BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00962.x
www.brjpharmacol.org

RESEARCH PAPER

Dapsone hydroxylamine induces premature removal of human erythrocytes by membrane reorganization and antibody binding

Luciana Bordin[1], Cristina Fiore[2], Francesco Zen[3], Michael D Coleman[4], Eugenio Ragazzi[5] and Giulio Clari[1]

[1]*Department of Biological Chemistry, University of Padova, Padova, Italy,* [2]*Endocrinology Unit – Department of Medical and Surgical Sciences, University of Padova, Padova, Italy,* [3]*Laboratory of Clinical Chemistry and Hematology, San Bortolo Hospital, Vicenza, Italy,* [4]*Mechanisms of Toxicity Group, Aston University, Birmingham, UK, and* [5]*Department of Pharmacology and Anesthesiology, University of Padova, Padova, Italy*

Correspondence
Luciana Bordin, Department of Biological Chemistry, University of Padova, Viale G. Colombo, 3, 35121 Padova, Italy. E-mail: luciana.bordin@unipd.it

Keywords
DDS-NHOH; human erythrocyte membrane alterations; band 3 Tyr-phosphorylation; band 3 aggregates; autologous IgG binding

Received
20 October 2009
Revised
23 June 2010
Accepted
30 June 2010

BACKGROUND AND PURPOSE

N-hydroxylation of dapsone leads to the formation of the toxic hydroxylamines responsible for the clinical methaemoglobinaemia associated with dapsone therapy. Dapsone has been associated with decreased lifespan of erythrocytes, with consequences such as anaemia and morbidity in patients treated with dapsone for malaria. Here, we investigated how dapsone and/or its hydroxylamine derivative (DDS-NHOH) induced erythrocyte membrane alterations that could lead to premature cell removal.

EXPERIMENTAL APPROACH

Erythrocytes from healthy donors were subjected to incubation with dapsone and DDS-NHOH for varying times and the band 3 protein tyrosine-phosphorylation process, band 3 aggregation, membrane alteration and IgG binding were all examined and compared with erythrocytes from two patients receiving dapsone therapy.

KEY RESULTS

The hydroxylamine derivative, but not dapsone (the parent sulphone) altered membrane protein interactions, leading both to aggregation of band 3 protein and to circulating autologous antibody binding, shown in erythrocytes from patients receiving dapsone therapy. The band 3 tyrosine-phosphorylation process can be used as a diagnostic system to monitor membrane alterations both *in vitro*, assessing concentration and time-dependent effects of DDS-NHOH treatment, and *in vivo*, evaluating erythrocytes from dapsone-treated patients, in resting or oxidatively stimulated conditions.

CONCLUSIONS AND IMPLICATIONS

DDS-NHOH-induced alterations of human erythrocytes can be directly monitored *in vitro* by tyrosine-phosphorylation level and formation of band 3 protein aggregates. The latter, together with antibody-mediated labelling of erythrocytes, also observed after clinical use of dapsone, may lead to shortening of erythrocyte lifespan.

Abbreviations

cdb3, cytoplasmic domain of band 3; DDS, dapsone; DDS-NHOH, dapsone hydroxylamine; G6PD, glucose 6 phosphate dehydrogenase; GSH, glutathione; GSSG: oxidized glutathione; HMWA, high molecular weight aggregate; RBC, red blood cell; Tyr-P, tyrosine phosphorylation

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Introduction

Dapsone has been used for over half a century in the treatment of leprosy, for anti-inflammatory conditions and, in the chlorproguanil-dapsone and artesunate–dapsone–proguanil combinations, for treating malaria. It is also a second-line treatment for AIDS-related *Pneumocystis* pneumonia (Sangiolo *et al.*, 2005), and is increasingly applied to a variety of immunologically related conditions (Bahadir *et al.*, 2004; Ujiie *et al.*, 2006), despite its well-documented toxicity, which is closely related to its routes of biotransformation. Mono- and diacetylated metabolites of dapsone (MADDS and DADDS) are not associated with toxicity (Coleman *et al.*, 1991), although N-hydroxylation of the parent drug and MADDS lead to the formation of the toxic hydroxylamines DDS-NHOH and MADDS-NHOH (Israili *et al.*, 1973; Coleman *et al.*, 1989). These species, formed either by one isoform of the cytochrome P450 (CYP) family, CYP2C9 (Winter *et al.*, 2000), or other oxidative enzyme systems, are linked with several immune-mediated hypersensitivity reactions (Vyas *et al.*, 2006). The hydroxylamines are also responsible for the clinical methaemoglobinaemia associated with dapsone therapy (Israili *et al.*, 1973; Schiff *et al.*, 2006). DDS-NHOH can also induce erythrocyte removal in rat (Grossman *et al.*, 1995; McMillan *et al.*, 1995) and morphological alteration in human erythrocytes (McMillan *et al.*, 1995). The drug has long been associated with shortening of the erythrocytic lifespan in man (Cream, 1970; Byrd and Gelber, 1991) and this remains a clinical problem (Wertheim *et al.*, 2006). Indeed, anaemia is already a major cause of mortality and morbidity in areas where dapsone is used to treat malaria (Gonazales *et al.*, 2000).

DDS-NHOH undergoes a coupled oxidation-reduction reaction with haemoglobin and molecular oxygen yielding methaemoglobin and reactive oxygen species (ROS) (ferryl haem and hydroxyl radicals), respectively (Bradshaw *et al.*, 1997). To date, no direct evidence of the mechanism whereby DDS-NHOH shortens the erythrocytic lifespan has been reported. Only a possible effect of DDS-NHOH on the integrity of the erythrocytic lipid bilayer has been excluded, since neither lipid peroxidation nor phosphatidylserine externalization has ever been detected (McMillan *et al.*, 1998; 2005).

In human erythrocytes, tyrosine phosphorylation (Tyr-P) of membrane proteins mainly involves the band 3 protein. This is the most abundant membrane protein of red blood cells (RBC) and is divided into three regions: an external domain, enriched in glycosyl chains that probably allow band 3 protein to be recognized as a specific antigen (Bratosin *et al.*, 1998); a transmembrane domain, representing the anionic exchanger of cells; and a cytosolic portion (Wang, 1994) containing all the sites of potential phosphorylation. Although serine/threonine (Ser/Thr)-phosphorylation of the band 3 cytosolic domain has been demonstrated to regulate the anion flux rate (Baggio *et al.*, 1993a,b), Tyr-P is involved in multiple functions, including regulation of glycolysis (Low *et al.*, 1993), alteration of erythrocyte morphology (Bordin *et al.*, 1995) and volume (Musch *et al.*, 1999), and senescence (Bordin *et al.*, 2009; Pantaleo *et al.*, 2009).

We have previously demonstrated that the level of band 3 Tyr-P can be useful as a diagnostic system in analysing erythrocyte membrane status. When triggered by oxidative (diamide) or hyperosmotic stress, the band 3 Tyr-P level can predict both pathological and particular physiological conditions in red blood cells. In glucose-6-phosphate dehydrogenase (G6PD) deficiency, the higher band 3 Tyr-P level, compared with normal control cells, correlates well with chronic impairment of cell anti-oxidative defences (Bordin *et al.*, 2005b); conversely, the lower band 3 Tyr-P level observed in pregnancy is synonymous of characteristically increased anti-oxidative defences (Bordin *et al.*, 2006).

