Redox Control of Platelet Aggregation†

David W. Essex*,‡ and Mengru Li§

Department of Medicine, Division of Hematology/Oncology, and Department of Biochemistry, State University of New York/Downstate Medical Center, Brooklyn, New York 11203, and Department of Medicine, Division of Hematology, University of Texas/Health Science Center at San Antonio, San Antonio, Texas 78229-3900

Received August 1, 2002; Revised Manuscript Received October 29, 2002

ABSTRACT: Sulfhydryl and disulfide metabolism in platelet function has recently reemerged as a focus of platelet research. In this study we tested the effect of redox buffer on platelet aggregation and the effect of reduced glutathione (GSH) and platelet activation on sulfhydryl exposure in the platelet fibrinogen receptor, αIIbβ3. In the presence of subthreshold concentrations of agonist, physiologic concentrations of GSH (10 μM) stimulated platelet aggregation and secretion. These effects were found with more than one platelet agonist and with different low molecular weight thiols, including homocysteine. The effect of low molecular weight thiols was reproduced with the peptide LSARLAF which directly activates platelets through αIIbβ3, suggesting that the mechanism is at the level of this integrin. After determining optimal sulfhydryl labeling conditions for αIIbβ3 (5 mM EDTA, 37 °C, 60 min), we found that GSH (10 μM) generated sulfhydryls in the β3 subunit. To determine if the requirement was for reducing equivalents or for a redox potential (ratio of GSH to 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 to GSH alone. This indicates that, for potentiation of aggregation, GSH is not simply reducing disulfide bonds; there is rather a requirement for a certain redox potential. Additional studies performed in the absence of added glutathione showed an increase in sulfhydryl labeling in the β3 subunit during platelet activation. Finally, we show that vicinal dithiols of platelet surface proteins are involved in the sulfhydryl-dependent pathways of platelet activation. In summary, these data imply that the redox potential of blood regulates activation of the αIIbβ3 integrin and together with other reports in the literature suggest that disulfide bond cleavage with sulfhydryl generation in β3 is involved in activation of this receptor.

Blood platelets are central to hemostasis, and reactions in platelets involving disulfide bonds, sulfhydryl groups, and protein disulfide isomerase play important roles in platelet function (*1*−*3*). Disruption of disulfide bonds in the αIIbβ3[1] integrin is associated with activation of this receptor so that it binds fibrinogen (*3*−*9*). Most recently, it has been shown that disruption of a disulfide bond involving Cys560 of the cysteine-rich domain of β3 activates the αIIbβ3 integrin (*6*) and that disruption of disulfide bonds in αIIbβ3 with generation of sulfhydryl groups occurs during activation of this receptor (*3*).

Sulfhydryl groups on the platelet surface are necessary for platelet aggregation (*2*, *10*). Current evidence from our work and that of Yan and Smith suggests that a thiol−disulfide exchange reaction at the level of αIIbβ3 is required for platelet responses (*2*, *3*). O'Neill et al. have also provided evidence for thiol−disulfide reactivity in αIIbβ3 (*11*). Additionally, protein disulfide isomerase (PDI),[2] a redox-sensitive enzyme that can catalyze thiol−disulfide exchange reactions, is on the platelet surface (*12*, *13*) where it mediates platelet aggregation and secretion (*1*) and activation of integrins (*14*−*16*).

Glutathione is a membrane-impermeant sulfur/sulfhydryl-containing molecule that can modulate reactions involving thiols, disulfides, and PDI. Glutathione is an important modulator of the cellular redox environment and is also found in blood. Cells contain 1−10 mM glutathione (*17*). The cytoplasmic ratio of reduced to oxidized glutathione (GSH/GSSG) of approximately 100/1 maintains the cysteine residues of intracellular proteins in the reduced form (*18*). In the endoplasmic reticulum the ratio of GSH/GSSG of 1/1 to 3/1 provides an oxidized redox state (*17*). In plasma the total glutathione concentration is only about 8−25 μM (*19*−*23*). Plasma also contains other low molecular weight thiols (cysteine, cysteinylglycine, and homocysteine), mostly in disulfide forms (*18*, *20*). Plasma glutathione differs markedly from other thiol components in that a large fraction is in the reduced form, with the GSH/GSSG ratio being in the range of 3.5/1 to 13/1 (*19*−*21*, *23*). One explanation for this is that glutathione, as opposed to the other thiols, is continuously secreted in the reduced form by the liver and rapidly utilized by the kidneys (*18*, *20*). Glutathione levels as well

† This work was supported by the Heritage Affiliate American Heart Association Grant-in-Aid for Research. D.W.E. is an American Heart Association Charles H. Leach Fellow.

* Correspondence should be addressed to this author at the University of Texas/Health Science Center at San Antonio, Mail Code 7880, 7703 Floyd Curl Drive, San Antonio, TX 78229. Fax: (210) 567-1956. E-mail: essex@uthscsa.edu.

‡ University of Texas/Health Science Center at San Antonio.

§ State University of New York/Downstate Medical Center.

[1] Integrin αIIbβ3 is also called glycoprotein IIb/IIIa.

[2] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, reduced glutathione; GSSG, oxidized glutathione; LSA, peptide LSARLAF; PDI, protein disulfide isomerase.

10.1021/bi0205045 CCC: $25.00
Published on Web 12/14/2002

as the ratio of GSH/GSSG are altered in disease states including fasting, alcoholism, cirrhosis, and malignancy (*22*−*25*). However, unlike cellular glutathione, a specific role for plasma glutathione in redox reactions has not been well studied.

In this study we tested the effect of GSH and mixtures of GSH with GSSG on platelet responses. At physiologic concentrations GSH potentiated platelet aggregation and secretion while at higher concentrations GSH inhibited these platelet responses. Physiologic levels of GSH increased free thiols in the β3 subunit of the αIIbβ3 integrin, suggesting a mechanism for facilitation of platelet activation. Additionally, activation of the platelets by itself also increased sulfhydryl labeling in the β3 subunit of the αIIbβ3 receptor.

