S-glutathionylation in human platelets by a thiol–disulfide exchange-independent mechanism

Isabella Dalle-Donne, Daniela Giustarini, Roberto Colombo, Aldo Milzani, Ranieri Rossi

Department of Biology, University of Milan, via Celoria 26, I-20133 Milan, Italy
Department of Neuroscience, University of Siena, Siena, Italy

Received 11 August 2004; revised 12 January 2005; accepted 9 February 2005
Available online 3 March 2005

Abstract

Protein–glutathione mixed disulfide formation was investigated in vitro by exposure of human platelets to the thiol-specific oxidant azodicarboxylic acid-bis-dimethylamide (diamide). We found that diamide causes a decrease in the reduced form of glutathione (GSH), paralleled by an increase in protein–GSH mixed disulfides (S-glutathionylated proteins), which was not accompanied by any significant increase in the basal level of glutathione disulfide (GSSG). The increase in the appearance of S-glutathionylated proteins was inversely correlated with ADP-induced platelet aggregation. Platelet cytoskeleton was analyzed by SDS–PAGE followed by Western immunoblotting with anti-GSH antibody. The main S-glutathionylated cytoskeletal protein proved to be actin, which accounts for 35% of the platelet total protein content. Our results suggest that neither GSSG formation nor a consequent thiol–disulfide exchange mechanism is involved in actin S-glutathionylation of human platelets exposed to diamide. Instead, a mechanism involving the initial oxidative activation of actin thiol groups, which then react with GSH to the protein–GSH mixed disulfides, makes it likely that platelet actin is S-glutathionylated without any significant increase in the GSSG content.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Cytoskeletal proteins; Actin; Glutathione disulfide; Diamide; Protein thiols; Protein–GSH mixed disulfides; Platelet aggregation; Free radicals

A wide range of protein modifications induced by oxidative stress, including formation of protein carbonyls; methionine sulfoxide, dityrosine, and tyrosine nitration; S-nitrosocysteine; cysteine sulfenic, sulfinic, and sulfonic acid; and intermolecular, intramolecular, and protein–glutathione mixed disulfides, have been identified (for recent reviews, see Refs. [1–5]).

Under normal conditions, mammalian cells contain 1–10 mM cytosolic glutathione (GSH), depending on cell type and metabolic factors. GSH represents approximately 95% of total nonprotein thiols and is the main modulator of the cellular redox environment [6]. 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. GSSG generation from GSH can be favored during mild oxidative stress conditions [6,7]. The oxidation of only a limited amount of GSH to GSSG can dramatically change the GSH/GSSG ratio and, consequently, the redox status within the cell. Under these conditions of moderate oxidative stress, thiol groups of cytosolic proteins can be modified by the reversible formation of protein–GSH mixed disulfides, a process known as S-glutathionylation (reviewed in Ref. [4]).

Protein S-glutathionylation is a dynamic process that is currently considered a mechanism of redox-mediated and reactive oxygen/nitrogen species (RONS)-mediated signal transduction as well as a way for cells to store GSH during oxidative stress and/or to protect critical protein cysteines...
from the irreversible oxidation to cysteine sulfenic and sulfonic acids, thus preventing permanent loss of function as a consequence of oxidative and/or nitrosative insult [8].

