pCMBS caused strong inhibition of LSA-induced aggregation and secretion. Therefore, although PDI is required for platelet aggregation and secretion, an additional sulfhydryl-dependent step or protein is also required. This latter reaction occurs at the level of αIIbβ3.

A recent report also documented a role of PDI and sulfhydryls in platelet aggregation and activation of αIIbβ3 (50). A monoclonal anti-PDI antibody was found to have a substantial inhibitory effect on platelet aggregation. These researchers also sought to distinguish between a role for PDI and sulfhydryls in the initial agonist-induced stimulatory events and the activation of αIIbβ3. The monoclonal anti-PDI antibody had an inhibitory effect on activation of αIIbβ3 [measured by fluorescein isothiocyanate (FITC)-PAC-1 binding or FITC-fibrinogen binding], with the expression of a non–integrin-dependent marker of platelet activation (P-selectin) being inhibited to a lesser degree. αIIbβ3 activation was almost completely inhibited by pCMBS, but expression of P-selectin was inhibited by pCMBS to a substantially smaller degree. Thus, activation of αIIbβ3 is preferentially dependent on a sulfhydryl-containing protein compared with the initial stimulatory events. Additionally, expression of an epitope on αIIbβ3 that results from binding of the ligand fibrinogen was dependent on thiols and PDI.

Besides the role of PDI and sulfhydryls in platelet aggregation, PDI and extracellular sulfhydryls groups have been implicated in integrin-dependent platelet adhesion (49). pCMBS gave strong inhibition and anti-PDI partial inhibition of platelet adhesion by both β1 and β3 integrins. Removal of pCMBS prior to adhesion resulted in the restoration of adhesion, indicating that new sulfhydryls are exposed in response to adhesion.

The involvement of PDI in activation of a second platelet integrin, α2β1, a collagen receptor, has been studied in detail (51). This study used a collagen-based synthetic peptide that contains the α2 I-domain recognition motif, GFOGER, in type I collagen (47) shown to be specific for binding to integrin α2β1 (21, 48). This allowed examination of platelet

adhesion via α2β1 separately from other adhesive receptors for collagen, such as glycoprotein VI. The non-integrin glycoprotein VI-specific synthetic collagen-related peptide (CRP) allowed glycoprotein VI to be tested as an integrin-independent substrate. Fab fragments of the polyclonal anti-PDI [previously shown to inhibit PDI activity and agonist-induced platelet aggregation (22, 24)] inhibited adhesion to GFOGER but not to CRP, as did a monoclonal anti-PDI antibody that blocks PDI activity (51). Adhesion to type I collagen or to GFOGER was also inhibited by sulfhydryl blocking agents, whereas adhesion to CRP was not (51). Together with our previous reports, these data suggest that PDI-catalyzed rearrangement of disulfide bonds is a general mechanism for members of the integrin family.

A summary of the major points thus far is as follows: (a) PDI or sulfhydryls are not generally required for primary or reversible platelet aggregation, but (b) PDI and sulfhydryls are required for irreversible aggregation and secretion by normal stimuli. The original observation that inhibition of PDI decreased the ability of αIIbβ3 to bind PAC-1 (an antibody recognizing the activated state of αIIbβ3) (22), suggested a causal link between these two proteins. A similar relationship is suggested in the report that integrin-mediated adhesion is dependent on PDI (49), and more recently by the evidence that PDI regulates the affinity of the α2β1 receptor for collagen, but does not affect the glycoprotein VI collagen receptor (51). The evidence that direct activation of αIIbβ3 on platelets by an agonist peptide (LSA) is inhibited by sulfhydryl reagents along with the evidence that thiol–disulfide exchange is required for activation of purified αIIbβ3 by DTT (83) suggests that a thiol–disulfide exchange reaction in αIIbβ3 is required for the activation of this receptor.

Thiols can also participate in other reactions besides thiol–disulfide exchange that could serve to modulate the sulfhydryl-dependent pathways of platelet activation. For example, nitric oxide (NO) reacts with certain thiols to form *S*-nitrosothiols. NO is a potent inhibitor of platelets and can inhibit PDI, presumably by reacting with thiols in the active sites of PDI (85). Furthermore, the combination of 3 m*M* reduced glutathione (GSH) and NO has been shown to inactivate purified αIIbβ3 (83). During platelet activation, NO is generated and released from platelets, providing a negative feedback inhibition against continuing platelet recruitment (28). Although the physiologic significance of the formation of *S*-nitrosothiols in platelet surface proteins is presently unclear, the formation of *S*-nitrosothiols in the active site of platelet PDI would inhibit PDI function. The formation of *S*-nitrosothiols in αIIbβ3 would be expected to modulate thiol–disulfide exchange reactions in αIIbβ3. Similarly, peroxynitrite ($ONOO^-$; a product formed when NO reacts with superoxide anion released from platelets) is a sulfhydryl oxidant known to affect platelet function (63). As peroxynitrite can react with both low-molecular-weight and protein thiols in platelets (66), it may also contribute to platelet sulfhydryl oxidization.