MATERIALS AND METHODS

Materials. ATP, Chemo-Lumo reagents, ADP, collagen, and epinephrine were purchased from Chronolog Corp. (Havertown, PA). Thrombin was a gift of Dr. John Fenton II (Division of Laboratories and Research, New York State Department of Health, Albany, NY). Reduced glutathione (GSH), oxidized glutathione (GSSG), cysteine, cysteinylglycine, homocysteine, and phenylarsine oxide (PAO) were from Sigma Chemical Co. (St. Louis, MO). Diphenyleneiodonium (DPI) and apocynin were obtained from Fluka Chemical Corp. (Milwaukee, WI). 2,3-Dimercapto-1-propanesulfonic acid (DMPS) was from ICN Biochemicals (Irvine, CA). Proteins A and G were from Oncogenic Science (Uniondale, NY). A monoclonal antibody to αIIb was obtained from Immunotec (clone SZ22; Westbrook, ME) and to the β3 subunit from Zymed Labs Inc. (clone Y2/51; San Francisco, CA). MOPC21 (Sigma) was used as an isotype-specific monoclonal control antibody not against platelet proteins. A polyclonal goat antibody to the β3 subunit of αIIbβ3 and a control goat antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A polyclonal rabbit antibody to PDI was prepared as previously described (*2*). Normal rabbit IgG was from Sigma.

The peptide LSARLAF which activates platelets through the integrin αIIbβ3 was synthesized as described (*2*). Evidence that this peptide activates directly through αIIbβ3 is that antibodies to the putative binding site, the sequence 315−321 of αIIb, inhibit LSARLAF activation of platelets (control antibodies do not) and that LSARLAF does not induce release of platelet factor 4 from thrombasthenic platelets (missing αIIbβ3) as it does from normal platelets (*26*−*28*).

Platelet Aggregation. Platelets were prepared as described previously (*2*). Briefly, blood was drawn into 1/7 of acid−citrate dextrose solution, and the platelet-rich plasma (PRP), obtained after centrifugation of whole blood, was gel filtered on a Sepharose 2B column equilibrated in tyrode−albumin solution at pH 7.35. Calcium (1 mM) was then added to the platelets. Platelet aggregation and release studies were performed using a Chronolog lumiaggregometer as described elsewhere (*2*). GSH or GSSG was added to platelet samples at 37 °C as indicated. For some assays blood was collected in 3.8% sodium citrate (final concentration 0.38%) and platelet-rich plasma (PRP) made by centrifuging the blood at 300*g* for 20 min.

Sulfhydryl Labeling of Intact Platelets. Sulfhydryl labeling was performed using the poorly membrane-permeant maleimide reagent, 3-*N*-maleimidylpropionyl-biotin (MPB) (Molecular Probes), as previously described (*2*). Specificity of MPB labeling was previously shown by inhibition of labeling with preincubation of the reagent with GSH or preincubation of the platelets with a different type of sulfhydryl reagent (*2*). In addition to labeling sulfhydryls with MPB we used an agent that labels primary amines, sulfosuccinimido-biotin (SSB), to control for translocation of proteins to the platelet surface from internal stores. In brief, SSB (500 μM) was incubated with platelet samples, and unreacted SSB was quenched with glycine (2 mM) for 30 min at room temperature, as described elsewhere (*29*). The samples were washed three times using Tris-buffered saline (NaC1, 0.150 mM; Tris base, 20 mM; EDTA, 2 mM; pH 7.4), and protein quantitation was performed on each sample using the BCA protein assay (Pierce, Rockford, IL). Reducing SDS sample buffer was added to the samples, and the samples were electrophoresed using 2% SDS−10% PAGE. The samples were transferred to a PVDF membrane, and the biotinylated proteins were detected using streptavidin−horseradish peroxidase with a chemiluminescent substrate, as described (*2*). For quantitation purposes standard curves made from dilutions of samples were used to ensure the film was not oversaturated. The development times for the film varied from about 15 s to 2 min.

Measurement of Sulfhydryls in Platelet Samples. In these experiments gel-filtered platelets (4×10^8/mL in 700 μL of Tyrode's buffer, pH 7.4, containing calcium) were incubated with or without glutathione at 24 °C. For sulfhydryl quantitation the membrane-impermeant reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (0.1 mM) was added for 15 min at 24 °C. The platelets were pelleted (12 000 rpm for 4 min in a microcentrifuge), and 2-nitro-5-thiobenzoic acid in the supernatant was measured for absorbance at 412 nM using an extinction coefficient of 14150 M^{-1} cm^{-1} (*30*).

Immunoprecipitation. Immunoprecipitation of MPB-labeled samples was performed using the monoclonal or polyclonal antibodies to αIIbβ3 or a polyclonal rabbit antibody to PDI. After Triton X-100 was added to MPB-labeled samples, antibodies to αIIb or β3 or control antibodies (all at 10 μg/mL) were incubated with the samples for 1 h at 24 °C. A mixture of protein G plus protein A was used to precipitate the primary antibodies overnight at 4 °C using a rotator. The samples were washed five times in Tris-buffered saline. After the addition of reducing sample buffer the samples were run on a 10% polyacrylamide gel, the proteins were transferred to a PVDF membrane, and the membrane was analyzed using the appropriate secondary antibody, streptavidin−peroxidase, and a chemiluminescent substrate.

RESULTS

Effect of Glutathione on Platelet Aggregation. GSH in high concentrations (millimolar) has been shown to inhibit platelet aggregation in plasma (*31*). We found that GSH at 30−50 μM inhibited aggregation induced by low dose collagen or the secondary or irreversible phase of ADP-induced aggregation (not shown). Secretion was also inhibited, indicating that GSH interrupts an outside-in signal.

