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Protein S-glutathionylation and platelet anti-aggregating activity of disulfiram

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Abbreviations:
ALDH, aldehyde dehydrogenase
DTT, dithiothreitol
GSH, reduced glutathione
GSSG, glutathione disulfide
mBrB, monobromobimane
MeDTC-SO, S-methyl-*N*,
N-diethylthiocarbamoyl sulfoxide
NEM, *N*-ethylmaleimide
PKC, protein kinase C
PPP, platelet-poor plasma
PRP, platelet-rich plasma
PSSG, protein-GSH mixed disulfides
(S-glutathionylated proteins)
SH group, thiol group
TCA, trichloroacetic acid
tGSH, total glutathione

ABSTRACT

Blood platelets are central to haemostasis, and reactions in platelets involving sulfhydryl groups play important roles in platelet function. Reduced glutathione (GSH) plays an important role in platelet aggregation and glutathione-depleting chemicals inhibit platelet aggregation. The lipophilic drug disulfiram, because of its affinity for sulfhydryl groups, is a highly thiol-reacting agent. As a consequence, GSH and sulfhydryl groups of protein cysteines in human platelets, in analogy to other components of human blood, are a potential target of disulfiram. In the present study, we have shown that exposure of human platelets to disulfiram causes the depletion of platelet GSH and augmentation of mixed disulfides between GSH and protein sulfhydryl groups to form protein-glutathione mixed disulfides (S-glutathionylated proteins). The depletion of platelet GSH and the increase in S-glutathionylated proteins occurred at concentrations of disulfiram that inhibited platelet aggregation, suggesting that protein S-glutathionylation is involved in the inhibition of platelet aggregation caused by disulfiram.

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1. Introduction

The dithiocarbamate drug disulfiram ([tetraethylthioperoxydicarbonic diamide, (*N*,*N*,*N*′,*N*′)-tetraethylthiuram disulfide]; Antabuse™ or Antabus™) is a thiuram disulfide long used clinically in the aversion therapy treatment of chronic alcoholism to produce intolerance to alcohol [1]. It exerts its pharmacological effect by irreversibly inhibiting hepatic (both mitochondrial and cytosolic isoforms) aldehyde dehydrogenase (ALDH), one of the key enzymes involved in alcohol metabolism, which is responsible for the oxidation of acetaldehyde produced by alcohol dehydrogenase after the intake of alcohol. Inhibition of ALDH gives rise to the accumulation of acetaldehyde upon alcohol ingestion, causing the acetaldehyde syndrome (or ''alcohol sensitivity''), characterized by facial flushing, nausea, vomiting, tachycardia, and hypotension [1,2]. Although disulfiram can be taken with relatively mild adverse side effects for months or years, two main side effects have been observed: hepatotoxicity (actually rare) and neurotoxicity, which is more prevalent and is typically the dose-limiting side effect of disulfiram administration [3,4].

Disulfiram and/or its metabolites, *S*-methyl-*N*,*N*-diethylthiocarbamoyl sulfoxide (MeDTC-SO) and *S*-methyl-*N*,*N*-diethylthiocarbamoyl sulfone (MeDTC-SO_2), have also been shown to induce apoptosis in tumour cells both in vitro and in vivo as well as to permanently inactivate the human multidrug resistance P-glycoprotein membrane pump that extrudes drugs from the cell to reduce angiogenesis, to inhibit nuclear factor-κB, matrix metalloproteinases, cancer cell invasiveness, and tumour growth in mice [5–12]. However, the mechanism(s) underlying the cytotoxic and anti-tumour effects of disulfiram has not been conclusively established. A number of different mechanisms have been proposed, among them is the redox-related processes involving copper and zinc [5,6,9, and citations therein]. Recent findings suggest that the anti-tumour and nuclear factor-κB inhibiting activity of disulfiram could be attributed, at least partly, to its 26S proteasome-inhibitory action [9].