Labeling of platelet surface thiols

The results described above show that platelet function is dependent on extracellular thiols and point to thiol-mediated reactions in αIIbβ3 and/or proteins associated with it. Both the αIIb and β3 subunits can be labeled with sulfhydryl reagents (23, 26, 83). Using a maleimide reagent, both subunits of purified αIIbβ3 were labeled, but only the β3 subunit was labeled, on intact platelets (83). Labeled sulfhydryls in the β3 subunit were localized to a 30-kDa fragment (amino acid residues 400–650) that contains the cysteine-rich region (83). We labeled intact platelets with three different membrane-impermeant sulfhydryl reagents, including the biotinylated maleimide MPB, and found similar labeling patterns of about nine proteins, including αIIb and β3 (26). Specificity of labeling for sulfhydryls was demonstrated by inhibition of the labeling by either preincubation of the labeling reagent with GSH or preincubation of the platelets with a different type of sulfhydryl blocking reagent.

Optimal labeling of αIIbβ3 on intact platelets with the reagent MPB was found to occur on intact platelets under conditions known to disrupt and inactivate the receptor (5 m*M* EDTA, 60 min, 37°C) (23). As EDTA does not by itself generate new sulfhydryls, the increased labeling in these studies suggests cryptic sulfhydryls that are sterically hindered from reacting with the labeling reagent we used.

The effect of platelet activation on labeling of thiols

When platelets are stimulated and allowed to aggregate in the presence of MPB, the sulfhydryl labeling increases in ~11 platelet surface proteins, including PDI (10). The increase in sulfhydryl labeling is not accounted for by an increase in surface exposure of the same proteins. This implies that sulfhydryls are generated in these proteins during platelet activation/aggregation. Importantly, only a small amount of the active sites on PDI were in the dithiol form on resting platelets, compared with 81% in the dithiol form on activated platelets. As the increase in labeling could not be accounted for by translocation of proteins from internal stores or by secretion of low-molecular-weight thiols (10), it suggests the possible action of an NAD(P)H oxidoreductase system similar to that found on cell membranes of other cells (43, 81).

We found a two- to threefold increase of labeling in the β3 subunit of αIIbβ3 on intact platelets with platelet activation (23). This increase could not be explained by receptor number and is therefore due to exposure or generation of new sulfhydryls. There was no detectable increase in labeling of the α subunit. Sulfhydryl labeling studies performed on the purified αIIbβ3 integrin found an increase of 2-sulfhydryls in the activated form of the integrin (83). As the activated form of the αIIbβ3 receptor has two additional thiols (83), and as sulfhydryls are generated in ~11 other platelet surface proteins with platelet aggregation (10), the increase we found in β3 with activation is likely to be due to generation of thiols during activation.

The effect of glutathione and other low-molecular-weight thiols on platelet aggregation

Glutathione is an important modulator of the cellular redox environment, but is also found in blood where it could modulate platelet function and integrin activation. Whereas

cells contain 1–10 m*M* total glutathione, almost all in the reduced form, plasma contains only ~10–25 μ*M* (2, 52, 53, 60, 61). However, a large fraction in plasma is also in the reduced form, with the GSH/glutathione disulfide (GSSG) ratio in plasma being in the 4:1 to 13:1 range (2, 52, 53, 60). Plasma also contains other low-molecular-weight thiols (cysteine, cysteine-glycine, and homocysteine), mostly in disulfide forms (31, 60). Plasma glutathione levels, as well as the ratio of GSH/GSSG, are altered in disease states, including fasting alcoholism, cirrhosis, and malignancy (26, 53, 61). Unlike cellular glutathione, a specific role for plasma glutathione in redox reactions has not been well studied.