We next tested the effect of low GSH levels on platelet aggregation using levels of collagen that were not sufficient

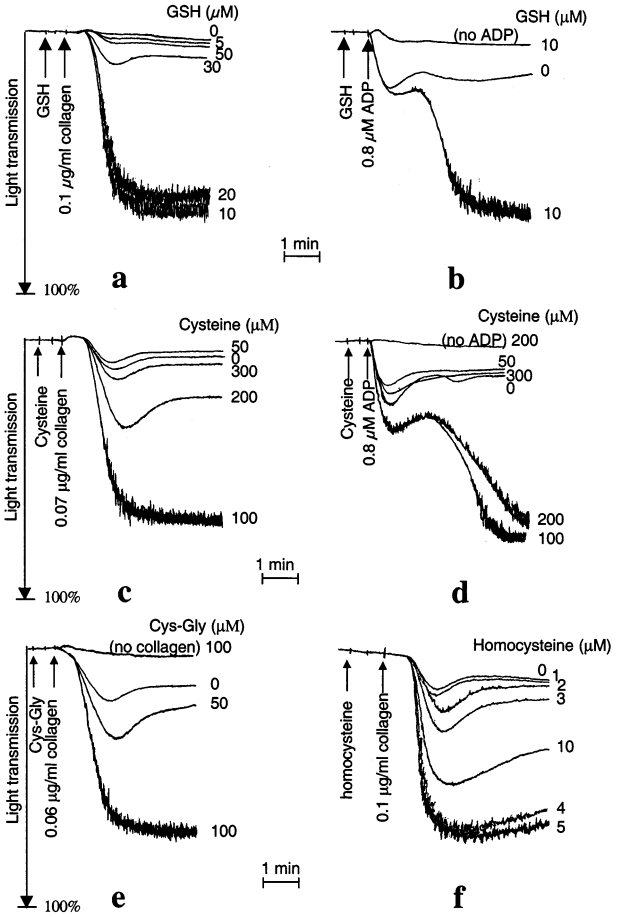

FIGURE 1: Stimulatory effect of GSH, cysteine, cysteinylglycine, and homocysteine on platelet aggregation. In these experiments GSH or one of the other low molecular weight thiols was added to platelets shortly before the addition of agonist. The agonist was used at a concentration insufficient to induce irreversible aggregation by itself. (a) shows the effect of various concentrations of GSH with collagen. (b) shows the effect of ADP (0.8 μM) or GSH (10 μM) alone or together. In (c) and (d), cysteine was incubated with platelets in the concentrations shown, and collagen (c) or ADP (d) was added. In (e) the effect of cysteinylglycine with collagen is seen. In (f) homocysteine was added to the platelets before the addition of collagen. Controls of the specific low molecular weight thiol in the absence of the agonist are shown in (b), (d), and (e).

alone to induce aggregation. At these subthreshold agonist concentrations, low concentrations of GSH (10−20 μM) stimulated aggregation; at higher concentrations (30−50 μM) this stimulatory effect was lost (Figure 1a) (as expected by the inhibitory effect of higher GSH concentration). GSH (10 μM) also potentiated irreversible aggregation by another agonist, ADP, at a concentration of ADP which by itself caused only primary aggregation (Figure 1b). Additionally, GSH potentiated irreversible aggregation induced by the low dose calcium ionophore (not shown). GSH by itself had little effect on platelets (Figure 1b) and even when tested at varying concentrations could not by itself stimulate aggregation (not shown). GSH was equally effective when incubated with the platelets for 10 min prior to the addition of collagen as when added simultaneously with collagen.

To determine if the activating effect of GSH was due to a general effect of sulfhydryls, other low molecular weight thiols, cysteine and cysteinylglycine, were tested. These thiols also potentiated irreversible aggregation. However, concentrations 10-fold higher than GSH were required (Figure 1c−e). With subthreshold collagen, 100 μM cysteine induced irreversible aggregation (Figure 1c); this effect was lost when the concentration was increased to 300 μM. Cysteinylglycine had a similar effect (Figure 1e). With subthreshold ADP, 100 μM cysteine induced secondary aggregation; this effect was also lost at 300 μM cysteine. Cysteine and cysteinylglycine, like GSH, by themselves had little effect on platelets (Figure 1d,e). A stimulatory effect of the sulfhydryl or reduced form of homocysteine was also found on aggregation at 1−3 μM concentrations and was maximal at 4−5 μM concentrations (Figure 1f).

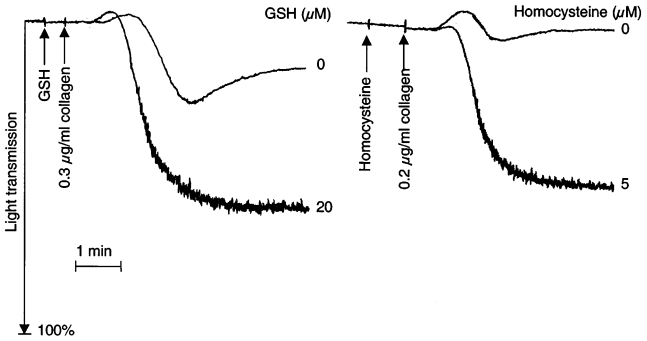

FIGURE 2: Stimulatory effects of GSH and homocysteine on platelet aggregation in plasma. The experiment was performed as in Figure 1 except the platelets were in citrated plasma instead of tyrode buffer.

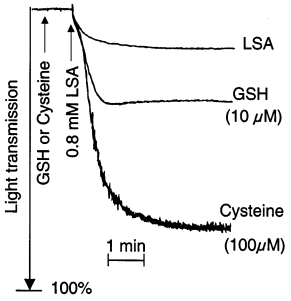

FIGURE 3: Effect of GSH and cysteine on platelets stimulated with the agonist peptide LSARLAF. The LSARLAF (LSA) concentration was reduced to a level (0.8 mM) that gave a minimal response. The potentiating effect of GSH (10 μM) or cysteine (100 μM) on LSA stimulation is evident.

The effects of GSH and homocysteine were reproduced in a plasma system by addition of similar concentrations of GSH or homocysteine (Figure 2). Similar effects were seen with cysteine (not shown). This indicates that these effects hold true in a physiologic system and that there is little modulation of the stimulatory effect by plasma components.

Since the effect of GSH is found with more than one platelet agonist and since GSH is membrane impermeant (i.e., is not effecting cytoplasmic events), 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. To further define the effect of GSH, we tested GSH with the agonist peptide LSARLAF that induces platelet aggregation by directly interacting with αIIbβ3 (*2*). Both GSH and cysteine had a stimulatory effect on activation by subthreshold levels of this peptide (Figure 3).