Our present data indicated that DDS-NHOH induced progressive alterations in erythrocytes, starting from cytosol, where it induces methaemoglobin formation (Israili *et al.*, 1973; Schiff *et al.*, 2006), to glutathione oxidation initial impairment of Tyr-protein kinase and then impairment of phosphatase activity. Later, the effect of DDS-NHOH progressed, with reorganization of membrane proteins, as shown by enzyme recruitment and the formation of high molecular weight aggregates (HMWA) of band 3 protein. Lastly, general membrane reorganization was achieved, with protein relocation from the Triton-soluble compartment to the cytoskeleton and with autologous antibody recognition. Notably, the same membrane reorganizations, as observed in cells treated *in vitro*, were found in the membranes of cells from two dapsone-treated patients, more markedly in the patient with glucose 6 phosphate dehydrogenase (G6PD) deficiency.

Methods

Treatment of erythrocytes

Following informed consent, erythrocytes were obtained from fresh blood collected from healthy volunteers, and from two middle-aged male patients, who were considered for dapsone therapy in the hospital's dermatological department. The choice of therapy was not influenced by the study

design; the two patients were diagnosed as suffering from dermatitis herpetiformis according to skin biopsies and cell surface deposition of IgA, and were given oral dapsone. At admission, both had normal blood and urine samples. Their treatment started with 100 mg·day^{-1} dapsone, the usual dose (Leonard and Fry, 1991).

Patient 1 remained successfully in treatment for the length of the study; blood was withdrawn before and during dapsone administration (after 14 days' treatment). (Relative blood values are listed in Table S1)

Patient 2 was hospitalized for a haemolytic episode following 3 days of 100 mg·day^{-1} dapsone ($P2_{100}$). His laboratory tests revealed that he had a deficiency in G6PD, class II, according to the WHO directive (Betke *et al.*, 1967). G6PD residual activity in red cells was <10%, measured spectrophotometrically at 340 nm on a Sigma diagnostic kit (Sigma-Aldrich, Italy). Dapsone was discontinued for a month, after which laboratory test results had returned to normal range. Dapsone treatment was later re-administered, starting with 2 days with 30 mg·day^{-1}, and then 50 mg·day^{-1}, with partial relief but not total remission of symptoms.

Blood samples from both patients were taken before and during treatments. Samples from patient 1 were called P1 and $P1_{100}$ to indicate samples before administration and during 100 mg·day^{-1} dapsone treatment; RBC from patient 2 were called P2, $P2_{30}$ and $P2_{50}$ to indicate samples withdrawn before and after 2 days at 30 mg·day^{-1}, or after 3 days at 50 mg·day^{-1} dapsone, respectively.

Human erythrocytes were recovered and pelleted at 3000× *g* for 3 min (Brunati *et al.*, 2000). After removal of the supernatant, packed RBC were washed three times at 3000× *g* for 3 min in nine volumes of Dulbecco's phosphate-buffered saline, containing 5 mM glucose (D-PBS), to avoid contamination by leukocytes and platelets.

For *in vitro* evaluation of the effects of dapsone and DDS-NHOH on normal erythrocytes, packed cells (50 µL) were resuspended (at 20% haematocrit) in D-PBS and incubated at 35°C for varying times in the presence or absence of increasing concentrations (from 0.15 to 0.6 mM) of dapsone or DDS-NHOH (or acetone as solvent). Parallel experiments were carried out in the same conditions but at 50% haematocrit in platelet poor-plasma (P-PP), diluted to 66% in D-PBS. In this case, blood was centrifuged at 180× *g* for 10 min, and the supernatant was further centrifuged at 1500× *g* for 15 min to obtain P-PP (Ciccoli *et al.*, 2004); RBC were purified as described above.

After incubation, each sample was centrifuged at 3000× *g*, and packed cells were washed in D-PBS and subjected to haemolysis in 1.5 mL of hypotonic buffer containing 5 mM sodium phosphate, pH 8; 0.02% NaN_3, 30 µM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, and a protease inhibitor cocktail.

For evaluation of RBC from patients receiving dapsone therapy, packed cells were incubated for 30 min at 35°C in D-PBS (20% haematocrit) in the presence or absence of 0.3 mM diamide, and then subjected to haemolysis as described above.

Membranes were separated from the cytosol by centrifugation (17 000 × g for 20 min) and washed once in hypotonic buffer. Aliquots of membranes (10 µg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gels) in reducing conditions unless otherwise indicated, transferred to nitrocellulose membranes, and immunostained with the appropriate antibody.

Preparation of band 3 proteolytic fragment (cdb3)

The 40/45-kDa fragment of the cytoplasmic domain of band 3 (cdb3) was obtained by α-chymotrypsin-promoted breakdown of inverted membrane vesicles derived from ghosts and isolated by DE 52 chromatography, according to Bennett and Stenbuck, (1980).

[^{32}P]-cdb3 was prepared by incubating 30 µg cdb3 for 10 min at 30°C, in 300 µL of the same incubation mixture as described below for the kinase assay except that the cell lysates were substituted by Syk immunoprecipitated (Syk-IP) protein from 3 mL of normal cell cytosol (Brunati *et al.*, 2000). [^{32}P]-cdb3 was recovered as supernatant after centrifuging 6 min at 6000× g, lyophilized, resuspended in imidazole buffer (25 mM, pH 7, mercaptoethanol 10 mM, dithiothreitol (DTT) 4 mM), and dialysed with the same buffer.

Protein kinase assay

Packed treated erythrocytes were subjected to several freeze–thaw cycles to yield a homogeneous cell lysate. 3 µL of each were assayed for tyrosine kinase activity at 30°C for 10 min in a 30-µL incubation mixture containing 50 mM Tris HCl, pH 7.5, 10 mM $MnCl_2$, 30 µM (γ-^{32}P)ATP (specific activity 1000 cpm·pmol^{-1}), 10 µM vanadate and 3 µg of cdb3 as tyrosine kinase activity substrate. After incubation, the reaction was stopped by the addition of 1% SDS and 1% β-mercaptoethanol (final concentrations), and heated for 5 min at 100°C. Solubilized proteins were subjected to SDS/(10%)PAGE, and the gels were stained with Coomassie blue and treated with 2 M NaOH for 60 min at 55°C to highlight Tyr-P.

Dried gels were autoradiographed for 2 days. Bands corresponding to [^{32}P]-cdb3 were then excised and counted in a liquid scintillation counter.

Protein phosphatase activity

Three µL of the total cell lysate obtained as above were assayed for tyrosine protein phosphatase activity in a 30-µL incubation mixture containing imidazole buffer and 3 µg [^{32}P]-cdb3, at 30°C for 10 min.

The reaction was stopped as described above and solubilized proteins were subjected to SDS-PAGE (10% gels). The gels were stained, dried and autoradiographed, and [^{32}P]-cdb3 was counted in a liquid scintillation counter.

Preparation of membrane skeletal and soluble fractions

Membranes, obtained from 200 µL of treated and untreated erythrocytes and recovered as described above, were extracted with 2 volumes of buffer A containing 50 mM Tris, pH 7.5, 1% (final) Triton X-100, 1 mM vanadate and protease inhibitor cocktail for 1 h at 4°C. After removal of one aliquot for Western blot analysis, the remainder was microfuged at 80 000× *g* for 40 min. Both supernatant, corresponding to the Triton-soluble fraction, and pellet, corresponding to the Triton-insoluble fraction (cytoskeleton), were then collected, and the pellet was resuspended to the same soluble fraction volume with buffer A. 10 µg of total membrane and the corresponding soluble and cytoskeleton fractions were then subjected to Western blot analysis and revealed with anti-band 3, anti-Syk or anti-SHP-2 antibodies.

