PLATELET SURFACE GLUTATHIONE REDUCTASE-LIKE ACTIVITY

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Abbreviations: DPI: diphenyleneiodonium; DTNB: 5,5'-dithiobis(2-nitrobenzoic acid); MPB: 3-N-maleimidylpropionyl biotin; SSB: sulfosuccinimido-biotin

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ABSTRACT

We previously found that reduced glutathione (GSH) or a mixture of GSH/GSSG potentiated platelet aggregation. We here report that glutathione disulfide, GSSG, when added to platelets alone, also potentiates platelet aggregation. Most of the GSSG was converted to GSH by a flavoprotein dependent platelet surface mechanism. This provided an appropriate redox potential for platelet activation. The addition of GSSG to platelets generated sulfhydryls in the beta subunit of the αIIbβ3 fibrinogen receptor, suggesting a mechanism for facilitation of agonist-induced platelet activation.

INTRODUCTION

Glutathione, an important modulator of the cellular redox environment, is found in blood where it could modulate the redox environment of platelets. Cells contain millimolar concentrations of glutathione, mostly in the reduced form. Plasma contains only 8-25 μM glutathione,\(^1\) also predominantly in the reduced form. This contrasts with other low molecular weight thiols that are mostly in disulfide forms.\(^2,6\) Plasma glutathione levels and GSH/GSSG ratios in plasma are altered in disease states.\(^4,5,7\) However, little is known about the role of plasma glutathione in the regulation of redox reactions. We recently found that physiologic concentrations of GSH generate thiols from disulfide bonds in αIIbβ3 and potentiate platelet aggregation.\(^7\)

Recent reports have also raised the possibility that platelets have a transplasma membrane oxidoreductase that like GSH can reduce extracellular disulfide bonds in redox sensitive membrane proteins.\(^7,9\) While there is little information about such systems in cells, a form of a
cell membrane NADH oxidase \(^{10,11}\) in plant cells has been reported to have disulfide reductase activity.\(^{12}\) Cells and platelets contain flavoprotein-electron transport systems such as NADPH-oxidase on neutrophils, however, the electron acceptor is molecular oxygen (not disulfide bonds) and the product is superoxide anion (\(O_2^-\)).\(^{13-15}\) There are no reports of transplasma membrane electron transport systems that reduce disulfide bonds in mammalian cells. We here report that non-stimulated platelets have the ability to reduce the disulfide bond in extracellular GSSG. This results in the production of GSH, the generation of thiols in the \(\alpha\)IIb\(\beta3\) integrin and facilitates platelet aggregation.

**STUDY DESIGN**

Platelet aggregation, measurement of extracellular sulfhydryls in platelet samples and sulfhydryl labeling of extracellular of platelets were as described elsewhere.\(^7\)

**RESULTS AND DISCUSSION**

We previously found that GSH or a mixture of GSH/GSSG potentiated platelet aggregation stimulated by agonists such as collagen.\(^7\) When GSSG (5 \(\mu\)M) was used alone as a control for the collagen-induced aggregation studies, surprisingly, it also enhanced platelet aggregation (Fig. 1). As expected, GSSG alone does not contain any GSH by the DTNB assay (not shown). When the concentration of agonist gave partial or sub-maximal aggregation, a low concentration of GSSG (2 \(\mu\)M) further stimulated aggregation (Fig. 1b). Thus, under appropriate conditions low concentrations of glutathione, similar to that found in blood, substantially potentiate aggregation.
We hypothesized that the GSSG was being converted to GSH by the platelets. To test this we incubated GSSG with gel-filtered platelets and measured total thiols in the sample. The combination of GSSG with platelets gave substantially more free thiols as detected by DTNB than could be accounted for by either platelets or GSSG alone (Fig. 1c). The amount of sulfhydryls on platelets alone corresponds to about $9.0 \times 10^{-18}$ moles of thiol per platelet (similar to what has been reported).\cite{16,17} Platelets with GSH (10 $\mu$M) gave the additive results of platelets alone plus GSH alone (column 3). Platelets with GSSG (5 $\mu$M) gave about double what was expected from platelets alone (about 6.5 $\mu$M additional SH, $P<0.05$, $n=5$). This suggests that 3-4 $\mu$M of the GSSG added is being converted to GSH by platelets. Therefore, the addition of GSSG to platelets would result in a ratio of GSH/GSSG of approximately 3/1 to 4/1. This ratio is similar to that found in blood and to the GSH/GSSG ratio that provides maximal potentiation of platelet activation.\cite{7} In contrast to the effect of platelets on GSSG, when cystine (the disulfide form of cysteine) was incubated with platelets no increase in sulfhydryls was found (Fig. 1d). This indicates some specificity of the reaction.

The conversion of GSSG to GSH requires the addition of two electrons to reduce the disulfide bond. In cells NADPH serves as the electron donor for GSSG in the following reaction: 

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+.$$ 

Therefore, the generation of GSH by platelets in the absence of external reducing agents implicates a transmembrane electron transport system that utilizes a cytoplasmic electron donor, presumably NAD(P)H. NADPH oxidase\cite{13,14} and NADH oxidase\cite{10,12,18,19} are plasma membrane oxidoreductase systems found in cells. To begin to explore these possibilities we tested the effect of diphenyleneiodonium (DPI), an inhibitor of flavin-containing enzymes, as well as a structurally unrelated inhibitor of NADPH oxidase,
apocynin. Both DPI (10 µM) and apocynin inhibited the GSSG reductase activity (Fig. 1e). Since the reaction is inhibited by relatively low concentrations of DPI, the mechanism likely involves a flavoprotein.