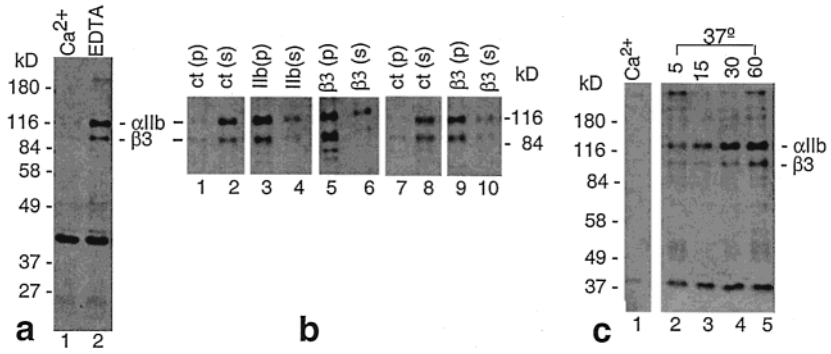

FIGURE 4: Effect of disruption of αIIbβ3 on sulfhydryl labeling of αIIbβ3. In (a), lane 1, the platelets were labeled with MPB in the presence of calcium (Ca^{2+}); in lane 2 MPB labeling was performed after incubation of the platelets with 5 mM EDTA for 60 min at 37 °C. (b) shows the results of immunoprecipitation of MPB-labeled samples by antibodies to αIIbβ3. The samples were run on a 10% polyacrylamide gel, the proteins were transferred to a PVDF membrane, and the membrane was analyzed using avidin−peroxidase and a chemiluminescent substrate. In (b) the 10 lanes contain the pellet (p) and supernatant (s) from samples incubated with one of five different antibodies. In lanes 1 and 2, a control mouse antibody MOPC21 (ct) was used; in lanes 3 and 4 a mouse antibody to αIIb (SZ22) and in lanes 5 and 6 a mouse antibody to β3 (SZ21) were used. Lanes 7 and 8 contain the sample using a control of normal goat IgG (ct); for the sample in lanes 9 and 10 a goat anti-β3 antibody was used. In (c) the platelets were incubated with EDTA for various times, up to 60 min, and then labeled with MPB. Lane 1 shows labeling in the presence of calcium (Ca^{2+}); lanes 2−5 show labeling with EDTA (5 mM) at 37 °C for 5, 15, 30, and 60 min.

These findings suggest that the effect of GSH is on the αIIbβ3 receptor. Previous studies labeling sulfhydryls of this receptor on intact platelets showed relatively little labeling of αIIbβ3 in the presence of calcium (*2*, *32*). Therefore, before studying the effect of GSH on sulfhydryl formation in αIIbβ3, we first determined optimal conditions for labeling sulfhydryls in this receptor. When we labeled the receptor on intact platelets under conditions that disrupt the receptor (5 mM EDTA, 60 min, 37 °C) (*33*), three major bands were found (Figure 4a, lane 2). A major increase in labeling was seen in bands the size of αIIb and β3 [longer exposure of the film gives the usual 9−11 bands (*2*)]. (We get variable labeling of the third 40 kDa band, which may be actin.) Using antibodies to either subunit of αIIbβ3 and immunoprecipitation, the larger bands were identified as αIIb and β3 (Figure 4b). Lanes 1 and 7 show that mouse and goat control antibodies failed to precipitate αIIb or β3. Mouse antibodies to αIIb or to β3, or a polyclonal antibody to β3, all precipitated the bands (lanes 3, 5, and 9, respectively). When the platelets were incubated with EDTA for various time intervals at 37 °C and then labeled, the sulfhydryl exposure of both the αIIb and β3 subunits increased progressively and in parallel for up to 60 min (Figure 4c). There was little or no increase in labeling under conditions in which calcium is chelated but the receptor is not disassociated (5 mM EDTA, at 24 °C), and no increase was seen after 60 min (data not shown). Similar results were found using EGTA instead of EDTA.

Effect of GSH on Sulfhydryl Labeling in αIIbβ3. Having determined optimal labeling conditions, we next examined the effect of GSH on sulfhydryl labeling in αIIbβ3 (Figure 5a). The results are expressed quantitatively for the β3 subunit as a ratio of labeling of β3 with GSH to labeling without GSH (Figure 5b). A concentration of GSH (10 μM) that potentiated platelet aggregation increased sulfhydryl labeling in the β3 subunit by over 2-fold (Figure 5b). Using an agent which labels primary amines, sulfosuccinimidobiotin (SSB) (*29*), no increase in labeling of β3 was seen in platelets treated with GSH (Figure 5b). The differences between MPB and SSB labeling for GSH is significant (P value < 0.05). Therefore, the increase in sulfhydryls in β3

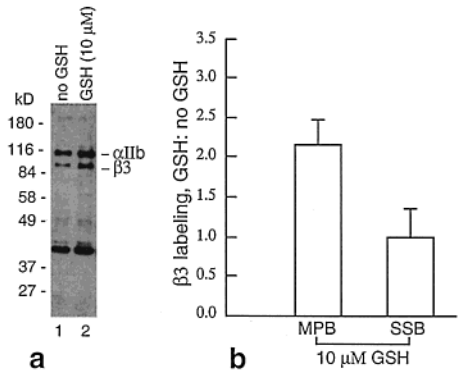

FIGURE 5: Effect of GSH on sulfhydryl labeling of αIIbβ3. In (a) platelets were incubated without GSH (lane 1) or with GSH (10 μM) (lane 2) for 5 min at 24 °C under nonstirring conditions. After incubation of the platelets under conditions which cause disruption of the αIIbβ3 receptor (5 mM EDTA, 60 min, 37 °C) the sulfhydryl reagent MPB (50 μM) was added in excess of the GSH, and labeling was performed. Equal amounts of protein were added to each lane. In (b) densitometry of the individual bands was used to obtain quantitative results. The first column gives the ratio of labeling of the β3 subunit with GSH to the β3 subunit without GSH (±1 SE, $n = 8$). The second column gives the ratio of labeling using SSB in the β3 subunit with GSH to the β3 subunit without GSH (±1 SE).

cannot be accounted for by translocation of cytoplasmic stores of αIIbβ3. A 1.6 ± 0.2 (1 SE) fold increase in labeling of αIIb was also found in the presence of GSH (this was not statistically significant compared to the SSB label).

We next studied the effect of adding GSSG to GSH on platelet aggregation. After finding a ratio of GSH/GSSG that worked well, we varied the concentration of the mixture. Figure 6a shows the effect of GSH alone in concentrations of 10−160 μM; Figure 6b shows the same concentrations of GSH combined with a 5-fold lower concentration of GSSG. The addition of GSSG to a specific concentration of GSH always enhanced aggregation relative to GSH alone.