The precise mechanisms in vivo leading to protein S-glutathionylation are still far from being well understood and under active investigation and may depend on several specific criteria including the tissue type, the nature of the oxidative stress, and its duration. Anyway, the reversible S-glutathionylation of a number of proteins has recently been reported and suggested as a candidate mechanism for regulating protein function (reviewed in Ref. [4]). Proposed mechanisms of RONS-induced protein S-glutathionylation can be attributed mainly to two distinct pathways [4,8,9]. Protein S-glutathionylation can occur in response to changes in the intracellular redox potential, i.e., a decrease in the GSH/GSSG ratio caused by an increase in the amount of GSSG that is able to exchange disulfide with available reactive protein thiols. Although the ratio of GSH to GSSG favors S-glutathionylation in the endoplasmic reticulum [7] and in the mitochondrion [10], GSSG is unlikely to be the mediator of S-glutathionylated protein formation based on typical redox potentials for cysteine residues [11]; furthermore, the rate of exchange for GSSG with the thiolate in a protein is slow and probably would need catalysis; finally, because the reaction would produce GSH, which is 1–10 mM in cells, it would be unfavorable both thermodynamically and kinetically [12]. Nevertheless, S-glutathionylation through thiol/disulfide exchange remains a likely mechanism in oxidative stress in which significant transient increases in intracellular GSSG occur, as we have observed in human erythrocytes [13].

Alternatively, redox-dependent S-glutathionylation can be triggered by the oxidation or S-nitrosation of GSH to glutathione sulenenate or S-nitrosoglutathione, respectively, which induce the incorporation of the GSH moiety into target cysteine residues, yielding the corresponding S-glutathionylated protein. Glutathione–thyl radical, which may be continuously produced at a low level when a redox signaling pathway is activated, has also been proposed as a potential alternative mediator of protein S-glutathionylation [14,15], and the transfer of the GS radical to form protein–GSH adducts (protein–SSG) is greatly enhanced by glutaredoxin (thiol transferase) [16]. The second proposed mechanism leading to S-glutathionylated proteins derives from the intriguing observation that S-glutathionylation can occur in various cell models of RONS generation without changes in the intracellular GSH/GSSG ratio. The occurrence of protein S-glutathionylation in hepatocytes, human neutrophils, and rat erythrocytes without any increase in cellular GSSG concentration [13,17,18] supports the latter mechanism. This has been explained by direct oxidation of reduced protein thiols generating a reactive protein thiol intermediate, such as protein sulenate and protein–thyl radical, which further reacts with GSH, leading to the mixed disulfide [8,9]. Reactive nitrogen intermediates derived from nitric oxide can react with protein thiols to form S-nitrosothiols, which can react with GSH, leading to protein S-glutathionylation as well [9,19].

In analogy to other components of the vascular system such as neutrophils, erythrocytes, and endothelial cells, platelets are a potential target of RONS, either produced by other cells under oxidative/nitrosative stress conditions, such as inflammation or ischemia–reperfusion, or released by platelets themselves upon appropriate stimulation [20,21]. The relationship between oxidative stress and platelet function has been investigated [20]. Studying the modulation of platelet soluble guanylate cyclase activity by thiol-oxidizing agents, including diamide, Ullrich and co-workers found that reversible activation of the enzyme was accompanied by a reduction in GSH and a concomitant formation of protein–SSG [22]. But they did not investigate these events further.

Our interest has mainly turned to what mechanism can lead to the formation of S-glutathionylated proteins in human platelets and what is the main target protein(s). To mimic oxidative stress conditions, we exposed resting human platelets to the thiol-specific oxidant diamide, which penetrates cell membranes within seconds and can oxidize both GSH and protein thiols [23]. Cells can tolerate prolonged exposure to millimolar concentrations of diamide and oxidation can be reversed. In the specific case of human platelets, diamide concentrations up to 1 mM have been shown not to be destructive to the cells [22].

Materials and methods

Materials

Diamide, ATP (disodium salt), and all other reagents of analytical grade were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Sephasil C18 HPLC column (250 × 4 mm) was purchased from Pharmacia (Uppsala, Sweden). Monobromobimane (mBrB) was obtained from Calbiochem (La Jolla, CA, USA) and HPLC-grade reagents from BDH (Poole, England). The slot-blotter (Bio-Dot SF apparatus) and the Opti-4CN Substrate Kit were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Monoclonal anti-GSH antibody was obtained from Virogen (Watertown, MA, USA), sheep antirat IgG, horseradish peroxidase conjugate, was obtained from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, England).