In the presence of subthreshold concentrations of agonist, physiologic concentrations of GSH (10 μ*M*) stimulated platelet aggregation and secretion (23). GSH by itself had little effect on platelets and, even when tested at varying concentrations, could not by itself stimulate aggregation. Other low-molecular-weight thiols, cysteine and cysteine-glycine, also potentiated irreversible aggregation, but concentrations 10-fold higher than GSH were required. A stimulatory effect of the sulfhydryl or reduced form of homocysteine was also found on aggregation at 1–3 μ*M* concentrations and was maximal at 4–5 μ*M* concentrations. The effect of homocysteine is of special interest, because homocysteine is a known risk factor for vascular disease. Levels of the reduced form of homocysteine that potentiate aggregation have been reported in end-stage renal disease, a disease with unusually high rates of cardiovascular morbidity and death (38).

As the effect of GSH is found with more than one platelet agonist (ADP and collagen), and as the primary agonist receptors have markedly different structures, it is likely that GSH stimulates the later phases of the platelet activation pathway. One possible mechanism is that the sulfhydryl compounds are inducing a partially activated state of the platelet integrin αIIbβ3, which lowers the threshold for other agonists to induce a fully active conformation. Both GSH and cysteine had a stimulatory effect on activation using subthreshold levels of the direct αIIbβ3 agonist peptide LSA (23). GSH (10 μ*M*) induced an over twofold increase in sulfhydryl labeling in the β3 subunit, confirming a mechanism in αIIbβ3. (A 1.6–1.7-fold increase in labeling of αIIb was also found with GSH). As GSH by itself does not cause platelet aggregation, this sulfhydryl generation does not by itself fully activate the receptor. It rather appears to prime the αIIbβ3 receptor for activation by a second step that is presumably a thiol–disulfide exchange reaction.

The effect of GSH on platelet aggregation that we found contrasts with the effect of the reducing agent DTT on platelet aggregation and activation of the αIIbβ3 receptor (57, 58, 87). DTT in the presence of fibrinogen causes a slow progressive platelet aggregation without secretion of α or dense granule contents. No agonist is needed. GSH by itself does not cause aggregation, and the aggregation tracings produced by the effect of GSH follow the pattern of the agonist used (for example, collagen or ADP) (23). Additionally, the effect of DTT is only found with concentrations over 1 m*M*, much higher than the concentrations of GSH used in our studies [despite DTT being a better reducing agent (32)]. Therefore, mechanistically the understanding of the role of thiol groups in our studies is an advancement on the studies with DTT. Most importantly, the effect of GSH appears to be physiologic. Not only is GSH normally found in plasma, but also its effect on platelets is found at physiologic GSH concentrations (23).

To determine if the requirement was for reducing equivalents or for a redox potential (ratio of GSH/GSSG), aggregation was further studied with the addition of low concentrations of GSSG to the GSH. With a ratio of GSH/GSSG of 5:1, similar to that of blood, the addition of GSSG potentiated the stimulatory effect as compared with GSH alone (23). This indicates that the effect of glutathione on aggregation is not simply by GSH reducing disulfide bonds; there is rather a requirement for a certain redox potential.

Although not our current focus, it should be mentioned that intracellular glutathione also has a role in platelet activation. Platelets contain 11–15 nmol of GSH per 10^9 platelets with >90–95% of intraplatelet glutathione being found in the reduced form (35, 37). Conversion of cytoplasmic GSH to GSSG (by diamide) inhibits platelet aggregation and induces disulfide cross-linking of cytoskeletal proteins (9, 62, 74). Thus, cytoplasmic GSH appears to have a fundamental role in platelet activation, probably by maintaining the sulfhydryl status of cytoplasmic proteins. As platelets do not secrete GSH (10), platelet sources of GSH do not appear to have a direct role in the extracellular reactions we are studying.

A role for vicinal thiols

Sulfhydryl groups that are in proximity to each other such that they undergo reversible dithiol/disulfide conversions are considered vicinal thiols (34). The active sites of PDI and similar redox enzymes contain cysteines that undergo these reversible conversions. The susceptibility of disulfide bonds in αIIbβ3 to low concentrations of GSH (10 μ*M*) is unusual and suggests the presence of a disulfide bond that can undergo such reversible dithiol/disulfide conversions. To test for a role for vicinal thiols of surface proteins in the sulfhydryl-dependent pathways of platelet activation, we used phenylarsine oxide (PAO), a reagent that binds to vicinal sulfhydryls. PAO was found to inhibit platelet aggregation (23). The membrane-impermeable sulfonic acid dithiol 2,3-dimercaptopropane sulfonic acid (DMPS) removes PAO from its target (8). Reversibility of the inhibitory effect of PAO by DMPS confirmed that PAO was not nonspecifically affecting platelets and that its effect is on vicinal thiols on the platelet surface.