Quantitative determination of total glutathione (GSSG+GSH) and oxidized glutathione (GSSG) content in erythrocytes

Total glutathione was determined according to the method of Tietze (1969). Briefly, 10 µL of cytosol, obtained from differently treated erythrocytes, was added to 2 mL of reaction mixture containing 1.9 mL of phosphate 0.1 M/ EDTA 0.6 mM buffer, pH 7.4, 30 µL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) 10 mM, 100 µL of NADPH 5 mM and glutathione reductase 10 µg, and analysed spectrophotometrically at 412 nm. The GSSG content was evaluated in 10-µL cytosol incubated in the glutathione assay mixture, to which 3 µL of 2-vinylpyridine (2-VP) was added (Teare *et al.*, 1993). The level of GSH was determined by calculating the difference between the two determinations.

Data analysis

Data are presented as means ± SEM of at least three individual experiments from six different blood donors. One-way or two-way ANOVA was used to analyze the variables under investigation. Following a significant ANOVA result, *post hoc* statistical analyses were carried out by the Tukey's honestly significant difference (HSD) test (Ruxton and Beauchamp, 2008). Differences were considered significant at $P < 0.05$.

Materials

For *in vitro* work, dapsone was supplied by Aldrich Chemistry (Milano, Italy), whereas for dapsone therapy, it was supplied by St. Antonio Bissone SA Pharmacy (Bissone/TI, Switzerland). D-PBS (Dulbecco's phosphate-buffered saline, containing 5 mM glucose), anti-P-Tyr and anti-Syk monoclonal antibodies were purchased from Sigma (Milan, Italy) and Upstate (Lake Placid, NY), respectively. Rabbit anti-SHP-2 (C-18) polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protease inhibitor cocktail was obtained from Calbiochem (Darmstadt, Germany). [γ-^{32}P]-ATP was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK), and dapsone hydroxylamine (DDS-NHOH) from Toronto Research Chemicals Inc. (North York, Ontario). Anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase (HRP) were from Bio-Rad Laboratorories (Hercules, California), anti-human IgG-HRP was purchased from Biodesign (TEMA Ricerca, Bologna, Italy). All other reagents were from Sigma.

Results

The action of DDS-NHOH was tested by incubating human erythrocytes with increasing concentrations of the hydroxylamine for 30 min. In these conditions, the membrane proteins, mainly band 3, exhibited Tyr-P, which increased concentration dependently up to 0.3 mM DDS-NHOH and drastically decreased to the control level at higher concentrations. However, the parent compound, dapsone, was not able to trigger erythrocyte band 3 Tyr-P at any concentration (Figure 1).

Comparing the ability of DDS-NHOH to induce band 3 Tyr-P with that of another oxidant, diamide, the maximum Tyr-P-level for DDS-NHOH (at 0.3 mM) was close to that obtained with 1 mM diamide.

Band 3 P-Tyr level and enzyme recruitment

To better characterize DDS-NHOH-induced alterations in human erythrocytes, we tested the same hydroxylamine concentrations described above at increasing times of incubation. As shown in Figure 2, DDS-NHOH induced band 3 Tyr-P which peaked after 30 min at 0.3 mM, and was completely

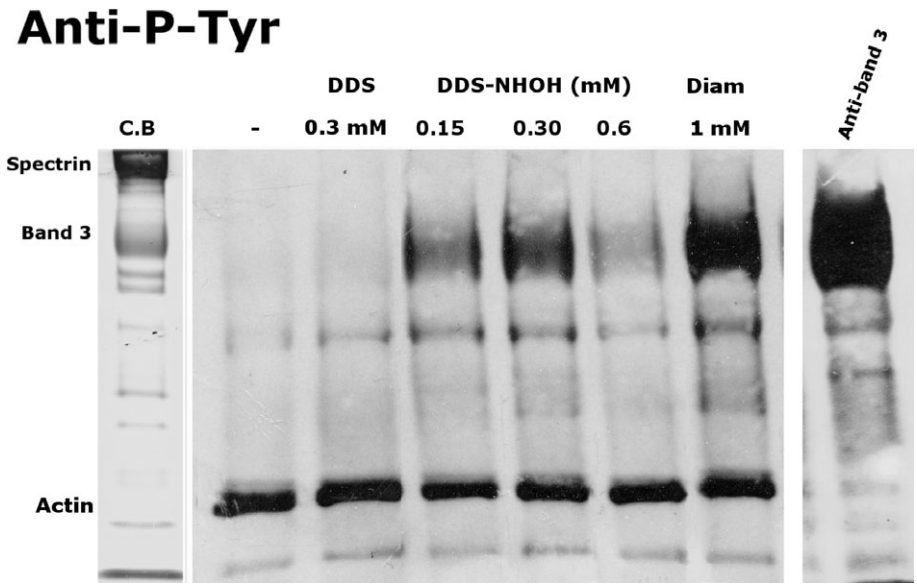

Figure 1

Erythrocytes were incubated with increasing concentrations of dapsone (0.15–0.6 mM), all ineffective in triggering band 3 Tyr-P (and thus only shown in one lane) or DDS-NHOH. Membranes (10 μg), obtained as described in Methods, were analysed by Western blotting and revealed with anti-actin for loading control and, successively, with anti-P-Tyr antibodies. Membrane band 3 P-Tyr pattern obtained from erythrocytes treated with 1 mM diamide (Diam) was used to compare band 3 Tyr-P level. Lane C.B. Coomassie Blue staining gel. Lane Anti-band 3 shows the last lane after being stripped and reprobed with anti-band 3 antibody. Results shown are representative of six separate experiments.

reversed after 45 min of incubation. This response was distinguishable from that previously observed with diamide, represented by concentration-dependent increase of band 3 Tyr-P (Bordin *et al.*, 2005a). A relatively low concentration of DDS-NHOH (0.15 mM) yielded effects after 15 min, but to a lower extent, and decreased more rapidly in 30 min of incubation. 0.6 mM only induced a slight band 3 Tyr-P level that almost completely disappeared in 30 min of incubation (panel A).

However, when analysed with anti-Syk (panel B) or anti-SHP-2 (panel C) antibodies, membranes revealed concentration and time-dependent recruitment of both enzymes (Figure S1). The content of both enzymes, after incubation with 0.6 mM, were almost four times greater than those in the control.

DDS-NHOH effect on enzyme activities

Tyr-kinase and phosphatase activities expressed by the cells after these treatments are shown in Table 1. For this purpose, erythrocytes were incubated with increasing DDS-NHOH concentrations and incubations were stopped at various incubation times. Cells were subjected to several freeze–thaw cycles as described in the Methods, and activities were assayed with cdb3 or ^{32}P-cdb3 as substrates for Tyr-kinases or Tyr-P-phosphatases, respectively. We found that both Tyr-kinase and phosphatase activities were promptly inhibited by DDS-NHOH, both concentration- and time-dependently and total inactivation was reached in both after 60 min incubation with 0.15 and 0.3 mM. At 0.6 mM, DDS-NHOH treatment produced almost complete inhibition after only 15 min of incubation.

Effect of DDS-NHOH on band 3 aggregation

In a previous study, we proposed band 3 alterations as biomarkers able to reveal the status of membranes. Band 3 aggregated in high molecular weight aggregate (HMWA) complexes following diamide treatment (oxidative stress), but disaggregated in hypertonic conditions (hyperosmotic stress) (Bordin *et al.*, 2006). We therefore analysed membranes from DDS-NHOH treated erythrocytes with anti-band 3 antibodies.