To determine if there was any oxidation of GSH to GSSG in our assays (which would change the redox potential), we incubated a mixture of 10 μM GSH and 2 μM GSSG with platelets for up to 10 min and used DTNB to measure the total sulfhydryls in the sample. No significant changes in

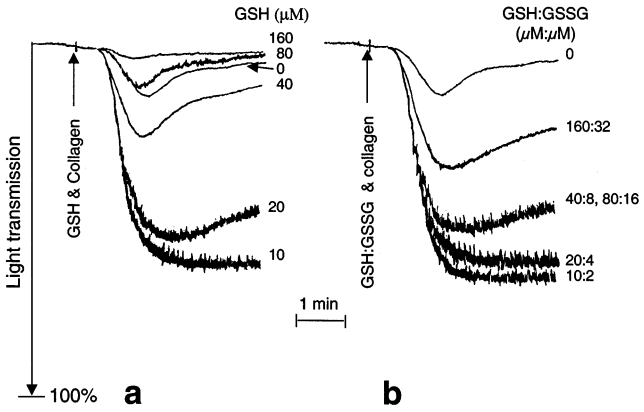

FIGURE 6: Addition of low concentrations of GSSG potentiates platelet activation by GSH. (a) shows the effect of GSH added simultaneously with a concentration of collagen (0.6 μg/mL) that by itself gave a minimal response. The GSH concentration was varied from 10 to 160 μM. (b) shows the effect of adding GSSG to the GSH so that the ratio of GSH/GSSG was kept constant at 5/1. The experiment was performed three times with similar results.

SH were found over time (data not shown). Similar results were found when GSH (10μM) alone was incubated with platelets, suggesting that minimal oxidation of GSH to GSSG occurs under our experimental conditions. These results are similar to those of studies using purified PDI and RNase where there was negligible (less than 5−10%) oxidation of GSH to GSSG exposed to air during 30−60 min assays (*34*).

Effect of Platelet Activation on Labeling of Thiols in αIIbβ3. We next determined the effect of platelet activation itself (in the absence of added GSH) on sulfhydryl exposure in αIIbβ3. Platelets were activated with collagen in the absence of fibrinogen or stirring. Absence of aggregation was confirmed by microscopic examination. A substantial increase of labeling in several proteins including the β3 subunit was seen with platelet activation (Figure 7a, lane 2) compared with nonactivated platelets (lane 1). When the platelets were labeled with aggregates, an increase in labeling of both subunits of αIIbβ3 was also seen (lane 3) [for these experiments, EDTA (5 mM) was added at the very beginning of aggregation]. Results using antibodies to precipitate the αIIb and β3 subunits from the nonactivated and aggregated samples are shown in Figure 7b.

When the results were expressed quantitatively as a ratio of labeling in nonactivated to activated platelets, a much larger increase in sulfhydryl labeling with activation (about 4−5-fold) was observed in the β3 subunit compared to the αIIb subunit (1.6-fold) (Figure 7c). Similar results were found when labeling was performed during aggregation with a 4.39 (±0.31 SE) increase in β3 versus a 2.29 (±0.33 SE) increase in αIIb ($P < 0.05$, $n = 3$). The disproportional increase in labeling of the β3 subunits compared to αIIb suggests that the increase in β3 is not due to translocation of αIIbβ3 from internal stores. Furthermore, labeling of amines with SSB, to control for translocation of additional αIIbβ3 receptor from internal stores, showed a 1.46 ± 0.20 (1 SE) fold increase with activation, consistent with the known increase in αIIbβ3 on the platelet surface with activation [of about 1.3−1.5-fold (*35*, *36*)]. The difference between the increase in MPB labeling of β3 in activated platelets versus the increase in SSB labeling resulting from translocation of αIIbβ3 is significant ($P < 0.05$, $n = 8$). Therefore, the increase in

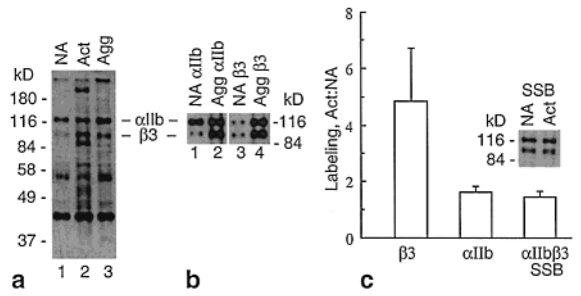

FIGURE 7: Sulfhydryl labeling with MPB of αIIbβ3 in activated platelets. In these studies platelets were activated, incubated under conditions that disrupt the αIIbβ3 subunits (as in Figure 4), and labeled with MPB (50 μM). The samples were washed three times, and equal amounts of protein were added to each lane. After electrophoresis and transfer, the PVDF membrane was developed as described in Materials and Methods. In (a) the labeling pattern of nonactivated platelets (NA) is seen in lane 1. In lane 2, the platelets were activated with collagen (Act) (10 μg/mL for 10 min) in the absence of fibrinogen or stirring. For the sample in lane 3, EDTA was added to platelets just at the start of aggregation (Agg), and then labeling was performed. (b) shows samples immunoprecipitated from nonactivated (NA) or aggregated (Agg) platelets. In lanes 1 and 2 the samples were immunoprecipitated with a monoclonal antibody to αIIb (SZ21) and in lanes 3 and 4 with the polyclonal antibody to β3. In (c) densitometry of the individual bands, performed on samples containing platelet lysate [as in (a)], was used to determine relative amounts of sulfhydryl labeling in αIIb and β3 ($n = 8$). The results are expressed as a ratio of labeling in activated to nonactivated platelets for each subunit (labeling, Act:NA) ±1 SE. The inset to (c) compares labeling with SSB (500 μM), used as a marker for protein translocation, of nonactivated to activated platelets. The third column in (c) shows the ratio of labeling with SSB of both the αIIb and β3 subunits of activated to nonactivated platelets (±1 SE). For the samples analyzed in (c) activation was induced by collagen in the absence of fibrinogen or stirring. Absence of aggregation was confirmed by microscopic examination. In several samples an antibody that blocks binding of any secreted fibrinogen, 10E5 (a gift from Dr. B. Coller), was added. The data for the two methods were apparently identical, so they were pooled.

sulfhydryl labeling in β3 with activation cannot be totally explained by an increase in receptor number. It must be due to exposure or generation of new sulfhydryls during activation.