Vicinal thiols in αIIbβ3

Possible targets of PAO on platelets include the known vicinal thiols of the PDI active site or potential vicinal thiols in αIIbβ3. The vicinal thiols in the active site of PDI are expected to react with PAO, and this has been demonstrated experimentally (19). To test for a possible effect of PAO on αIIbβ3, we incubated platelets with PAO under different conditions and looked for inhibition of the labeling with MPB. Lanes 1 and 4 of Fig. 3 show the labeling patterns of nonactivated and activated platelets. The bands at approximately 100 and 130 kDa were previously shown by immunoprecipitation to be the β3 and αIIb subunits of αIIbβ3, respectively (23). As previously reported, the β3 subunit shows an increase in

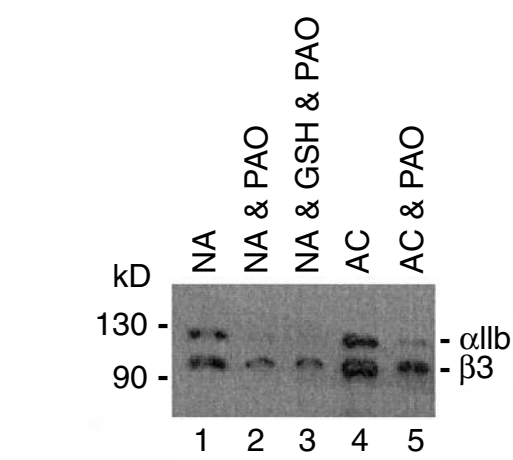

FIG. 3. Inhibition of MPB labeling of αIIbβ3 by PAO. Gel-filtered platelets were prepared and labeled with MPB under conditions that maximize labeling of αIIbβ3, as described elsewhere (23). Lanes 1 and 4 show labeling of nonactivated (NA) and activated platelets (AC), respectively. Lanes 2 and 5 show labeling of nonactivated and activated platelets incubated with PAO (100 μ*M*, 10 min, 24°C) prior to MPB labeling. Lane 3 shows platelet samples treated with GSH (10 μ*M*, 10 min, 24°C), and then with PAO before labeling with MPB. The samples were run on sodium dodecyl sulfate (10%)–polyacrylamide gel electrophoresis under reducing conditions, blotted onto a polyvinylidene difluoride membrane, and developed as described (26).

sulfhydryl labeling with platelet activation (lane 4) (23). PAO substantially inhibited the labeling of the αIIb and β3 subunits in both the nonactivated and activated platelets (lanes 2 and 5). We have also previously shown that a low concentration of GSH (10 μ*M*) causes a two- to threefold increase of thiols in the β3 subunit (23). This effect is also inhibited by PAO (lane 3). These findings indicate that both αIIb and β3 contain vicinal thiols and that platelet activation results in exposure or generation of vicinal thiols in the β3 subunit. As vicinal thiols are in equilibrium with disulfide bonds, these findings imply that there are redox-sensitive sites in both αIIb and β3 that can be regulated by platelet redox mechanisms or by low-molecular-weight thiols found in the external redox environment.

A role for sulfhydryls in the platelet ADP receptor

In an interesting recent development, a platelet receptor for ADP, P2Y12, was found to contain two extracellular thiols that are involved in platelet activation by this receptor (18). Thiols at both Cys17 and Cys270 of the P2Y12 ADP receptor are the targets of thiol reagents like pCMBS, although Cys270 appears to be the most important. The active metabolites of a commonly used platelet inhibitor, clopidogrel, have a reactive sulfhydryl group that forms disulfide bridges with Cys17 and/or Cys270. This results in inactivation of the receptor, apparently by blocking the binding of ADP to the P2Y12 receptor (interference of redox exchange between Cys17 and Cys270 was also a postulated mechanism). In contrast to the P2Y12 ADP receptor, the corresponding extracellular cysteines of a homologous platelet ADP receptor, P2Y1, are disulfide-linked and therefore not inhibited by thiol reagents like pCMBS or clopidogrel. As ADP secreted from platelets plays a role in platelet activation by other agonists (such as collagen or thrombin) through the P2Y12 receptor (40, 44), this receptor may provide an additional site for regulation of platelet function by redox reactions.