Platelet preparation

Plateletswere obtained from healthy volunteer donors who had not received any medications in the 2 weeks before blood collection. An acid/citrate/dextrose buffer in a 9:1 (v/v) ratio was used as an anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation at 200g for 20 min at room temperature and platelet content was adjusted to about 5 ×
platelets/ml. PRP samples were treated with different concentrations of diamide at 37°C; aliquots were periodically removed for required determinations.

**Platelet aggregation**

Platelet aggregation was monitored at 37°C on a dual-channel aggregometer (Elvi Logos, Milan, Italy) with continuous stirring at 1000 rpm. Aliquots of 440 μl PRP were incubated for various times with 0.2, 0.4, and 0.75 mM diamide. Twenty microliters of ADP (10 μM final concentration) was then added and changes in light absorbance were monitored for 10 min. The extent of platelet aggregation was expressed as the percentage of the light transmission of plasma deprived of platelets.

**Isolation of the platelet cytoskeleton**

PRP was washed three times in PBS (145 mM NaCl, 10 mM Na+/K+ phosphate buffer, pH 7.4) containing 5 mM EDTA and 2 mM NEM. The platelet cytoskeleton was obtained according to the procedure described by [24]. Washed platelet suspensions were centrifuged for 1 min at 3000g and the supernatant was discarded. Pellets were treated with 200 μl of 2% (v/v) Triton X-100, 100 mM Tris–HCl (pH 7.4), 10 mM EGTA, 4 mM EDTA, 2 mM PMSF, 1 μg/ml leupeptin, and 1 mM benzamidine (lysis buffer). Triton-insoluble residues, corresponding to the cytoskeletal fraction, were isolated by centrifugation at 12,000g for 5 min at 4°C in a tabletop centrifuge. Cytoskeletal fractions were washed twice with lysis buffer without Triton X-100 (washing buffer) at 4°C, solubilized with washing buffer containing 2% SDS, and heated at 100°C for 5 min. Samples were frozen at −20°C until used in a 12% SDS–PAGE under reducing or nonreducing conditions.

**Determination of GSH and GSSG**

One-milliliter aliquots of PRP were centrifuged for 1 min at 3000g and the plasma was discarded; pellets were then treated with 200 μl of 5% (w/v) trichloroacetic acid (TCA). After protein separation by centrifugation, GSH and GSSG were determined on the clear supernatant by HPLC as previously described [25].

**Determination of protein SH groups**

One-milliliter aliquots of PRP were washed three times in PBS (145 mM NaCl, 10 mM Na+/K+ phosphate buffer, pH 7.4) containing 5 mM EDTA, then proteins were precipitated by 5% (w/v, final concentration) TCA and centrifuged (15,000g for 5 min). Pellets were then resuspended with a glass rod in 1 ml of 1.5% (w/v, final concentration) TCA and then pelleted by centrifugation (15,000 g for 5 min); this step was repeated three times. Finally, the protein pellet was resuspended in 0.2 M Na+/K+ phosphate buffer, pH 7.4, containing 1% (w/v, final concentration) SDS, being gently stirred with a glass rod until the proteins were dissolved. Protein SH groups were then measured spectrophotometrically at 410 nm with 5,5′-dithio-bis(2-nitrobenzoic acid) as previously described [13].

**Detection of protein S-glutathionylation by slot-immunoblotting**

Whole platelet lysates were mixed with an equal volume of 2% SDS supplemented with 5 mM NEM to block unreacted thiol groups and then subjected to slot-blotting. Parallel lysate samples were treated with 5 mM DTT before slot-blotting. A polyvinylidene difluoride (PVDF) membrane was prepared by wetting it with 100% methanol and then soaking it in a 20% methanol–80% Tris-buffered saline solution (20 mM Tris-base, pH 7.4, 500 mM NaCl) for 5 min. Two hundred fifty microliters of diluted protein solutions (10 μg total proteins) was applied to each slot. After three washes with PBST [10 mM Na phosphate, pH 7.2, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20] for 5 min each, blots were blocked for 1 h in 5% (w/v) commercial nonfat dried milk powder in PBST and incubated for 2 h with monoclonal mouse anti-GSH antibody (1:10,000 dilution) in 5% milk/PBST. After three washes with PBST for 5 min each, the membrane was incubated for 1 h with a 1:10,000 dilution of a horseradish peroxidase-conjugated anti-mouse antibody in PBST containing 5% (w/v) milk. S-glutathionylated proteins were visualized using the Opti-4CN Substrate Kit. Total protein loading was evidenced by amido black staining after the blots were washed extensively in PBS.