As shown in Figure 3, DDS-NHOH induced net band 3 reorganization, shown by the appearance of a band at 180–200 kDa, forming at DDS-NHOH concentrations higher than 0.15 mM (panel A). In addition, this rearrangement became more marked as incubation time was prolonged (panel B), thus partially confirming the hypothesis of a progression of the DDS-NHOH effect.

DDS-NHOH-induced reorganization of cytoskeleton

To better evaluate band 3 aggregation, band 3 HMWA location in membrane organization was

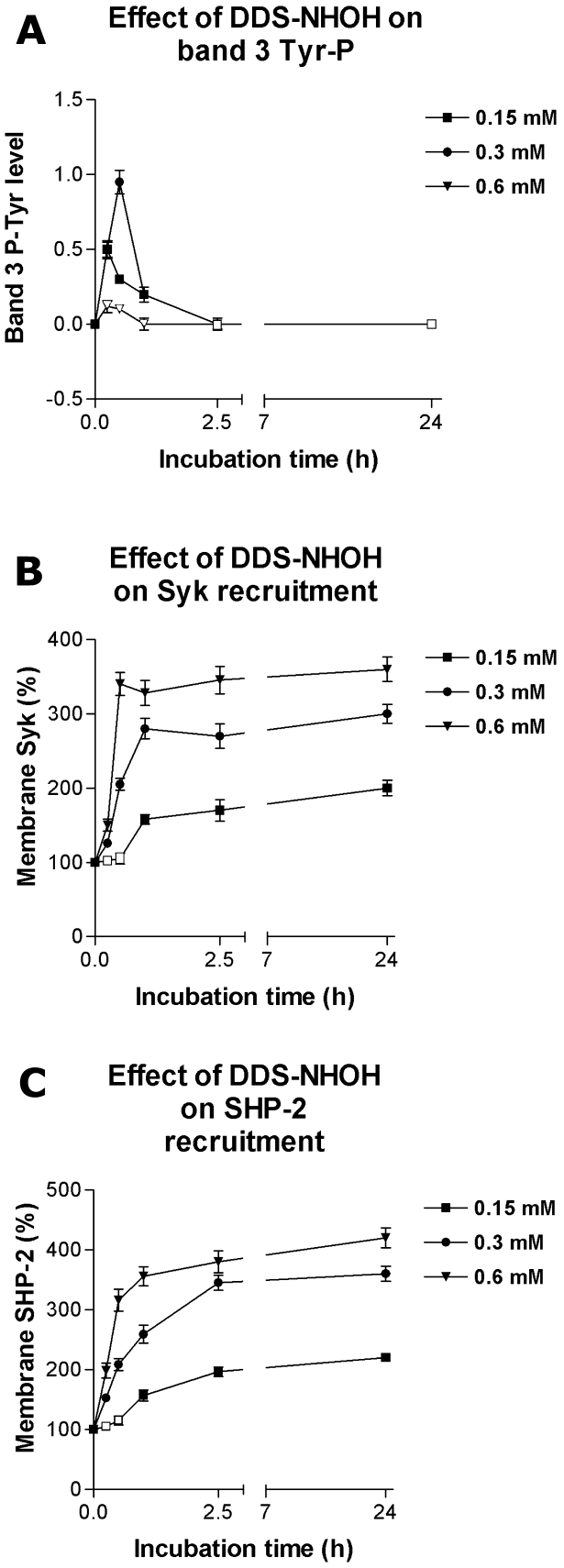

Figure 2

Concentration- and time-dependent effect of DDS-NHOH on the membrane Tyr-phosphorylative process. Erythrocytes were treated with increasing DDS-NHOH concentrations and incubated at 35°C. Aliquots were taken at various incubation times, and membranes (10 μg), obtained as described in Methods, were analysed by Western blotting and revealed with anti-P-Tyr (panel A), anti-Syk (panel B) or anti-SHP-2 (panel C) antibodies. Densitometric analysis. Bands corresponding to band 3-Tyr-P, Syk and SHP-2 were counted in a densitometer. Amounts of Syk and SHP-2 in unstimulated erythrocytes were chosen as 100% value. For band 3-Tyr-P evaluation, an arbitrary unit was chosen, corresponding to band 3-Tyr-P level obtained with 1 mM diamide. Results represent means ± SEM of four separate experiments in duplicate. In each experiment, data analysis was carried out by two-way ANOVA, giving significant differences among concentrations in the time course of experiment ($P < 0.001$). *Post-hoc* Tukey's test was performed; filled symbols indicate significant difference versus respective basal value ($P < 0.01$).

studied. Cells were treated with 0.3 mM DDS-NHOH for 30 min – conditions which induced the highest band 3 Tyr-P level – and for prolonged incubation times – when band 3 Tyr-P level was absent. Corresponding membranes were extracted with Triton X-100 and the membrane-soluble compartment was separated from the Triton-insoluble cytoskeleton (Bordin *et al.*, 2005a). The pattern of Figure 4 shows that, following 30 min DDS-NHOH treatment, the HMWA content of band 3 markedly increased (about five times that of the control), but only in the Triton-soluble fraction, whereas the corresponding cytoskeleton (Triton-insoluble fraction) showed the same amount of band 3 HMWA as the control. By prolonging incubation time, not only was the content of band 3 HMWA further increased (up to about seven times the control), but complete membrane reorganization also occurred, the cytoskeleton containing almost all the band 3 HMWA complexes formed, including some of those previously present in the soluble fraction.

When erythrocytes were treated with 0.6 mM DDS-NHOH the band 3 HMWA were detected almost completely in the cytoskeleton even after 30 min incubation (data not shown).

Effect of DDS-NHOH on enzyme redistribution

Following the rearrangement of band 3 HMWA complexes within RBC membranes, we also analysed enzyme distribution between Triton-soluble and insoluble fractions of the membranes (Figure 5). Interestingly, both Syk (panel A) and SHP-2 (panel B) were engaged by DDS-NHOH to the cytoskeleton, while the amounts of both enzymes present in the membrane extract (Triton-soluble fraction) remained similar to that present in the control Triton-soluble fraction. Only treatment with 0.3 mM

Table 1

Tyr-protein kinase and phosphatase activities

	Tyr-kinase activity (% cpm on cdb3)	Tyr-phosphatase activity (% on ^{32}P-cdb3)
Control	100	100
0.15 mM DDS-NHOH 15 min	95 ± 2 *(ns)*	90 ± 5 *(ns)*
30 min	80 ± 4*	75 ± 7*
60 min	n.d.	n.d.
2 h30 min	n.d.	n.d.
0.3 mM DDS-NHOH 15 min	70 ± 2*	74 ± 3*
30 min	53 ± 4*	50 ± 5*
60 min	n.d.	n.d.
2 h30 min	n.d.	n.d.
0.6 mM DDS-NHOH 15 min	13 ± 4*	15 ± 4*
30 min	n.d.	n.d.
60 min	n.d.	n.d.
2 h30 min	n.d.	n.d.

n.d. not detected.

Erythrocytes were treated with increasing concentrations of DDS-NHOH and recovered by centrifuging at 3000 g for 3 min. Packed cells were subjected to several freeze–thaw cycles and aliquots (3 μL) were incubated with purified cdb3 in presence of (γ-^{32}P)ATP for kinase activity or [^{32}P]-cdb3 for PTP-ase activity, as described in Methods. Values are expressed as % means ± SEM of corresponding control (n = 3). * $P < 0.05$, significantly different versus control (ANOVA followed by Tukey's test); *ns,* not significant.