Disulfiram possesses a reactive disulfide bond (Fig. 1), which reacts readily with both protein and low-molecular-mass thiols forming mixed disulfides, disulfides and dithiocarbamates. Disulfiram has been reported to undergo thiol–disulfide exchange with select protein sulfydryls. For instance, in vitro inhibition of liver mitochondrial ALDH is caused by the formation of an intramolecular disulfide bond between two out of the three adjacent cysteines within the active site of the enzyme [13]. Differently, in vivo, ALDH is inhibited by covalent adducts (i.e., carbamoyl derivatives) on Cys302, one of the three adjacent essential cysteines in the active site of ALDH, formed by one of the disulfiram metabolites, most likely MeDTC-SO [14]. Direct interaction of disulfiram, forming protein mixed disulfides, has also been demonstrated with caspases (both in T-cells and in a cell-free model system) [15,16], DNA topoisomerase I and II [17], and protein kinase C (PKC) isozymes, which are differentially regulated by disulfiram through thiol–disulfide exchange reactions [18]. Disulfiram does not deplete total glutathione but significantly decreases the reduced (GSH) to oxidized (GSSG) glutathione ratio, oxidizing the intracellular glutathione to its disulfide [6,19–22].

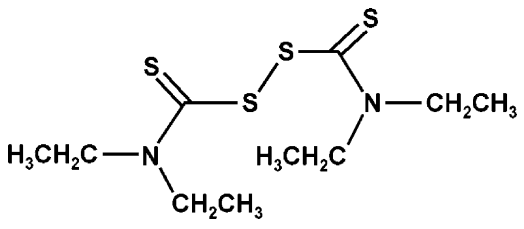

Fig. 1 – Disulfiram [(*N*,*N*,*N*′,*N*′)-tetraethylthiuram disulfide].

The effect of disulfiram on circulating blood components is still unknown. Platelet function has been shown to be greatly influenced by the thiol/disulfide redox state of both intracellular and extracellular compartments [23,24]. In particular, S-glutathionylation of cytoskeletal proteins has been shown to reversibly inhibit the ADP-induced platelet aggregation. Thus, since one of the main molecular actions of disulfiram is to induce the formation of mixed disulfides on various target proteins, we have investigated human platelet aggregation in relation to changes in the thiol redox state changes produced by disulfiram.

2. Materials and methods

2.1. Chemicals and materials

HPLC column Sephasil C18 (250 mm × 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, UK). Disulfiram and all other reagents of analytical grade were purchased from Sigma–Aldrich (Milan, Italy).

2.2. Platelet preparation

Platelets were obtained from healthy volunteer donors who had not received any medications in the 2 weeks before blood collection. A 0.1 M citrate buffer, pH 6.5, containing 25 mM glucose was used as an anticoagulant (1:10 ratio). Platelet-rich plasma (PRP) was prepared by centrifugation at 200 × *g* for 20 min at room temperature and platelet content was adjusted to 0.35×10^6 platelets/μl. PRP samples were treated with different concentrations of disulfiram at 37 °C. Aliquots were periodically removed for required determinations. Disulfiram was dissolved in DMSO to a final concentration of 75 mM. Equal volumes of DMSO were added to control samples.

2.3. Measurement of GSH, GSSG and PSSG

One milliliter-aliquots of PRP were centrifuged for 30 s at 3000 × *g* and plasma was discarded; pellets were treated with 200 μl of 5% (w/v, final concentration) trichloroacetic acid

(TCA). After protein separation by centrifugation, GSH and GSSG were determined on the clear supernatant by HPLC as previously described [23,25]. To quantify S-glutathionylated proteins (PSSG), protein pellets were washed three times with 0.5% (w/v) TCA, resuspended in 200 μl of 0.5 mM DTT and slightly alkalinized (pH 7.8–8.0) with solid $NaHCO_3$. After 10 min, samples were deproteinized by addition of 5% TCA (final concentration); thiols released from the disulfide bond by DTT reduction were conjugated with 2 mM (final concentration) mBrB after alkalinization (pH 7.8–8.0) with solid $NaHCO_3$. After a 10-min incubation at room temperature in the dark, samples were acidified with 10 μl 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. For all HPLC measurements, a Hewlett Packard HPLC Series 1100, equipped with both diode array and fluorescence detector, was utilised. Derivatized thiols were analysed by fluorescence detection (excitation, 380 nm; emission, 480 nm) and quantified using authentic GSH similarly derivatized with mBrB.