**Determination of protein S-glutathionylation by reversed-phase HPLC**

Proteins were periodically isolated from low-molecular-weight reagents by gel filtration on PD10 columns. Samples were then incubated with 0.5 mM DTT and neutralized by addition of solid NaHCO₃ until saturation. After 20 min, samples were deproteinized by 5% (w/v, final concentration) TCA and the thios, released from the disulfide bond by DTT reduction, were conjugated with 2 mM mBrB (final concentration) at neutral pH. After a 15-min incubation at room temperature in the dark, samples were acidified with an adequate amount of 37% (v/v) HCl and injected into a Sephasil C18 HPLC column. Solvent A was 0.25% (v/v) acetic acid, adjusted to pH 3.09 with 1 N NaOH, and solvent B was methanol. The elution profile was as follows: 0–8 min 20% B, 8–15 min 20–40% B, 15–25 min 40–100% B. A Bio-Tek Instruments KromaSystem 2000 HPLC was used. Derivatized thios were analyzed by fluorescence detection (excitation, 380 nm; emission, 480 nm) and quantified using authentic GSH similarly derivatized with mBrB.
Detection of cytoskeletal protein S-glutathionylation by Western blot immunoassay

Protein S-glutathionylation was detected by Western blot immunoassay as recently reported [26]. Protein samples were mixed with 2× SDS sample buffer, without reducing agents and supplemented with 5 mM NEM to block unreacted thiols, and then subjected to 10% SDS-PAGE followed by electroblotting onto PVDF membrane. In a parallel set of samples, 10 mM DTT was added to the 2× SDS sample buffer. Immunodetection of protein S-glutathionylation was performed as reported for the slot-immunoblotting.

Bovine cardiac myofibrillar proteins (utilized as apparent molecular weight markers in SDS–PAGE) were prepared as previously reported [27].

Results

Protein–GSH mixed disulfide formation

To characterize the effects of oxidative stress on platelet intracellular proteins, human platelets were challenged with varying concentrations of the highly specific thiol oxidant diamide. After different times of incubation, platelet extracts were prepared in the presence of 5 mM NEM, to prevent artifactual S-thiolation during the extraction procedure, or 5 mM DTT, to reduce any S-thiolated protein. Immunochemical evidence for protein S-glutathionylation in whole platelet lysate samples was obtained by a sensitive solid-phase immunochemical method, using monoclonal antibodies raised to GSH, as we have recently reported [12,25].

Fig. 1 shows that the basal level of protein S-glutathionylation in unstressed platelets (time 0 min), if any, was undetectable by slot-immunoblotting probed with anti-GSH, but increased after treatment with diamide. Amido black staining (right strip) is displayed in comparison with immunostaining using anti-GSH antibody (left strip). Exposure of platelets to 0.4 or 0.75 mM diamide induced similar high levels of protein S-glutathionylation that peaked at 10 and 2 min of incubation, respectively (Figs. 1B and 1C), whereas S-glutathionylation from 0.2 mM diamide was much lower and hardly detectable (Fig. 1A). Treatment of parallel samples with the reducing agent DTT abolished S-glutathionylation, as judged by the disappearance of the immunostained bands (central strip).