DDS-NHOH for 30 min incubation showed an increase (about 30% more than in the control) of the amount of Syk present in the Triton-soluble fraction. As these were the conditions for the maximum level of band 3 Tyr-P, the favoured substrate-kinase combination resulting in the band 3 Tyr-phosphorylating process may be ascribed to this Syk recruitment to the soluble fraction.

DDS-NHOH-induced autologous antibody binding

Removal of human erythrocytes involves cells undergoing significant alterations, induced either by normal ageing or by toxic/disease injury, and is mediated by antibody recognition (Lutz *et al.*, 1987; Bratosin *et al.*, 1998; Arese *et al.*, 2005; Kay, 2005).

When we analysed membranes from erythrocytes exposed to concentration- and time-dependent DDS-NHOH treatments in the presence of autologous plasma (P-PP), immunostaining with anti-human IgG revealed net enhancement of the number of autologous antibodies with increasing both DDS-NHOH concentration (Figure 6, panel A) and incubation time (panel B).

Interestingly, in this case too, the progression of the DDS-NHOH effect was clearly distinguishable, with an initial slight antibody recognition which occurred only at 0.3 and 0.6 mM, and was increased greatly after 24 h treatment.

Membrane reorganization, IgG binding and phosphorylative process in RBC from patients receiving dapsone therapy

In order to confirm that the DDS-NHOH-induced membrane reorganization described above was the mechanism effectively leading to RBC denaturation/removal *in vivo*, we analysed membranes from two patients receiving dapsone therapy for dermatitis herpetiformis. Quantitative analysis obtained by densitometric scanning of the anti-band 3 and anti-IgG immune-blots, evidenced that dapsone therapy in patient 1 (P1) induced a slight increase in band 3 HMWA (12.3 ± 0.9%) (Figure 7, lane b compared with lane a, representing P1 before dapsone), which was correlated with an increase in bound IgG (11 ± 0.8%) (Figure 7, anti-human IgG panel). Instead, patient 2, characterized by G6PD deficiency, was clearly intolerant to the same 100 mg·day^{-1} oral dapsone (Table S1) so that the dose was reduced to 30 mg·day^{-1} and then to 50 mg·day^{-1}. However, even when corrected and reduced, dapsone was not completely well tolerated by P2, who showed slight signs of intolerance (Table S1). RBC membranes from patient 2 showed a higher level of band 3 HMWA (Figure 7, anti-band 3 panel) even before dapsone (P2) (lane c). This band 3 aggregation increased (22 ± 0.8%) during the 30 mg·day^{-1} dapsone ($P2_{30}$, lane d), but reached a dramatic level at 50 mg·day^{-1} ($P2_{50}$, lane e). The effect was

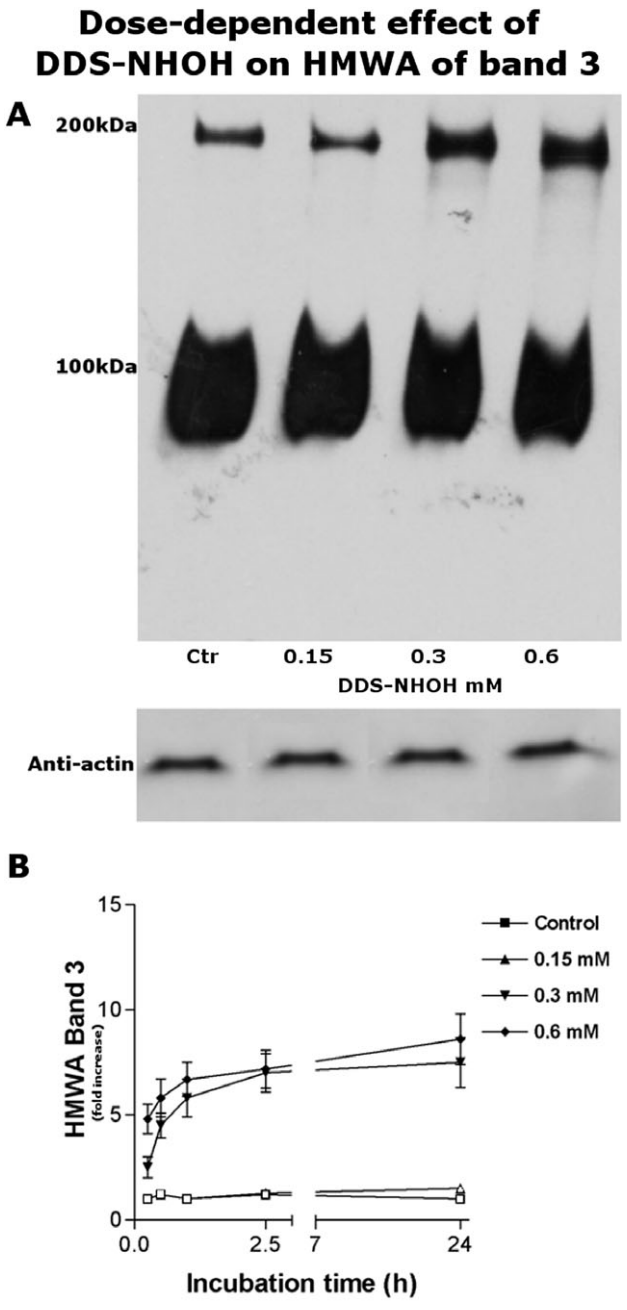

Figure 3

Erythrocytes were treated with increasing concentrations of DDS-NHOH or acetone (control) for 30 min (panel A), or for various incubation times (panel B). Membranes (1 μg), recovered as described in Methods, were analysed by SDS/8%PAGE in non-reducing conditions, blotted to nitrocellulose paper and revealed with anti-band 3 antibodies (anti-actin as loading control). A is representative of six separate experiments. B. Densitometric scanning of band 3 HMWA provided the values shown, expressed as fold-increase over the amount of band 3 HMWA in unstimulated cells. Results represent means ± SEM of six separate experiments in duplicate. Data analysis was carried out by two-way ANOVA, indicating significant differences among concentrations in the time course of experiment ($P < 0.001$). *Post-hoc* Tukey's test was performed; filled symbols indicate significant difference versus respective basal value ($P < 0.01$).

correlated with a 30% increase in bound IgG in $P2_{30}$ and with more than 200% in $P2_{50}$ (Figure 7, anti-human IgG panel, lanes d, e, compared with lane c, corresponding to P2 before dapsone).

In addition, when analysed for their Tyr-P level extent, membranes from RBC of both patients showed that the basal level of band 3 Tyr-P was negligible (Figure 8, lanes a, c, e–g). Successive analysis of glutathione content (Table 2) disclosed that dapsone treatment induced a decrease in total GSH content in both patients. However, whereas $P1_{100}$ maintained about 85% of total glutathione in the reduced form (GSH), P2 showed progressive depletion of glutathione, with an alarming rise in GSSG which, at $P2_{50}$, reached almost 60% of total glutathione. When erythrocytes from both patients were subjected to 0.3 mM diamide to induce oxidative stress, $P1_{100}$ showed a reduction in total glutathione content and a rise of GSSG. P2 and $P2_{30}$ also displayed a net reduction in the amount of total glutathione which at $P2_{50}$ was only 50%, compared with the glutathione content of P2. The diamide-induced net increase in the GSSG form reached almost 100% glutathione at $P2_{50}$.