Time course studies on the conversion of GSSG to GSH were performed at 37°C, the conditions under which GSSG enhanced platelet aggregation. The platelet reaction with GSSG was complete by 1 minute (Fig. 2a). This suggests that the effect of GSSG on platelet aggregation is after some GSSG is converted to GSH, and thus after an appropriate redox potential for platelet activation is reached. To test for this possibility we compared the effect of GSSG added 1 minute before collagen to GSSG added simultaneously with collagen. Figure 2b shows that GSSG (5 µM) added simultaneously with collagen did not potentiate aggregation (the 0 sec tracing). In contrast to the lack of effect of GSSG added with collagen, GSSG incubated for 1 minute with platelets as expected facilitated aggregation (the 60 sec tracing). As expected from previous studies, controls of GSH (10 µM) or a mixture of GSH (10 µM) and GSSG (2 µM) potentiated aggregation when added simultaneously with collagen (not shown). These results imply that platelets convert GSSG to GSH and this can be part of the stimulus for platelet activation.

Labeling of platelet surface thiols. The effect of GSH (10 µM) and GSSG (5 µM) on sulphydryl generation in αIIbβ3 is shown in Figure 2c. Using immunoprecipitation the bands shown were previously identified as αIIb and β3. GSH, previously found to generate sulphydryls in the β3-subunit, increased sulphydryl labeling in β3, by 2-2.5 fold. A concentration of GSSG (5 µM) that potentiated platelet aggregation increased sulphydryl labeling in the β3-
subunit by almost 3-fold (Fig. 2d). This is similar to the effect of GSH (10 µM) and confirms a mechanism in αIIbβ3. A 1.6-1.7 fold increase in labeling of αIIb was also found with both GSH and GSSG (not shown). Since GSH by itself does not cause platelet aggregation the sulfhydryl generation in β3 apparently does not by itself activate the receptor. An additional stimulus is necessary.

In summary, the conversion of GSSG to GSH by platelets implicates a novel surface flavoprotein glutathione reductase activity that may be a marker for a more general disulfide reductase activity. This activity appears to provide the appropriate redox potential for platelet activation. This reaction may modulate the local redox environment at sites of vascular injury where platelets are found in high concentrations. Redox sensitive protein on platelets whose function may be modulated by the redox environment include αIIbβ3 and protein disulfide isomerase.21
REFRENCES


LEGENDS

Figure 1. Effect of GSSG on platelet aggregation and effect of platelets on GSSG. In a) gel filtered platelets were incubated for 1 minute at 37°C with the indicated concentration of GSSG. Collagen was then added at a concentration insufficient to induce aggregation by itself. In b) collagen alone caused partial aggregation that a low concentration of GSSG (2 µM) further potentiated. These experiments were performed three times with similar results. In c, d & e gel filtered platelets were incubated with GSSG, or under other conditions, and total SH in the sample determined using DTNB as described.7 c) Shows the results of platelets incubated with GSSG. Platelets alone are in column 1. Column 2 shows the results of platelets preincubated with GSSG (5 µM) for 15 min at 24°C before the DTNB was added. Column 3 shows the results for GSH (10 µM) with platelets (Plat/GSH). For these experiments n=5. In d) the lack of effect of platelets on cystine (disulfide of cysteine) is seen. Column 1 again shows the amount of SH in platelets. Column 2 shows the SH of platelets incubated with 5 µM cystine (Plat/cystine). Also shown are platelets with cysteine (10 µM) (Plat/cysteine). For these experiments n=7. In e) the effect of NADPH oxidase inhibitors on conversion of GSSG to GSH is seen. The first column shows the SH of platelets. Column 2 is the SH of the platelet/GSSG mixture; columns 3 and 4 are the same as column 2 except the flavoprotein NADPH oxidase inhibitors DPI (10 µM) or apocynin (100 µM) (Apo) were added to the platelets for 30 min prior to the addition of GSSG. For these experiments n=2 to 4 for each sample. The results are ± 1SE.
Figure 2. Time course studies on GSSG and platelets and the effect of GSSG on sulfhydryl labeling of αIIbβ3. a) Shows the time course of GSSG conversion to GSH at 37°C. In these studies GSSG was added to platelets for the indicated time and then DTNB was added. Total SH in the sample was measured as described in Figure 1c. Each point in the graph represents the mean of duplicate samples. In b) GSSG (5 µM) was added either simultaneously (0 sec) with a subthreshold concentration of collagen (0.1 µg/ml) or 1 minute (60 sec) before the subthreshold dose of collagen. (Controls not shown demonstrated the stimulatory effect of GSH (10 µM) or GSH (10 µM) with GSSG (2 µM), added simultaneously with the same dose of collagen). These experiments were performed a minimum of 2 times with similar results. In c) platelets were incubated without glutathione (lane 1), with GSH (10 µM) (lane 2), or GSSG (5 µM) (lane 3), for 5 minutes at 24°C under non-stirring conditions. The sulfhydryl reagent 3-N-maleimidylpropionyl biotin (MPB) (50 µM) was added in excess of the GSH, and the labeling performed under conditions which cause disruption of the αIIbβ3 receptor (5 mM EDTA, 60 min, 37°C). Equal amounts of protein were added to lanes 1, 2 and 3. In d) densitometry of the individual bands was used to obtain quantitative results. The first two columns show the ratio of labeling of the β3 subunit with GSH or GSSG to the β3 subunit without glutathione (± 1SE, n=8). As a control for possible translocation of αIIbβ3 the third and fourth columns give the ratio of labeling using a label for primary amines, sulfosuccinimido-biotin (SSB) in the β3 subunit with GSH or GSSG to the β3 subunit without glutathione (± 1SE).
Figure 1
Figure 2
Platelet Surface Glutathione Reductase-Like Activity

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