The time course of S-glutathionylation of total proteins was quantified by reversed-phase HPLC with fluorescence detection after mBrB derivatization of released bound thiols [25,26] (Fig. 2). Quantitative analysis confirmed the qualitative assessment of the slot-immunoblotting. As expected from the immunochemical evidence, no or negligible protein S-glutathionylation was detected in unstressed platelets (time 0), whereas a moderate or high S-glutathionylation, depending on the oxidant concentration, was measured after platelet exposure to oxidative stress (Fig. 2). We measured a maximum extent of ~14 nmol protein–GSH mixed disulfides/10⁹ platelets (i.e., ~20% of protein SH groups), reached within 10 min after addition of the oxidant, when platelets were exposed to 0.4 and 0.75 mM diamide (Figs. 2B and 2C). Total protein S-glutathionylation in platelets treated with 0.2 mM diamide peaked at 10 min and then declined and recovered to basal levels over the course of 120 min (Fig. 2A). When platelets were exposed to stronger oxidizing conditions (0.4 mM diamide, Fig. 2B), protein–GSH mixed disulfides started to recover toward initial values after 120 min, and a complete deglutathionylation occurred within 4–5 h (not shown). Conversely, irreversible formation of mixed disulfides (PSSG) was observed at 0.75 mM diamide (Fig. 2C).
The effect of diamide-induced protein S-glutathionylation on platelet aggregation

Incubation of human blood platelets with diamide influences the aggregation behavior considerably [28]. Experiments to verify the ADP-induced aggregation of diamide-treated platelets were carried out. Addition of 10 μM ADP to platelets caused an immediate aggregation response, as judged by the increase in light transmission of the platelet control sample (not shown). ADP-induced platelet aggregation was shown to be reversibly inhibited by 0.2–0.4 mM diamide, with a maximum inhibition after 10–30 min from diamide exposure (Figs. 3A and 3B). Afterward, platelets tended to recover their initial ability to aggregate in response to ADP stimulus depending on the oxidant bolus administered. In contrast, oxidation of platelets with 0.75 mM diamide was accompanied by irreversible inhibition of aggregation (not shown), indicating that, at that concentration, diamide exerted an irreversible (lethal) effect on human platelets. The reversible increases in S-glutathionylated proteins (Figs. 1a and 2) proved to be inversely correlated with platelet aggregation (Figs. 3A' and 3B').

GSH and GSSG status in platelets treated with diamide

We next determined whether/how the increases in protein S-glutathionylation correlated with changes in the cellular redox state. GSH is the most abundant low-molecular-weight sulfhydryl, and differences in the ratio GSH/GSSG can be used as a sensitive indicator of the overall redox balance. Unstressed human platelets have a mean GSH/GSSG ratio of ~45, indicating that most glutathione is maintained in the reduced form (Fig. 4A, time of incubation with diamide 0 min). Treatment with diamide reduced the GSH/GSSG ratio by decreasing readily GSH solely without increasing the levels of GSSG, at all the diamide concentrations and incubation times tested (Fig. 4). Variations in the GSH concentration paralleled those in the level of total protein S-glutathionylation shown in Fig. 2. The initial (basal) thiolic homeostasis, i.e., the GSH/GSSG ratio, recovered completely within 3 or 5 h from platelet exposure to 0.2 or 0.4 mM diamide, respectively. In contrast, the drastic reduction (>95%) in the GSH level occurring in platelets challenged with 0.75 mM diamide remained steady (Fig. 4D), indicating that, at that concentration, diamide exerted an irreversible (lethal) effect on human platelets, preventing the restoration of an appropriate GSH/GSSG ratio and, as a consequence, not allowing protein thiols to be returned to the cellular thiol pool by the reaction of GSH with protein–SSG.

Results shown in (Figs. 1, 2, and 4) suggest that the appearance of S-glutathionylated proteins induced by oxidative stress conditions correlates with the time-dependent decrease in GSH. The loss of GSH is totally due to GSH conversion into mixed disulfides (protein–SSG), as no extracellular total glutathione (GSH + 2GSSG) increase was found (not shown). This is also evidenced by the fact that the concentration of the intracellular total glutathione (GSH + 2GSSG + protein–SSG), obtained by data from Figs. 2 and 4, remains constant with time after diamide treatment.