A Role for Vicinal Dithiols. The active sites of PDI contain vicinal dithiols, and the increase in sulfhydryls in the β3 subunit of αIIbβ3 induced either by GSH or by platelet activation raises the possibility that vicinal dithiols are being formed in the cysteine-rich domain. To probe for a role for vicinal dithiols in platelet aggregation, we tested the effect of phenylarsine oxide (PAO), a reagent which binds to vicinal dithiols. PAO inhibited platelet aggregation (Figure 8). The dithiol 2,3-dimercaptopropanol (DMP) or its membrane-impermeable sulfonic acid analogue 2,3-dimercaptopropanesulfonic acid (DMPS) removes PAO from its target (*37*, *38*). Reversibility of the inhibitory effect of PAO by DMPS (Figure 8b) confirms that PAO is not nonspecifically affecting platelets and that its effect is on vicinal dithiols on the platelet surface. In preliminary studies we have found substantial inhibition of MPB labeling of several bands in activated platelets by PAO, including αIIb, β3, and PDI.

DISCUSSION

In this study we found that GSH at concentrations normally found in blood potentiates platelet aggregation. The stimu-

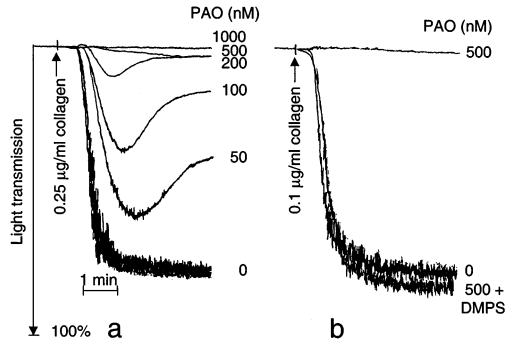

FIGURE 8: Effect of PAO on platelet aggregation. In (a) the platelets were preincubated with PAO at the concentrations indicated for 30 s before the addition of collagen. In (b) the effect of the vicinal dithiol DMPS (10 μM for 10 min) on PAO inhibition is seen.

latory effect of other thiol donors shows that the effect of GSH is due to its sulfhydryl moiety. However, the effect of other low molecular weight thiols was at concentrations over 10-fold more than that of the thiol forms of these molecules in blood (*20*). This implies that the glutathione redox system is the important physiologic regulator in blood. Additionally, the effect of GSH was found with more than one platelet agonist. This suggests that it stimulates the later phases of the platelet activation pathway, since it is unlikely that GSH would similarly affect different primary agonist receptors.

The stimulatory effect of homocysteine on platelet aggregation is of special interest in that homocysteine is a known risk factor for vascular disease. The normal concentrations of total homocysteine in blood are about 10 μM; however, only a small fraction of homocysteine, about 0.2−0.3 μM, is in the sulfhydryl or reduced form (*20*, *39*). The concentrations of the sulfhydryl or reduced form of homocysteine that potentiate platelet aggregation in our studies are higher than those normally found in blood. However, it is possible that in conditions with hyperhomocysteinemia an effect of homocysteine is on platelets. For example, levels of the reduced form of homocysteine that may potentiate aggregation have been reported in end stage renal disease, a disease with unusually high rates of cardiovascular morbidity and death (*40*). In support of this concept, continuous infusion of reduced homocysteine into baboons has been found to increase platelet consumption and cause platelet-mediated intimal proliferation (*41*).

To determine the effect of GSH on αIIbβ3, we first determined optimal sulfhydryl labeling conditions for αIIbβ3. Conditions that disrupt or dissociate the subunits of the αIIbβ3 receptor result in sulfhydryl exposure in each subunit. Since EDTA does not by itself generate new sulfhydryls, the increased labeling in these studies implies cryptic sulfhydryls that are sterically hindered from reacting with MPB. The approximately parallel increase in labeling of the two subunits over time (Figure 4) may suggest a spatial proximity in the subunits of the sulfhydryls being labeled.

We found that GSH (10 μM) increased labeling of thiols in the β3 subunit of the αIIbβ3 receptor. While EDTA-dependent disruption of αIIbβ3 likely exposes existing thiols, the effect of GSH, which can reduce disulfide bonds, is likely by generating new thiols. The cysteine-rich repeat region of this subunit contains sulfhydryls (*3*), and it is possible that low amounts of GSH facilitate a shift in certain disulfide bonds in this region to sulfhydryls. Since GSH by itself does not cause platelet aggregation, this sulfhydryl generation does not by itself activate the receptor to a fibrinogen binding state but rather primes the receptor for activation by a lower threshold of agonist. It is possible that generation of thiols by GSH could by increasing available thiols potentiate a thiol−disulfide exchange reaction.

The effect of GSH on platelet aggregation that we have found contrasts with the effect of the reducing agent dithiothreitol (DTT) on platelet aggregation and activation of the αIIbβ3 receptor (*4*, *42*, *43*). DTT in the presence of fibrinogen causes a slow progressive platelet aggregation without secretion. 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; see Figure 1). Additionally, the effect of DTT is only found with concentrations over 1 mM, much higher than the concentrations of GSH used in our studies [despite DTT being a better reducing agent (*44*)]. Therefore, mechanistically the understanding of the role of thiol groups in our studies is an advancement on the earlier studies with DTT.

The effect of GSH in our studies also contrasts with the effect of GSH on αIIbβ3 in the studies of Yan and Smith (*3*). These authors found that a combination of a high concentration of GSH (3 mM) together with nitric oxide decreased the free cysteine residues in the activated form of αIIbβ3 (possibly by S-nitrosylation), deactivating the receptor.