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

Protein S-glutathionylation was detected by slot immunoblotting as recently reported [23].

2.5. *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 PRP were incubated for various times with 0.1, 0.3, and 1 mM disulfiram. Ten micromolars (final concentration) ADP 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, taking as 100% the light transmission of the plasma deprived of platelets (platelet-poor plasma, PPP).

3. Results

3.1. *Glutathione redox status in human platelets treated with disulfiram*

Potential effects on thiol/disulfide redox balance of disulfiram on human platelets were studied in PRP exposed to various concentrations of the drug by measuring levels of GSH and GSSG after different times of incubation (Fig. 2). Unstressed human platelets have a mean GSH/GSSG ratio of ~45, indicating that most glutathione is maintained in the reduced form [23]. Treatment with disulfiram reduced the GSH/GSSG ratio by readily depleting GSH without increasing the level of GSSG (Fig. 2). The intraplatelet redox status returned to the baseline level within 2 or 4 h from platelet exposure to 0.1 mM (Fig. 2A) or 0.3 mM (Fig. 2B) disulfiram, respectively. In contrast, the drastic reduction (>95%) in the GSH level occurring in platelets treated with 1 mM disulfiram (Fig. 2C) remained steady.

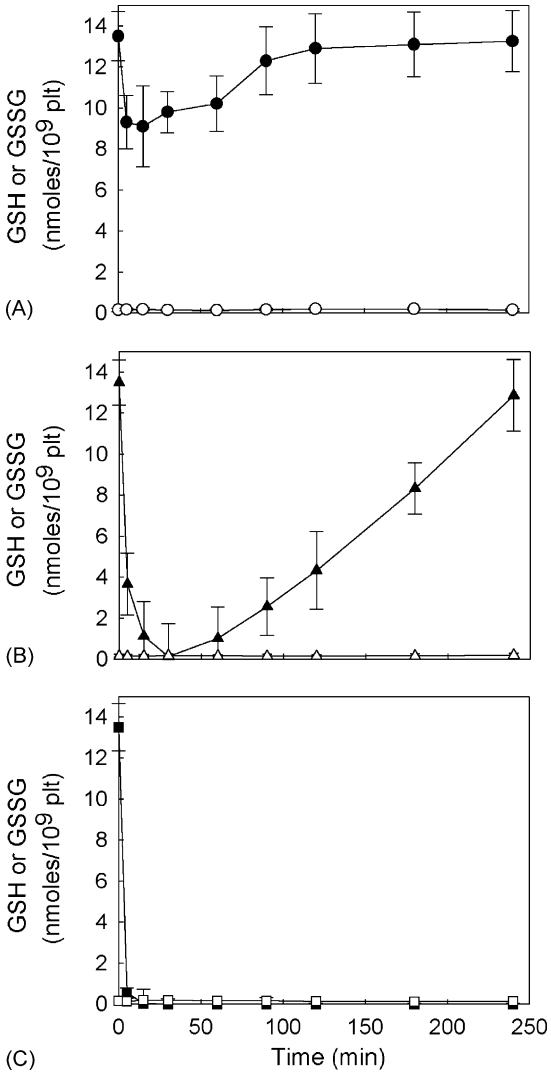

Fig. 2 – Measurement of GSH and GSSG. GSH (black symbols) and GSSG (white symbols) changes were measured in platelets exposed to (A) 0.1 mM (circles), (B) 0.3 mM (triangles), and (C) 1 mM (squares) disulfiram for different times. Results represent the mean ± S.D. of three replicate measurements.