These data and the absence of any evidence of oxidation of GSH into GSSG are clearly in contrast with a possible mechanism of redox-dependent protein S-glutathionylation involving the oxidative modification of GSH to GSSG.
S-glutathionylation of cytoskeletal proteins

Platelet cytoskeletal proteins were obtained by lysing platelets with Triton X-100, a detergent that solubilizes most platelet proteins except actin filaments and proteins associated with them. Cytoskeletal proteins can be sedimented at low g forces and then detected and quantified after SDS–PAGE of the detergent-insoluble material [24,29]. The platelet cytoskeleton consists mainly of ABP, talin, myosin, α-actinin, gelsolin, actin, and tropomyosin (Fig. 5, left). The most abundant cytoskeletal protein is actin, which accounts for 35% of the total platelet protein content [30].

To demonstrate S-glutathionylation, cytoskeletal proteins were separated by nonreducing (−DTT) and reducing (+DTT) SDS–PAGE and analyzed by Western blot utilizing monoclonal antibodies raised to GSH (Figs. 5A–5C). Incubation of platelets with increasing concentrations of diamide led to the formation of disulfide bonds between cytoskeletal proteins (intermolecular cross-linking), promoting the formation of large molecular mass aggregates, which did not enter the separating gel in nonreducing electrophoresis (Figs. 5A–5C, −DTT lanes). These aggregates were resistant to denaturation by heating in SDS, whereas they could be reduced by treatment with DTT (Figs. 5A–5C, +DTT lanes).

Consistent with the ability of diamide to induce total protein S-glutathionylation (Figs. 1 and 2), exposure of human platelets to varying concentrations of diamide resulted in a concentration-dependent increase in cytoskeletal protein S-glutathionylation, as determined by Western blot analysis with monoclonal anti-GSH antibody (Figs. 5A–5C). Actin proved to be the protein most readily S-glutathionylated as well as to the highest extent. Note that increased S-glutathionylation staining on the blots was not a result of loading different amounts of cytoskeletal proteins, as Coomassie brilliant blue staining of SDS–PAGE showed equal protein loading in all lanes (Figs. 5A–5C). With the increase in oxidative stress burden, the number of cytoskeletal proteins becoming S-glutathionylated also increased (Figs. 5B and 5C). No S-glutathionylation was detected after treatment of cytoskeletal protein samples with DTT (not shown).

Discussion

Under inflammatory conditions and in other pathological situations, platelets may be subjected to external oxidative stress by exposure to RONS released by granulocytes, endothelial cells, and/or monocytes/macrophages [6]. Furthermore, human platelets generate and
release RONS in response to physiological stimulation [21,31]. Thus, platelets are likely to face physiological oxidative stress conditions, particularly in situations that require modulation of platelet function, suggesting a regulatory role for oxidative signals [20,32].

Diamide has been shown to affect platelet morphology, aggregation, and serotonin transport mechanisms [28]; the content of α-tocopherol [33]; and soluble guanylyl cyclase activity [22]. Diamide impairs thrombin- and serotonin-mediated calcium signaling in human platelets as well [34].

Fig. 4. Measurement of GSH and GSSG. (B–D) Changes in GSH (white bars) and GSSG (gray bars) were measured in platelets exposed to (B) 0.2, (C) 0.4, and (D) 0.75 mM diamide for various times. Results represent the means ± SD of five independent determinations. (A) The GSH/GSSG ratio of platelets exposed to 0.2 (triangles), 0.4 (squares), and 0.75 mM (circles) diamide.