A recent report on PDI-like activity in αIIbβ3 also bears on our work. Using a reduced RNase assay, O'Neill et al. demonstrated intrinsic thiol−isomerase activity in platelet αIIbβ3 in the presence of EDTA (*11*). In 100 μM Ca^{2+} this activity is lost, suggesting that dissociation of the αIIb and β3 chains is required for isomerase activity. The isomerase activity implies a catalytic amount of free sulfhydryls in αIIbβ3, consistent with the free thiols found in labeling studies of αIIbβ3 (*2*, *3*; Figure 4). This thiol−isomerase activity raises the possibility that αIIbβ3 may autocatalyze thiol−disulfide exchange within itself. However, since this activity is a reflection of the thiol−disulfide reactivity of αIIbβ3, it is equally plausible that this isomerase activity in αIIbβ3 supports a role for αIIbβ3 as a substrate for a PDI-catalyzed event.

We found that the addition of a small amount of GSSG to GSH enhanced aggregation relative to GSH alone. This indicates that the platelet requirement is not simply for reducing equivalents from GSH but rather for a redox potential (ratio of GSH to GSSG). The ratio of GSH to GSSG used of 5/1 is within the range normally found in blood (3.5/1 to 13/1) (*19*−*21*, *23*). A ratio of GSH to GSSG of 10/1 gave similar results, but when we used a mixture of 10 μM GSH with 5 μM GSSG, the stimulatory effect was lost (data not shown). This suggests that modulation of platelet aggregation by redox potential is over a relatively tight physiologic range of GSH/GSSG ratios.

In addition to αIIbβ3, PDI is a second platelet surface protein known to be redox sensitive. We did not show in the present study an effect of GSH on platelet PDI; however, the composition of the glutathione redox buffer is known to effect catalysis of the folding of the PDI substrate by PDI. For example, it was shown that the addition of a narrow range of low GSSG concentrations to GSH sharply enhanced

the rate of PDI-catalyzed folding of reduced ribonuclease A (*34*). The redox buffer could have two effects on catalysis by PDI. First, changes in the glutathione redox buffer composition could affect the redox state of PDI itself, so that PDI activity is expressed only under the appropriate redox conditions. In this regard, the concentration of GSH that promotes platelet aggregation (10 μM) begins to generate sulfhydryls in the active sites of PDI, converting PDI to its active form (*45*). It may be that a slight increase in active PDI contributes to the lowering of the threshold for agonist-induced platelet aggregation. Second, changes in the composition of the glutathione redox buffer are known to alter the distribution of thiol−disulfide redox isomers of the PDI substrate (*46*). Therefore, the redox buffer will affect not only PDI on the platelet surface but also the potential substrate(s) of platelet PDI. As discussed above, αIIbβ3 is a redox-sensitive protein, and there is evidence that PDI can modulate the affinity state of αIIbβ3 (*1*) or other integrins (*15*, *16*).

In addition to the effects of the redox buffer on platelet aggregation we also demonstrated that sulfhydryls increase in β3 with platelet activation (Figure 7). Although our studies do not exclude exposure of existing thiols as a mechanism, it is likely that a component of this increase is due to generation of new thiols from reduction of disulfide bonds. This is because sulfhydryls are known to be generated in αIIbβ3 as well as other platelet surface proteins during platelet aggregation. Yan and Smith labeled purified αIIbβ3 with sulfhydryl agents under denaturing condition and found an increase in labeled cysteines in the active form of the receptor (*3*). Also, Burgess et al. showed an increase in sulfhydryls in at least 11 platelet surface proteins with aggregation (*29*), suggesting that a platelet surface mechanism exists for reducing disulfide bonds. Therefore, it is likely that a component of the increase we found in β3 is by cleavage of disulfide bonds. Our studies localize the increase in sulfhydryls to the β3 subunit of the receptor (after adjustment for translocation of αIIbβ3 from internal stores no increase in αIIb was found). Taken together, these findings suggest that disruption of disulfide bonds with concomitant sulfhydryl generation in β3 is part of the activation events in the αIIbβ3 receptor.

The increase in thiols in β3 occurred in activated platelets even in the absence of aggregation. This is significant because aggregation can itself be a stimulus for a variety of platelet responses [for example, aggregation can stimulate certain phosphorylation events (*47*) and cytoskeletal changes (*48*)]. The increase in sulfhydryl labeling in β3 in platelets that had been activated but that had not undergone aggregation (Figure 7) shows that the sulfhydryl increase can be induced by the activation process itself.

Vicinal dithiols are involved in certain activation events of rabbit platelets (*49*); however, whether these thiols are cytoplasmic or on the platelet surface is not known. Our results show that vicinal dithiols of platelet surface proteins are involved in the sulfhydryl-dependent pathways of platelet activation. These potential vicinal dithiols include those of the active site of PDI or those in αIIbβ3.

In summary, the present report together with earlier reports indicates a role for reactions involving thiols and disulfides on the platelet surface in the activation of αIIbβ3. The redox potential of the platelet environment is a controlling factor for platelet responses with a physiologic redox potential facilitating platelet aggregation, apparently by an effect on αIIbβ3. Furthermore, sulfhydryl changes in the β3 subunit of αIIbβ3 appear to be involved in activation of this receptor. More work is needed to further define both the sulfhydryls and the reactions involved in the sulfhydryl-dependent pathways of platelet activation.