The generation of thiols by a redox mechanism

In regard to the role of PDI one question is, how is PDI activity controlled? The thiols of the active site of PDI are in equilibrium with a disulfide bond. A shift to the thiol form increases PDI activity. As the mechanism of thiol–disulfide exchange requires nucleophilic attack of a free thiol as the first step, a redox mechanism may be required to generate or maintain free thiols in the active site of PDI. The demonstration that activated/aggregated platelets show an increase in surface sulfhydryls and, in particular, PDI sulfhydryls in the absence of secreted low-molecular-weight thiols (10) supports this concept. These findings together with the increase in thiols in the activated form of αIIbβ3 (83) point to a redox system in the platelet membrane similar to those found on the plasma membrane of other cells (43, 81). Additional evidence for such a system is that whereas, using purified proteins, added GSH is generally required in assays for PDI-catalyzed thiol–disulfide exchange or reductive activities (to shift the active site of PDI to the thiol form), added GSH is not required for PDI activity in platelet aggregation (22) or for cell surface PDI activity in other cells (41, 59, 71, 85). This suggests that GSH plays a role in these *in vitro* studies that some cellular mechanism normally provides.

In addition to possible cellular mechanisms for activating PDI, extracellular low-molecular-weight thiols are also likely to affect PDI activity. Plasma contains a concentration of GSH (~10 μ*M*) that begins to generate thiols in purified PDI (30). Therefore, in plasma GSH may work together with cellular redox mechanisms to control activity of platelet PDI.

SUMMARY AND WORKING MODEL

Although we traditionally think of disulfide bonds as structural components in proteins, current evidence also implicates thiol–disulfide rearrangement as a dynamic process in stimulus–response coupling. Numerous lines of evidence now confirm early findings that sulfhydryl groups in platelet surface proteins are required for platelet responses. Accumulating evidence supports the original report (22) that PDI mediates activation of αIIbβ3 to a high-affinity/activity state (50). PDI also plays a role in the activation of other platelet integrins, including the α2β1 collagen receptor (49, 51). GSH at concentrations (and a ratio of GSH/GSSG) normally found in blood potentiates platelet aggregation (26). As the effect of GSH was found with several platelet agonists, the effect is likely on the later phases of the platelet stimulation pathway. The effect of low-molecular-weight thiols on LSA-induced aggregation localizes this effect to the level of αIIbβ3. The finding that GSH increases sulfhydryls in the β3 subunit substantiates this.

Several lines of evidence suggest that a transplasma membrane oxidoreductase generates thiols from disulfides on the platelet surface. Such a mechanism would both stimulate PDI activity and prime other proteins such as αIIbβ3 for thiol–disulfide exchange reactions. Vicinal thiols in equilibrium with disulfides in αIIbβ3 and PDI provide specific redox sites able to respond either to a transplasma membrane oxidoreductase or to changes in thiols in the external redox environment. Other reactions that thiols are involved in, such as the formation of *S*-nitrosothiols, are likely to modulate this system.

Mechanistically, at least two lines of evidence imply that a thiol–disulfide exchange reaction is required in αIIbβ3 in order for this receptor to attain a full ligand binding conformation. First, reagents like pCMBS or MPB that prevent thiols from being involved in thiol–disulfide exchange reactions inhibit both activation of the purified integrin and platelet aggregation induced by direct activation of the integrin (26, 83). Second, GSH generates sulfhydryls in β3, but does not by itself induce aggregation. This implies that a second thiol-dependent reaction in αIIbβ3 is required (23).

Working model

The working model from the above data (Fig. 4) focuses on interrelations of PDI and the integrin αIIbβ3. It is likely that the PDI/sulfhydryl surface events work together with cytoplasmic pathways (not shown here) that lead to conformational changes in αIIbβ3 through inside-out signaling (54, 73). The major points are as follows: After agonist stimulation, cytoplasmic events lead to a low-affinity binding of fibrinogen to αIIbβ3 (Fig. 4; **1**); this is followed by further inside-out changes in αIIbβ3 (**2**); these changes are facilitated on the platelet surface by a PDI-catalyzed event (**3**). The PDI-catalyzed event is probably a thiol–disulfide exchange reaction, although reduction of a disulfide bond could be involved. Thiol–disulfide rearrangement, depicted in αIIbβ3, facilitates formation of the high-affinity/avidity state. It is not yet clear whether the interaction of PDI with αIIbβ3 is direct or indirect. Rearrangement of disulfide bonds or sulfhydryl generation may be part of a cascade of events that couples platelet stimulation to the various responses, including aggregation and secretion. External GSH or a transplasma membrane NAD(P)H-dependent reductase would generate sulfhydryls in PDI or αIIbβ3. This would facilitate the reactions, as well as provide mechanisms for controlling the reactions.