Fig. 5. S-glutathionylation of cytoskeletal proteins. Human platelets were challenged with (A) 0.2, (B) 0.4, and (C) 0.75 mM diamide for 60 min. Cytoskeletal proteins were analyzed by 12% SDS–PAGE run under either reducing (+DTT) or nonreducing (−DTT) conditions. Samples under nonreducing conditions were then blotted to PVDF and probed with anti-GSH antibody (right strip in each panel). Data are representative of three independent experiments. Left: CP, platelet cytoskeletal proteins; MP, bovine cardiac myofibrillar proteins. Western blot probed with anti-GSH antibody shows that there are no S-glutathionylated cytoskeletal proteins in unstressed (control) platelets [CP (−DTT)].
Diamide also causes intracellular protein S-glutathionylation, as shown in human T cell blasts [35,36], endothelial cells [37], immortalized ECV 304 endothelial-like cells [38], human and rat erythrocytes [25], and human platelets [22].

Diamide induces platelet protein S-glutathionylation (Figs. 1 and 2) and the formation of disulfide bonds between cytoskeletal protein SH groups (intermolecular cross-linking) (Fig. 5).

Among cytoskeletal proteins, actin remains the main and more easily S-glutathionylated protein [39]. Actin has been found to be a substrate for S-glutathionylation in endothelial cells [37,40], gastric mucosal cells [41], human neutrophils [18], human T cell blasts [35,36], and human ECV 304 endothelial-like cells [38] and during reperfusion of the ischemic rat heart [42]. The cysteine at position 374 is the S-glutathionylation site both in cells [43] and in the isolated protein [26]. Accordingly, modification of actin at Cys\textsuperscript{374} with NEM completely inhibits actin S-glutathionylation [12].

The thiol-oxidizing agent diamide can evoke a number of actions due to conversion of protein SH groups to disulfides: PSSG and/or inter- and/or intramolecular protein disulfides. Oxidation of GSH into GSSG may also be of prime importance, making GSH unavailable for some reactions that could be involved in platelet homeostasis and physiological processes leading to platelet aggregation. However, GSH depletion alone seems not to be sufficient for influencing platelet aggregation, as experiments in which GSH was specifically decreased by platelet treatment with 1-chloro-2,4-dinitrobenzene showed that platelets aggregated normally [44]. Thus, it remains to evaluate if diamide inhibits platelet aggregation via protein disulfides and/or protein–SSG formation. We cannot exclude that a main role could be played by intermolecular protein disulfides, and this will be the subject for further analyses using, for instance, proteomics with diagonal electrophoresis [45]. However, in accordance with what we found in in vitro experiments of actin polymerization, in which the formation of F-actin was shown to be inhibited by S-glutathionylation of its Cys\textsuperscript{374} residue [26], and considering that the rearrangement of cytoskeletal proteins, particularly of actin, is fundamental in platelet adhesion and aggregation processes, the relationship between actin–SSG formation and platelet function regulation may be hypothesized.

Usually, treatment of cells with diamide leads to the oxidation of GSH into GSSG, because diamide is more likely to react with a small thiol like GSH than with protein SH groups, GSH having less steric hindrance [23]. Diamide can react with GSH and/or protein thiols, forming GSSG and/or S-glutathionylated proteins, respectively, according to the reactivity of each thiol species (GSH and protein thiol). The reaction rate of small thiols (i.e., GSH, cysteine, or N-acetylcysteine) with diamide is usually well correlated with the pK\textsubscript{a} of the sulfhydryl group, which normally lies between pH 8.5 and pH 9.5, whereas for protein thiols, accessibility as well as the dissociation state of the sulfur must be considered [46,47].

The diamide-induced protein S-glutathionylation can follow two distinct pathways that mimic, at least partly, the main mechanisms proposed to mediate protein S-glutathionylation. One pathway involves the addition of GSH to the double bond of the diazene, forming a sulfenylhydrazine, which can react either with another GSH, giving GSSG and a hydrazine, or directly with protein thiols, forming protein–GSH mixed disulfides [23]. The second pathway involves the initial modification of a protein sulfhydryl group to diamide-activated protein species, which may then react with GSH, forming the mixed disulfide.