Interestingly, when RBC were incubated with 0.3 mM diamide (conditions that triggered band 3 Tyr-P in G6PD deficient patients, but not in normal subjects; (Bordin *et al.*, 2006), $P1_{100}$ exhibited a slight trace of band 3 Tyr-P. In contrast, P2 showed net band 3 Tyr-P (Figure 8, lane h) – as expected, due to his G6PD deficiency, which dramatically escalated on increasing dapsone treatment (lanes i, j). Syk and SHP-2 content in membranes from P2 also rose after dapsone, in both the absence (lanes e-g) and presence (lanes h-j) of diamide incubation.

Discussion and conclusions

Oxidative stress occurs when ROS, which are intermediaries of normal oxygen metabolism, are produced faster than the endogenous antioxidant defence systems can neutralize them. The excessive production of ROS can damage proteins, lipids, nucleic acids and matrix components in a number of cells, and is often related to both metabolic and inflammatory diseases. We have recently shown that, in G6PD deficient erythrocytes, characterized by chronic lowering of anti-oxidant defences, membrane status seems similar to that of control cells, but diamide-induced oxidative assault results in an abnormal increase in the Tyr-P level of membrane band 3 (Bordin *et al.*, 2005b). Similarly, in endometriosis, typified by excessive ROS production due to inflammatory status, analysis of the diamide-induced abnormally higher erythrocyte band 3 Tyr-P

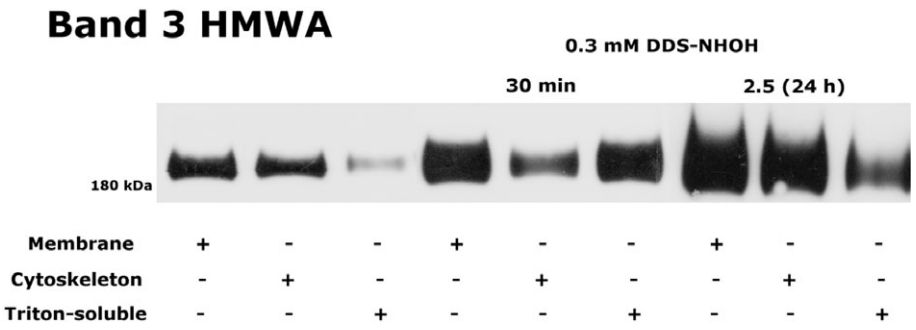

Figure 4

Erythrocytes were treated with 0.3 mM DDS-NHOH or solvent, for 30 min or prolonged time incubation (2 h30 min and 24 h, which showed similar patterns) as indicated. Membranes (10 μg) were extracted with Triton X-100. Membranes, Triton-soluble or –insoluble (cytoskeleton) fractions, collected by ultracentrifugation (see Methods) were analysed by SDS/8%PAGE in non-reducing conditions and revealed with anti-band 3 antibodies. Results shown are representative of five separate experiments.

level can discriminate endometriotic from non-endometriotic patients. In addition, the sensitivity of this parameter also allows it to detect post-surgical systemic improvement, also represented by decreased diamide-induced Tyr-P of band 3 (Bordin *et al.*, 2010).

Diamide is known to trigger band 3 Tyr-P by partially inhibiting Tyr-protein phosphatases (Bordin *et al.*, 2005a) and by inducing disulfide bond formation, leading to band 3 aggregation (Fiore *et al.*, 2008). The band 3 Tyr-phosphorylative mechanism has also been considered in the evaluation of DDS-NHOH-induced erythrocyte alterations. That DDS-NHOH did not act as an oxidant like diamide was indicated by the fact that SHP-2 recruitment (Figure 2C) was not Tyr-P-dependent. This observation represents a novel event for SHP-2 recruitment because we had previously observed that diamide-induced SHP-2 translocation to membranes occurred only after Lyn-catalysed phosphorylation of band 3 residue 359, which is the docking site for this phosphatase (Bordin *et al.*, 2002). DDS-NHOH modifies membranes in such a way as to uncover new docking sites for SHP-2. Together with the massive Syk translocation, which is indifferent to Tyr-P level (Bordin *et al.*, 2005a), SHP-2 relocation can also be used as a further parameter of the altered status of membranes.

That this compound affected oxidative status of the cell was previously demonstrated by its ability to induce oxyhaemoglobin, GSH content decrease and conversion to oxidized glutathione (GSSG) and GS-S-protein (Table 2) through a ROS-generating system, yielding nitrosoarene and methaemoglobin (McMillan *et al.*, 1995; 2005). A number of active oxygen species and sulphur-free radicals are produced as a consequence of this redox cycle and are certainly more or less involved in the erythrocyte damage leading to their removal from circulation. This damage is indicated by the time- and concentration-dependent increase of IgG binding to the membranes, as an indicator of reduced erythrocyte lifespan (Lutz *et al.*, 1987; Arese *et al.*, 2005; Kay, 2005).

We observed that DDS-NHOH triggered Tyr-P of band 3 protein in intact erythrocytes. This was expected by an oxidant, such as diamide, directly oxidising cysteine located in the catalytic domain of the enzyme (Hecht and Zick, 1992) and thus inhibiting protein tyrosine phosphatase activities. In 30 min incubation, DDS-NHOH induces band 3 aggregation in HMWA mainly located in the Triton-soluble part of the membrane.

With increasing incubation times, the effects of DDS-NHOH were clearly differentiated from those of diamide. These effects of DDS-NHOH were amplified, leading to further increases in band 3 HMWA, but, more interestingly, also to their total relocation within the membrane, accompanied by reorganization of both recruited Tyr-protein kinases and phosphatases, which were markedly inhibited (Table 1). This new membrane organization was easily recognized and marked by autologous IgG, and is representative of damaged cells.

In addition, since band 3 Tyr-P gradually tails off within the first 45 min (Figure 2), which may represent the time threshold between the formation of two differently located band 3 aggregates – Triton-soluble, and, successively, cytoskeleton bound – we can postulate that the Tyr-phosphorylative process is a cellular defence against the incoming oxidative modifications induced by DDS-NHOH. In this process, introduction of negative charges, represented by phosphate groups, to band 3 protein would slow down its aggregation, at least up to the total arrest of the phosphorylative process. Subse-