3.2. *Protein-GSH mixed disulfide formation in human platelets treated with disulfiram*

The time course of S-glutathionylation of platelet total proteins was quantified by reversed-phase HPLC with fluorescence detection after derivatization with mBrB of released bound thiols [25,26] (Fig. 3). Negligible protein S-glutathionylation was detected in unstressed platelets (time = 0 min), whereas a moderate to high S-glutathionylation, depending on the disulfiram concentration, was measured after platelet exposure to the drug. The maximum extent of S-glutathionylation was ~4, ~12, ~14 nmol protein-GSH mixed disulfides (PSSG)/10^9 platelets, when platelets were treated with 0.1, 0.3, 1 mM of disulfiram, respectively. When platelets were exposed to 1 mM disulfiram, irreversible formation of PSSG was observed.

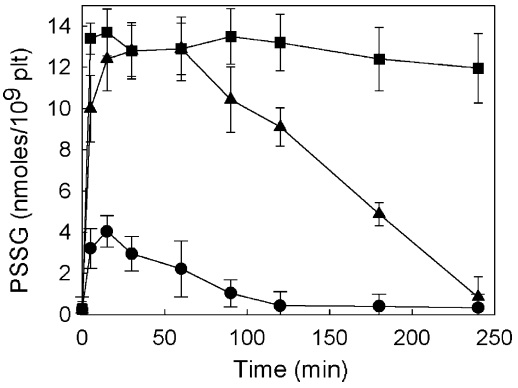

Fig. 3 – HPLC detection of protein S-glutathionylation. Human platelets were exposed to 0.1 mM (circles), 0.3 mM (triangles), and 1 mM (squares) disulfiram for different times. Protein S-glutathionylation in whole platelet lysates was quantitatively analysed by reversed-phase HPLC after derivatization of protein-bound thiols with mBrB. Data represent the mean ± S.D. of three replicate measurements.

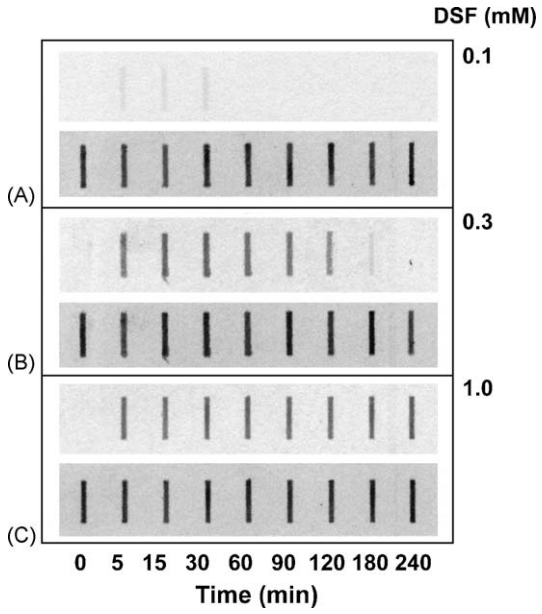

Fig. 4 – Immunochemical detection of protein S-glutathionylation. Slot-blot probed with monoclonal anti-GSH antibody showing PSSG generated after incubation of human platelets with 0.1 mM (A), 0.3 mM (B), and 1 mM (C) disulfiram for different times. The upper strip in each panel displays immunostaining under nonreducing (−DTT) conditions. The lower strip shows the corresponding duplicate slot-blot stained for proteins with amido black. Each panel shows a single representative experiment of a triplicate determination.