REFERENCES

1. Essex, D. W., and Li, M. (1999) *Br. J. Haematol. 104*, 448−454.
2. Essex, D. W., Li, M., Miller, A., and Feinman, R. D. (2001) *Biochemistry 40*, 6070−6075.
3. Yan, B., and Smith, J. W. (2000) *J. Biol. Chem. 275*, 39964−39972.
4. Zucker, M. B., and Masiello, N. C. (1984) *Thromb. Haemostasis 51*, 119−124.
5. Kouns, W. C., Jutzi, J., Jennings, L. K., and Steiner, B. (1993) *Thromb. Haemostasis 69*, 785a (abstract).
6. Ruiz, C., Liu, C. Y., Sun, Q. H., Sigaud-Fiks, M., Fressinaud, E., Muller, J. Y., Nurden, P., Nurden, A. T., Newman, P. J., and Valentin, N. (2001) *Blood 98*, 2432−2441.
7. Liu, C. Y., Sun, Q. H., Wang, R., Paddock, C. M., and Newman, P. J. (1998) *Blood 92*, 344a (abstract).
8. Kashiwagi, A., Shinozaki, K., Nishio, Y., Maegawa, H., Maeno, Y., Kanazawa, A., Kojima, H., Haneda, M., Hidaka, H., Yasuda, H., and Kikkawa, R. (1999) *Am. J. Physiol. 277*, E976−E983.
9. Yan, B., and Smith, J. W. (2001) *Biochemistry 40*, 8861−8867.
10. Aledort, L. M., Troup, S. B., and Weed, R. I. (1968) *Blood 31*, 471−479.
11. O'Neill, S., Robinson, A., Deering, A., Ryan, M., Fitzgerald, D. F., and Moran, N. (2000) *J. Biol. Chem. 24*, 36984−36990.
12. Chen, K., Detwiler, T. C., and Essex, D. W. (1995) *Br. J. Haematol. 90*, 425−431.
13. Essex, D. W., Chen, K., and Swiatkowska, M. (1995) *Blood 86*, 2168−2173.
14. Essex, D. W., Miller, A., Swiatkowska, M., and Feinman, R. D. (1999) *Biochemistry 38*, 10398−10405.
15. Lahav, J., Gofer-Dadosh, N., Luboshitz, J., Hess, O., and Shaklai, M. (2000) *FEBS Lett. 475*, 89−92.
16. Lahav, J., Wijnen, E., Hess, O., Essex, D. W., Barnes, M., and Farndale, R. (2001) *J. Thromb. Hemostasis*, 896a (abstract).
17. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science 257*, 1496−1502.
18. Gilbert, H. F. (1990) *Adv. Enzymol. Relat. Areas Mol. Biol. 63*, 69−172.
19. Anderson, M. E., and Meister, A. (1980) *J. Biol. Chem. 255*, 9530−9533.
20. Mansoor, M. A., Svardal, A. M., and Ueland, P. M. (1992) *Anal. Biochem. 200*, 218−229.
21. Lash, L. H., and Jones, D. P. (1985) *Arch. Biochem. Biophys. 240*, 583−592.
22. Martensson, J. (1986) *Metabolism 35*, 118−121.
23. Lauterburg, B. H., and Velez, M. E. (1988) *Gut 29*, 1153−1157.
24. Chawla, R. K., Lewis, F. W., Kutner, M. H., Bate, D. M., Roy, R. G., and Rudman, D. (1984) *Gastroenterology 87*, 770−776.
25. Beutler, E., and Gelbart, T. (1985) *J. Lab. Clin. Med. 105*, 581−584.
26. Derrick, J. M., Taylor, D. B., Loudon, R. G., and Gartner, T. K. (1997) *Biochem. J. 325*, 309−313.
27. Derrick, J. M., Loudon, R. G., and Gartner, T. K. (1998) *Thromb. Res. 89*, 31−40.
28. Basani, R. B., French, D. L., Vilaire, G., Brown, D. L., Chen, F., Coller, B. S., Derrick, J. M., Gartner, T. K., Bennett, J. S., and Poncz, M. (2000) *Blood 95*, 180−188.
29. Burgess, J. K., Hotchkiss, K. A., Suter, C., Dudman, N. P., Szollosi, J., Chesterman, C. N., Chong, B. H., and Hogg, P. J. (2000) *J. Biol. Chem. 275*, 9758−9766.
30. Balazs, M., Matko, J., Szollosi, J., Matyus, L., Fulwyler, M. J., and Damjanovich, S. (1986) *Biochem. Biophys. Res. Commun. 140*, 999−1006.
31. Thomas, G., Skrinska, V. A., and Lucas, F. V. (1986) *Thromb. Res. 44*, 859−866.
32. Xie, L., Chesterman, C. N., and Hogg, P. J. (2000) *Thromb. Haemostasis 84*, 506−513.

33. Peerschke, E. I. B., and Lopez, J. A. (1998) in *Thrombosis and Hemorrhage* (Loscalzo, J., and Schafer, A. I., Eds.) pp 229−260, Williams & Wilkins, Baltimore, MD.
34. Lyles, M. M., and Gilbert, H. F. (1991) *Biochemistry 30*, 613−619.
35. Berndt, M. C., and Shattil, S. F. (2001) in *Hemostasis and Thrombosis: Basic Princeples and Clinical Practice* (Colman, R. W., Hirsh, J., Marder, V. J., and George, J. N., Eds.) pp 479−491, Lippincott Williams & Wilkins, Philadelphia, PA.
36. Woods, V. L., Jr., Wolff, L. E., and Keller, D. M. (1986) *J. Biol. Chem. 261*, 15242−15251.
37. Bennett, T. A., Edwards, B. S., Sklar, L. A., and Rogelj, S. (2000) *J. Immunol. 164*, 4120−4129.
38. Le Cabec, V., and Maridonneau-Parini, I. (1995) *J. Biol. Chem. 270*, 2067−2073.
39. Araki, A., and Sako, Y. (1987) *J. Chromatogr. 422*, 43−52.
40. Hoffer, L. J., Robitaille, L., Elian, K. M., Bank, I., Hongsprabhas, P., and Mamer, O. A. (2001) *Kidney Int. 59*, 372−377.
41. Harker, L. A., Ross, R., Slichter, S. J., and Scott, C. R. (1976) *J. Clin. Invest. 58*, 731−741.
42. MacIntyre, D. E., and Gordon, J. L. (1974) *Biochem. Soc. Trans. 2*, 1265−1269.
43. MacIntyre, D. E., Grainge, C. A., Drummond, A. H., and Gordon, J. L. (1977) *Biochem. Pharmacol. 26*, 319−323.
44. Gilbert, H. F. (1995) in *Methods in Enzymology*, pp 8−30, Academic Press, New York.
45. Gilbert, H. F. (1989) *Biochemistry 28*, 7298−7305.
46. Konishi, Y., Ooi, T., and Scheraga, H. A. (1982) *Biochemistry 21*, 4734−4740.
47. Clark, E. A., Shattil, S. J., Ginsberg, M. H., Bolen, J., and Brugge, J. S. (1994) *J. Biol. Chem. 269*, 28859−28864.
48. Fox, J. E. (2001) *Thromb. Haemostasis 86*, 198−213.
49. Sugatani, J., Steinhelper, M. E., Saito, K., Olson, M. S., and Hanahan, D. J. (1987) *J. Biol. Chem. 262*, 16995−17001.

BI0205045