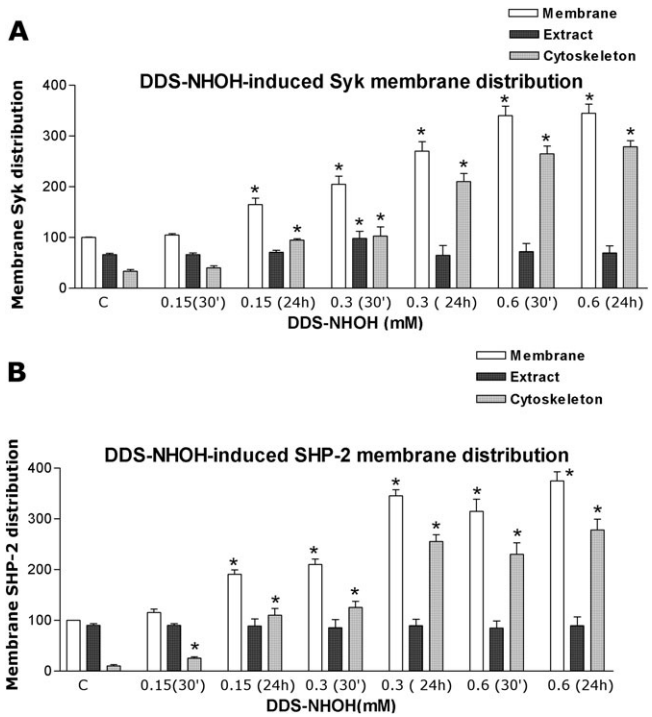

Figure 5

Enzyme distribution following DDS-NHOH treatment. Erythrocytes were treated with increasing DDS-NHOH concentrations or solvent (control), for 30 min or prolonged incubation (2 h30 min and 24 h patterns being similar are shown in same panel). Membranes (10 μg) were extracted with Triton X-100. Membranes, Triton-soluble (extracted) or insoluble (cytoskeleton) fractions, collected by ultra-centrifugation (see Methods) were analysed by SDS-PAGE and revealed with anti-Syk (panel A) or anti-SHP-2 (panel B) antibodies. Bands corresponding to Syk and SHP-2 were counted in a densitometer. Amounts of Syk and SHP-2 in unstimulated cells were chosen as 100% value. Results are means ± SEM of three separate experiments in duplicate. Data analysis was carried out by one-way ANOVA, showing significant differences in means among different treatments for membrane and cytoskeleton fractions ($P < 0.001$); for extract fraction, no significant difference was found. *Post-hoc* Tukey's test was performed versus corresponding basal value. *$P < 0.01$, significant difference versus corresponding basal value considered as control.

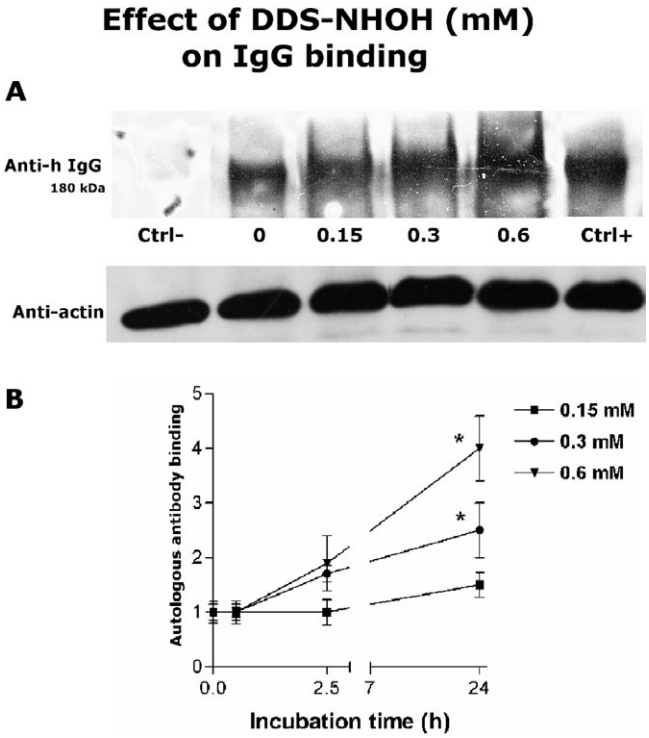

Figure 6

Erythrocytes were incubated in D-PBS-P-PP with increasing DDS-NHOH concentrations and incubated at 35°C for 24 h (panel A), or for varying incubation times (panel B). Membranes (15 μg), obtained as described in Methods, were analysed by Western blotting under non-reducing conditions and revealed with anti-human IgG HRP (or anti-actin as loading control). A: Lane Ctrl- represents erythrocytes incubated in D-PBS; Ctrl+ was obtained by incubating erythrocytes in D-PBS-P-PP in presence of 2 mM diamide. Results shown are representative of three separate experiments. B. Band corresponding to autologous antibody was counted in a densitometer. Amount of IgG bound to unstimulated cells was chosen as arbitrary unit. Results are means ± SEM of six experiments in duplicate. Data analysis was carried out by two-way ANOVA, indicating significant differences among concentrations in the time course of experiment ($P < 0.001$). *Post-hoc* Tukey's test was performed; asterisks indicate significant difference (*$P < 0.01$) versus basal value considered as control.

quently, modifications would continue more profoundly, inducing not only more marked clustering of band 3 but also totally redistributing HMWA from soluble to insoluble (cytoskeleton) membrane fractions (Figure 4). This is further suggested by total rearrangement of band 3 HMWA at 0.6 mM DDS-NHOH: in these conditions, band 3 Tyr-P is very slight (Figures 1 and 2), and band 3 HMWA were located in the cytoskeleton even after 30 min incubation (data not shown).

This may fit the hypothesis that reactive radicals also generate a second species of radicals, probably thiyl radicals (McMillan *et al.*, 2005), more reactive and efficacious in generating many and drastic alterations in membrane structure and composition.

This is in line with evidence from patients receiving dapsone therapy. In normal subjects, therapy leads to weakening of anti-oxidant defences (as indicated by decreased GSH content) and triggers membrane reorganization, as indicated by increased band 3 HMWA formation and higher sensitivity towards diamide-induced oxidative stress (Figure 8). When dapsone was administered to our G6PD deficient patient (P2), falls in both haemoglobin content and haematocrit were observed at $P2_{50}$, suggesting the onset of the haemolytic process. This cannot be explained by a simple fall in GSH content (see Table 2) since, even at 50 mg·day^{-1} dapsone ($P2_{50}$), almost one-third of glutathione was in reduced form, but incapable of preventing dapsone-induced erythrocyte modification. One explanation

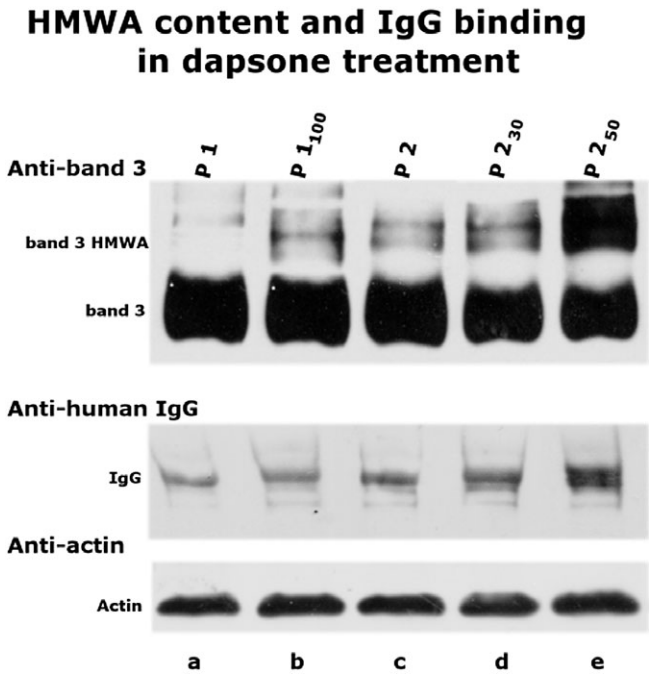

Figure 7

RBC were collected from Patient 1 before (P1) and during 100 mg·day^{-1} of dapsone therapy ($P1_{100}$), and patient 2 before (P2) and after 30 mg·day^{-1} ($P2_{30}$) and during 50 mg·day^{-1} ($P2_{50}$) of dapsone therapy. Membranes (1 μg), recovered as described in Methods, were subjected to Western blotting in non-reducing conditions and revealed with anti-band 3 antibodies. For analysis of human IgG, the same membranes (10 μg) were subjected to separate Western blotting in non-reducing conditions and revealed with anti-human IgG. Anti-actin panel, representing loading control, is referred to anti-human IgG panel. Results shown are representative of two separate experiments in duplicate.