The formation of S-glutathionylated proteins was confirmed in whole platelet lysates by slot-blotting, using monoclonal antibodies raised to GSH [23,25]. Fig. 4 shows immunostaining using anti-GSH antibody (upper strip in all panels) in comparison with amido black staining (lower strip in all panels). The basal level of protein S-glutathionylation in untreated platelets (time = 0 min) was undetectable, but it increased after treatment with disulfiram. S-Glutathionylation induced by 0.1 mM disulfiram was hardly detectable (Fig. 4A), whereas 0.3 and 1 mM disulfiram induced higher levels of PSSG (Fig. 4B and C), with the increase in the intensity of the bands paralleling the amount of PSSG measured by HPLC analysis. Treatment of aliquot of the same samples with the reducing agent DTT abolished S-glutathionylation, as judged by the disappearance of the immunostained bands (not shown).

Results shown in Figs. 2–4 suggest that the appearance of S-glutathionylated proteins induced by platelet exposure to disulfiram correlates with the time-dependent decrease in GSH. Interestingly, the decrease in GSH is not due to its conversion into GSSG but to the formation of protein mixed disulfides. Neither evident export of GSH nor its transformation into higher oxidized forms (e.g., GSOH) occurred, since the concentration of the intra-platelet total glutathione (tGSH = GSH + 2GSSG + PSSG), obtained by data from Figs. 2 and 3, remained constant with time after exposure to the drug (Table 1). Furthermore, the drastic reduction (>95%) in the GSH level occurring in platelets challenged with 1 mM disulfiram remained steady (Fig. 2C), indicating that, at that concentration, disulfiram exerted an irreversible 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 PSSG (Fig. 4).

3.3. *The effect of disulfiram-induced protein S-glutathionylation on platelet aggregation*

It has previously been reported that GSH plays an important role in platelet aggregation, and that GSH-depleting chemicals inhibit platelet aggregation significantly [27–29]. However, the association between the depletion of GSH and inhibition of platelet aggregation has not been reported for disulfiram.

Table 1 – Intraplatelet total glutathione

Disulfiram (mM)	Time (min)	[GSH]	2[GSSG]	[PSSG]	Total GSH
0.1	15	9.10 ± 1.97	0.32 ± 0.06	4.03 ± 0.76	13.45 ± 2.79
0.3	15	1.12 ± 1.70	0.30 ± 0.06	12.40 ± 1.54	12.4 ± 3.30
1.0	15	0.02 ± 0.07	0.40 ± 0.05	13.70 ± 1.13	14.12 ± 1.25

Concentration of the intraplatelet total glutathione (tGSH = GSH + 2GSSG + PSSG) after exposure to disulfiram, obtained by data from Figs. 2 and 3. Concentration of GSH, GSSG, PSSG, and tGSH were expressed in nmoles/10^9 platelets.

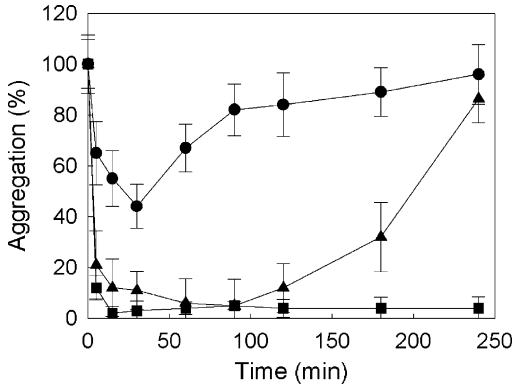

Fig. 5 – Effect of disulfiram on platelet aggregation. Human platelets were exposed to 0.1 mM (circles), 0.3 mM (triangles), and 1 mM (squares) disulfiram for different times. Platelet aggregation was initiated by the addition of ADP (10 μM, final concentration) and was monitored for 10 min by continuous recording of light transmission in a platelet aggregometer. The maximal extent of aggregation was expressed as the percentage change in light transmission by considering the transmission through platelet-poor plasma (PPP) as 100% and that of platelet-rich plasma (PRP) as 0%. Data represent the mean ± S.D. of three replicate measurements.

