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Original Contribution

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

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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.

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

Abbreviations: diamide, azodicarboxylic acid-bis-dimethylamide; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, glutathione disulfide; mBrB, monobromobimane; NEM, *N*-ethylmaleimide; RONS, reactive oxygen and nitrogen species; SH group, thiol group; protein–SSG, protein–GSH adducts.

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from the irreversible oxidation to cysteine sulfinic 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 sulfenate or *S*-nitrosoglutathione, respectively, which induce the incorporation of the GSH moiety into target cysteine residues, yielding the corresponding S-glutathionylated protein. Glutathione–thiyl 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 sulfenate and protein–thiyl 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 anti-mouse IgG, horseradish peroxidase conjugate, was obtained from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, England).

Platelet preparation

Platelets were 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 200*g* for 20 min at room temperature and platelet content was adjusted to about 5 ×