In unstimulated human platelets, diamide causes a rapid decrease in the GSH content paralleled by a concomitant increase in protein mixed disulfides, without any significant increase in the basal level of GSSG (Figs. 1, 2, and 4). This evidence suggests that proteins are unlikely to be S-glutathionylated by a thiol–disulfide exchange mechanism with GSSG within the platelet context. Diamide could activate GSH to sulfenylhydrazine, which could react with reduced protein SH groups, yielding the corresponding mixed disulfide. Alternatively, diamide could react directly with platelet protein thiols and then with GSH to form the S-glutathionylated protein. We have no data to support strongly one mechanism over the other. Anyway, some evidence could be taken into consideration.

Actin is readily S-glutathionylated in vitro by glutaredoxin-mediated glutathione–thiyl-radical transfer [16]. This could be an explanation for the presence of S-glutathionylated actin under some conditions under which GSSG does not accumulate sufficiently to support a simple thiol–disulfide exchange mechanism, as we have here demonstrated in human platelets. In fact, physiological levels of GSSG induce very little actin S-glutathionylation [12]. Instead, actin with the exposed cysteine thiol activated by diamide reacts with physiological levels of GSH, incorporating about 0.7 mol GSH/mol protein [12]; this may offer another explanation for the presence of actin–GSH adducts in platelets. Moreover, thiol–disulfide exchange is a rather slow reaction that requires high GSSG concentrations and probably would need catalysis [9], both of which seem unlikely in platelets. Thus, a GSH-dependent trapping mechanism in which protein SH groups are activated, as either a thiyl radical or sulfenic acid, may be a primary mechanism of formation of S-glutathionylated proteins in human platelets. These activated protein intermediates can react with the pool of platelet GSH to produce a mixed disulfide adduct of protein and GSH. Such a mechanism depends on a substantial supply of GSH to effectively trap partially oxidized protein thiols (thiyl radical or sulfenic acid form).

The cell concentration of protein SH groups (10 to 30 mM) is much greater than that of GSH (1–10 mM). Protein thiols can be present as reduced thiols, thiolates, disulfides,
or (protein–GSH) mixed disulfides. The dynamic, reversible process of protein S-thiolation is an early cellular response to mild oxidative stress that occurs under physiological conditions in cells and at different rates, depending on the protein and the nature of its thiol groups, and could be a reflection of the redox state of the GSH system in the cell. The importance of protein SH groups as critical buffers to maintain the intracellular redox state has been suggested by reports of protein thiols having reactivity similar to or greater than that of GSH [13,15,48].

How well protein sulphydryls serve as a redox buffer will depend on their reactivity with GSH [46,47]. For instance, the reactivity of protein thiols in erythrocytes from different sources has been found to depend on the pKₐ of the thiol and structural features, such as accessibility [46]. The rapid decrease in the GSH concentration without any increase in the GSSG level, paralleled by increasing protein S-glutathionylation, which we have observed in human platelets exposed to diamide, represents a peculiar biochemical behavior, because in all the different cells or tissues treated with diamide, with the only exception of rat red blood cells [46], most GSH is converted into GSSG [23,49–51], and S-glutathionylated proteins constitute only a small percentage of the oxidized forms of glutathione. In the case of rat erythrocytes, we have identified that the abnormal high reactivity of hemoglobin Cys3125 [13] coupled with the high concentration of hemoglobin in red blood cells (8–10 mM as a monomer) is the main cause of the dominant S-glutathionylation. In the case of human platelets, it could be hypothesized that actin, bearing the highly reactive Cys374 SH group, is present at a sufficient concentration (it accounts for 35% of the platelet total protein content [30]) to compete with GSH in the metabolism of oxidizing substances.

Acknowledgments

This work was supported by FIRST 2004 (Fondo Interno Ricerca Scientifica e Tecnologica), University of Milan, and by the Fondazione Monte dei Paschi di Siena.

References