Our earlier results have shown that exposure of human platelets to the highly SH-reacting agent diamide causes the formation of protein-GSH mixed disulfides, which resulted to be inversely correlated with the ADP-induced platelet aggregation [23]. Therefore, experiments to verify the capability of platelets to aggregate after agonist addition were carried out. ADP-induced platelet aggregation was shown to be reversibly inhibited by 0.1–0.3 mM disulfiram, with a maximum inhibition after ~30 or 60 min from disulfiram exposure, respectively (Fig. 5). Afterwards, platelets tended to recover their initial ability to aggregate in response to ADP stimulus depending on the amount of administered drug. In contrast, treatment of platelets with 1 mM disulfiram was accompanied by irreversible inhibition of aggregation, confirming that, at that concentration, disulfiram exerted an irreversible effect on human platelets. The formation of S-glutathionylated proteins measured after treatments with disulfiram, reported in experiments shown in Figs. 3–5, was finally plotted against the platelet aggregation measured at each experimental time-point (Fig. 6). An inverse correlation ($r = -0.963$) was found between PSSG and platelet aggregation (Fig. 6).

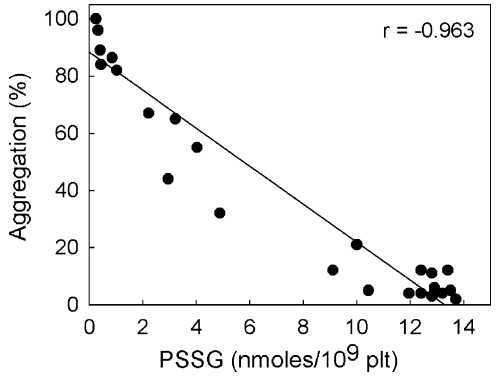

Fig. 6 – Correlation between PSSG concentration and the percentage of platelet aggregation, measured after exposure to 0.1, 0.3, and 1 mM disulfiram. Data from experiments reported in Figs. 3 and 5.

4. Discussion

Both protein and non-protein thiols are efficient antioxidants, and depletion of thiols in tissues under oxidative stress has been reported [30]. Reduced glutathione (GSH) and glutathione disulfide (GSSG) constitute the free glutathione forms and, together with the protein-bound fraction (PSSG), represent the total glutathione (tGSH). The reversible formation of protein-glutathione mixed disulfides (S-glutathionylation) is a dynamic process that is currently considered a mechanism of redox-mediated signal transduction and protein activity as well as a way for cells to store GSH during oxidative stress and/or to protect critical protein cysteines from the irreversible oxidation, thus preventing permanent loss of function as a consequence of severe oxidative insult [31,32]. Recently, a possible role for S-glutathionylation of proteins in human disease was discussed [33].

In vivo reports suggested that disulfiram induces intracellular pro-oxidative conditions in various cell types significantly shifting the prooxidant–antioxidant balance represented by the GSH/GSSG ratio [6,19,20,34].

Disulfiram is normally taken orally as a daily standard 250-mg dose [35]. Pharmacokinetic data on disulfiram are limited due to complex routes of metabolism (reviewed in Ref. [9]). Anyway, they suggest that 80–90% of an ingested dose is absorbed from the gastrointestinal tract and is rapidly distributed to tissues and organs [36]. In practice, however, it has been difficult to measure disulfiram levels in fresh human blood samples due to its rapid conversion to other metabolites [1]. However, studies indicate that micromolar concentrations of disulfiram can be achieved in man (reviewed in Ref. [9]). Disulfiram metabolites have been detected in fresh blood samples in micromolar concentrations after patients were given daily 300-mg oral doses for 2 weeks [7].

Because of its affinity for sulfhydryl groups, disulfiram is non-specific, highly SH-reacting agent. As a consequence, analogously to other components of the human blood, GSH and protein sulfhydryl groups in human platelets are potential targets of disulfiram.