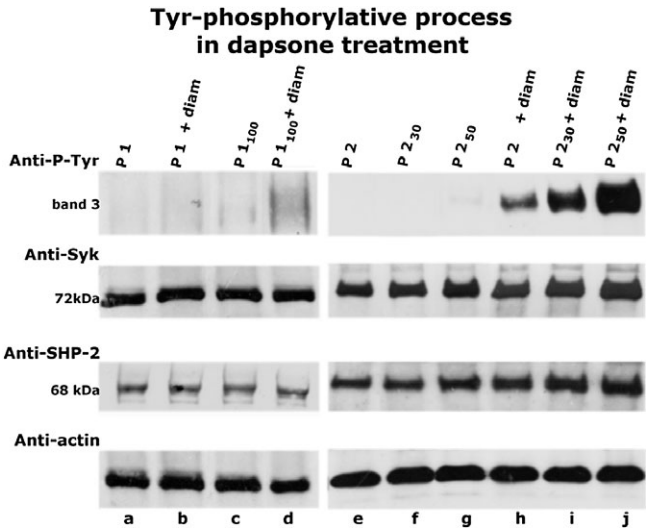

Figure 8

RBC from patients 1 and 2 receiving different doses of dapsone (100, 50 or 30 mg) were incubated in absence or presence of 0.3 mM diamide (diam). Membranes (10 μg), obtained as described in Methods, were subjected to Western blotting and revealed with anti-P-Tyr (uppermost panel), anti-Syk or anti-SHP-2 (successive panels) antibodies. Anti-actin panel represents loading control (lowest panel).
Results shown are representative of two separate experiments in duplicate.

matches expectations with the *in vitro* effect of DDS-NHOH on membrane alterations: HMWA and IgG binding were clearly present (Figure 7), showing that DDS-NHOH induced membrane reorganization had occurred. However, band 3 Tyr-P was not detected, even in $P2_{50}$ erythrocytes, where the effects of DDS-NHOH were enhanced by the concomitant defect in anti-oxidant defences. During dapsone treatment, DDS-NHOH does not irreversibly inhibit Tyr-protein kinases and/or phosphatases, as indicated by the diamide-induced band 3 Tyr-P of patients' erythrocytes (especially in P2) (Figure 8). This was probably because the concentration of this effector is too low to have immediate effects on the enzymes, like those observed in our *in vitro* experiments, which would represent high, toxic levels of this metabolite.

DDS-NHOH cannot be directly detected in human plasma, as it is rapidly taken up by erythrocytes prior to its redox cycling with haemoglobin, forming methaemoglobin (Coleman and Jacobus, 1993). In any case, the major metabolic elimination of dapsone is by N-hydroxylation, which accounts for between 30% and 40% of an oral dapsone dose, and the efficiency of N-hydroxylation is related to dapsone clearance (May *et al.*, 1990; 1992; Bluhm *et al.*, 1999). Dapsone therapy includes a daily administration of 50–100 mg for leprosy and 100–300 mg for dermatitis herpetiformis (Leonard and Fry, 1991), leading to serum concentrations of 0.5–5 mg·L^{-1} (equivalent to 2–20 μM); therapeutic doses up to 400 mg have been reported (Elonen *et al.*, 1979; Zuidema *et al.*, 1986), as well as some cases of intoxication with dapsone, such as after an overdose with 10 g of dapsone, leading to serum concentrations of 120 mg·L^{-1} (about 0.5 mM, comparable to those used in our *in vitro* experiments). Another case of intoxication produced methaemoglobinemia at serum concentrations of 18.8 mg·L^{-1} (76 μM) (Woodhouse *et al.*, 1983). The acetylation ratio (MADDS : DDS) shows a genetically determined bimodal distribution allowing the definition of 'slow' and 'rapid' acetylators (Zuidema *et al.*, 1986); therefore the amount of dapsone available for N-hydroxylation can vary accordingly, and blood cell damage can be more prominent, notably in the presence of G6PD deficiency. Our study confirms previous reports, stressing that sensitiveness to the compound is clearly idiosyncratic and dependent on the patho/physiological patient status (May

Table 2

Glutathione content

Blood samples	Glu_{tot} (mM) basal	GSSG (mM)	Glu_{tot} (mM) 0.3 mM diamide	GSSG (mM)
DDS-NHOH (mM): 0	2.15 ± 0.03	0.15 ± 0.01		
0.15	1.87 ± 0.02[a]	0.25 ± 0.02*		
0.3	1.58 ± 0.02[a]	0.35 ± 0.02*		
0.6	1.25 ± 0.02[a]	0.65 ± 0.02*		
P1	2.12 ± 0.03	0.15 ± 0.01	1.92 ± 0.03	0.26 ± 0.01
$P1_{100}$	1.89 ± 0.02†	0.25 ± 0.02†	1.68 ± 0.02†	0.35 ± 0.02†
P2	1.83 ± 0.03	0.22 ± 0.04	1.56 ± 0.03	0.35 ± 0.04
$P2_{30}$	1.36 ± 0.03†	0.45 ± 0.03†	1.18 ± 0.03†	0.73 ± 0.03†
$P2_{50}$	0.92 ± 0.03†	0.52 ± 0.03†	0.76 ± 0.03†	0.67 ± 0.03†

Control erythrocytes were incubated with increasing DDS-NHOH concentrations, haemolysed, cytosol was recovered and total glutathione (Glu_{tot} = GSH + GSSG) and oxidized glutathione (GSSG) were determined, as described in Methods. * $P < 0.01$, significant effect of DDS-NHOH (ANOVA followed by Tukey's test).

When patients receiving dapsone therapy were analysed, erythrocytes, withdrawn at different stages of therapy, were haemolysed, both untreated or following treatment with 0.3 mM diamide. Cytosol was recovered and total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) determined. Values are means ± SEM of six separate experiments. † $P < 0.01$, significant effect of dapsone treatment (ANOVA followed by Tukey's test) versus corresponding values before dapsone treatment.

et al., 1990; 1992; Wertheim *et al.*, 2006) who may also be at risk of the dapsone hypersensitivity syndrome (Sener *et al.*, 2006) even at a dose of 100 mg·day^{-1}. Alterations observed in the RBC membranes of P2 were very similar to those obtained *in vitro* with DDS-NHOH treatments, which were representative of a larger dapsone intake and may account for the toxic adverse effects reported after dapsone therapy. In patients receiving dapsone therapy, erythrocyte band 3 Tyr-P is not present in non-stimulated conditions, and this would be in agreement with the hypothesis that band 3 Tyr-P is related to the eryptosis (Bottini *et al.*, 1997; Bordin *et al.*, 2009), and, for this, is not compatible with patient tolerance. Diamide treatment can trigger this process to abnormally high levels, thus reflecting dapsone treatment-induced membrane alterations. Monitoring of band 3 alterations during dapsone intake in patients would be useful approach in preventing potential drug adverse effects due to incorrect dosage.

In conclusion, DDS-NHOH may be considered responsible for the adverse effects of dapsone on red blood cells. Net reorganization of membranes, leading to the autologous antibody binding seen following DDS-NHOH treatment of red blood cells *in vitro*, has also been demonstrated to characterize membranes from erythrocytes of patients receiving dapsone therapy. In addition, this alteration process is both concentration- and time-dependent, and involves oxidation of cytosol and membrane-bound proteins in a progressive mechanism which, in the end, causes shortening of the erythrocyte cell lifespan.

Acknowledgements

This work was supported by the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST).

The authors thank Claudio Bettella and Giancarlo Ruffato for supplying fresh blood from volunteers.

Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Dose- and time-dependent effect of DDS-NHOH on Syk and SHP-2 recruitment to membrane.
Table S1 Test values.

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