A more general model takes into account the role of PDI in activation of the α2β1 integrin (51). In this model, an initial interaction of an extracellular ligand with the α2β1 or αIIbβ3 integrin initiates a PDI-catalyzed event that induces a full ligand binding conformation in the integrin:ligand→αβ→PDI→αβ*. Although this model again focuses on extracellular events, transmembrane signaling events may also be involved.

In summary, stimulus–response coupling in platelets and, in particular, PDI modulation of the integrin αIIbβ3 can be seen as models for other systems in cell biology. PDI regulation of the platelet α2β1 collagen receptor suggests that affinity regulation of integrins by PDI is a general mechanism. Both the generation of thiols and thiol–disulfide exchange appear to be mechanisms in the activation of integrins. The characterization of the role of thiols, disulfide exchange, and PDI in platelet responses could lead to the development of a new class of therapeutic platelet or integrin inhibitors.

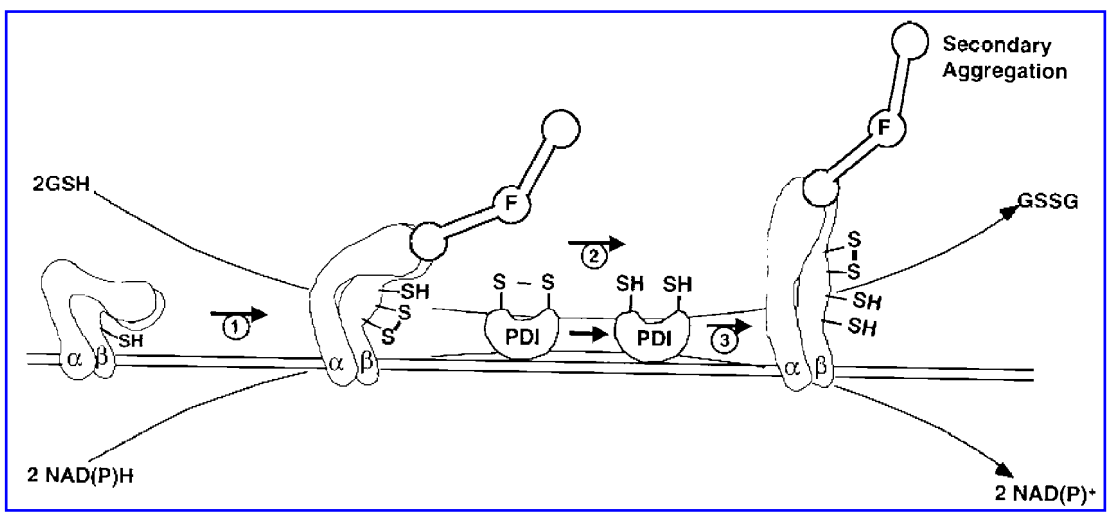

FIG. 4. Working model of the role of sulfhydryls in platelet function. The platelet fibrinogen receptor αIIbβ3 (αβ) is shown in three different activation states. The nonactivated state on the left side contains free thiols. Agonist-induced stimulation leads to cytoplasmic events resulting in inside-out signaling and an initial ligand-binding interaction of fibrinogen (F) with the receptor (**1**). Further inside-out signaling leads to the high-affinity conformation of αIIbβ3 (**2**). This change is facilitated by a PDI-catalyzed event (**3**). The high-affinity conformation is represented by secondary platelet aggregation. During platelet activation, sulfhydryls are generated in the active site of PDI as well as in αIIbβ3 from cytoplasmic reducing equivalents represented by NAD(P)H. Factors in the external redox environment, such as GSH, also influence the sulfhydryl status of both PDI and αIIbβ3 to facilitate the reactions shown.

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ABBREVIATIONS

CRP, collagen-related peptide; DMPS, dithiol 2,3-dimercaptopropane sulfonic acid; DTNB, 5, 5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; FITC, fluorescein isothiocyanate; GSH, reduced glutathione; GSSG, glutathione disulfide; LSA, peptide LSARLAF; MPB, 3-(*N*-maleimidylpropionyl)biocytin; NO, nitric oxide; PAO, phenylarsine oxide; pCMBS, *p*-chloromercuribenzene sulfonate; PDI, protein disulfide isomerase; RNase, ribonuclease A.

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