Little is known about the role of intracellular GSH in physiological functions of platelets, such as aggregation, one of the three critical events of primary haemostasis. Platelets contain a relative low GSH level of about 2 mM [22], corresponding to 11–15 nmol of GSH/10^9 platelets, with more than 90–95% of intraplatelet glutathione being maintained in the reduced form [37]. Consistently, the GSH/GSSG ratio, which is often used as an indicator of the redox state, is ~45 in unstressed human platelets under normal physiological conditions [23]. Thiol–disulfide reactions and redox control play an important role in platelet function. Conversion of

cytoplasmic GSH to GSSG (by diamide) inhibits platelet aggregation and induces disulfide cross-linking of cytoskeletal proteins [38,39]. Cytoplasmic GSH appears to have a fundamental role in platelet activation, probably by maintaining the sulfhydryl status of cytoplasmic proteins. However, it should be noted that irreversible GSH depletion by alky(ari)lating agents elicits an effect on platelet aggregation only when almost all GSH is being alky(ari)lated, suggesting that the thiol/disulfide homeostasis of platelets might be more important than the absolute GSH concentration [40]. Furthermore, although not our current focus, it should be mentioned that thiols and disulfides on the platelet surface also have a role in platelet activation. In particular, protein thiol groups regulated by protein disulfide isomerase appear to be associated with the platelet responses of aggregation and secretion, as well as activation of the platelet fibrinogen receptor, the αIIbβ3 integrin [24,41,42].

A specific role for protein S-glutathionylation in human platelets has not been well studied. In non-stimulated human platelets, 0.1–0.3 mM disulfiram causes a rapid reversible increase in S-glutathionylated proteins paralleled by a concomitant rapid reversible decrease in the GSH content, without any significant increase in the basal level of GSSG (Figs. 2–4). These results suggest that neither GSSG formation nor a consequent thiol–disulfide exchange mechanism is involved in protein S-glutathionylation of human platelets exposed to disulfiram. Instead, a mechanism involving the initial oxidative activation of protein SH groups, which then react with GSH to the protein-GSH mixed disulfides, makes likely platelet proteins are S-glutathionylated without any significant increase in the GSSG content.

A plausible mechanism of intraplatelet reactions of disulfiram could be as follows. Disulfiram may react with protein thiols but not with GSH producing protein mixed disulfides, which may then react with GSH producing PSSG and diethyldithiocarbamate as final products. Later, reduction of PSSG by thioredoxin to reduced protein thiols and GSH could explain time-dependent and parallel formation of these products. This scheme would also explain the absence of GSSG in these reactions.

Disulfiram-induced reversible GSH decrease might be of prime importance making GSH unavailable for some important reactions concerning the processes of haemostasis, thrombosis, and wound healing, which platelets are involved in, including aggregation. Depletion of intraplatelet GSH has been correlated with inhibition of platelet aggregation by several compounds. These include helaniline, 1-chloro-2,4-dinitrobenzene, and quinones, which deplete GSH [27,29,43], *N*-ethylmaleimide, which alkylates GSH [40], besides diamide, which decreases GSH and increases PSSG [23]. 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 (which also inhibits GSH peroxidase activity) showed that platelets aggregated normally [40], as well as platelets from long-term smokers, which have an intraplatelet GSH level significantly lower than that of age-matched non-smokers, show augmented aggregability [44,45]. Our current findings show that disulfiram does not increase GSSG content and that protein S-glutathionylation is closely related with platelet aggregation during the course of exposure to disulfiram (Figs. 5 and 6), confirming the key role of protein sulfhydryl groups for platelet activity. Therefore, protein S-glutathionylation, but not decrease in GSH (or GSH/GSSG ratio), could be responsible, at least partly, for inhibition of platelet aggregation. Finally, our results suggest that 1 mM disulfiram exerted an irreversible (lethal) effect on human platelets, preventing the restoration of an appropriate GSH/GSSG ratio and, as a consequence, not allowing S-glutathionylated proteins to be returned to the cellular thiol pool by the reaction of GSH with PSSG (Figs. 2C and 3).

Previous research has shown that GSH depletion by helenalin and 11α,13-dihydrohelenalin was associated with inhibition of platelet aggregation and suggested that this possibly caused cytoskeletal protein alterations [29]. Our recent research has shown that GSH depletion by diamide induces cytoskeletal protein (mainly, but not only, actin) S-glutathionylation and also caused inhibition of platelet aggregation [23]. It is possible that disulfiram induces S-glutathionylation of cytoskeletal proteins, which could be the key mechanism for platelet aggregation.

Other possible protein targets of disulfiram, which can be likewise crucial in platelet aggregation, may be (some of) those involved in the large number of platelet inside-out and outside-in signalling pathways [46–48], such as PKC isozymes, proven targets of disulfiram [18] and regulated by S-thiolation-triggered mechanisms as well [49–52]. The PKC family has long been known to be involved in a number of platelet processes, most importantly in regulating aggregation and secretion. At least seven PKC isoforms (α, β, δ, ε, η, θ, and ζ) are expressed in platelets, and it is becoming clear that each isoform may play different roles in platelet function and may have different modes of activation and downstream targets, essential in aggregation and secretion [47,48,53].

Another obvious possibility is that membrane receptors expressed on the surface of circulating platelets may play important role in the inhibition of platelet aggregation induced by disulfiram. Human platelets express two G protein-coupled ADP receptors that contribute to various agonist-induced physiologic responses: the platelet ADP receptor coupled to stimulation of phospholipase C ($P2Y_1$) via heterotrimeric GTP-binding protein G_q, and the platelet ADP receptor coupled to inhibition of adenylyl cyclase ($P2Y_{12}$) via heterotrimeric GTP-binding protein G_i. The $P2Y_1$ and $P2Y_{12}$ ADP receptors act in synergy to trigger platelet aggregation, each of them being ineffective alone: co-activation of both of the receptors is essential for ADP-induced activation of platelet fibrinogen receptor [54,55]. Therefore, inhibition of only one receptor type produces a potent inhibition of ADP-triggered platelet aggregation.

Unlike the $P2Y_1$ receptor, which has two essential disulfide bridges linking its extracellular domains, the $P2Y_{12}$ receptor has two free cysteines in its extracellular domains (Cys17 and Cys270), both of which are targets of thiol reagents [55,56].

Ticlopidine and clopidogrel are irreversible $P2Y_{12}$ antagonists and have been repeatedly proven as clinical antithrombotic agents [46,57,58]. In addition, another $P2Y_{12}$ antagonist, prasugrel (CS-747), a new thienopyridine derivative that is ~10 times more potent than clopidogrel, shows promise as a future

antithrombotic drug [59–61]. The active metabolites, containing a free thiol group, of clopidogrel and ticlopidine form disulfide bridges with both Cys17 and Cys270 in the $P2Y_{12}$ receptor, and thereby inactivate the receptor. Prasugrel also neutralizes the $P2Y_{12}$ receptor by the same mechanism. Differently, the $P2Y_1$ receptor is insensitive to the thiol reagents and to the active metabolites of clopidogrel, ticlopidine, and CS-747 because of the lack of free cysteines in the extracellular domains [55,56,58].

Therefore, we cannot rule out that disulfiram might act in the same manner as well, forming an initial mixed disulfide with the two free thiol groups of the $P2Y_{12}$ receptor, stimulating platelet aggregation. However, more work is needed to further identify the PSSG involved in the inhibition of platelet aggregation.

In conclusion, the present study, to the best of our knowledge, provides the first evidence that disulfiram causes imbalance of the intraplatelet glutathione redox state and induces reversible protein S-glutathionylation. Furthermore, in accordance with previous data [23], it indicates that protein S-glutathionylation attenuates platelet aggregation, whereas alterations in the GSH/GSSG ratio do not affect platelet aggregation.

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