Interactions between cell surface protein disulphide isomerase and S-nitrosoglutathione during nitric oxide delivery.

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INTERACTIONS BETWEEN CELL SURFACE PROTEIN DISULPHIDE ISOMERASE AND S-NITROSOGLUTATHIONE DURING NITRIC OXIDE DELIVERY

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Abstract

In this study, we investigated the role of protein disulphide isomerase (PDI) in rapid metabolism of S-nitrosoglutathione (GSNO) and S-nitrosoalbumin (albSNO) and in NO delivery from these compounds into cells. Incubation of GSNO or albSNO (1 μM) with the megakaryocyte cell line MEG-01 resulted in a cell-mediated removal of each compound which was inhibited by blocking cell surface thiols with 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB) (100 μM) or inhibiting PDI with bacitracin (5 mM). GSNO, but not albSNO, rapidly inhibited platelet aggregation and stimulated cyclic GMP (cGMP) accumulation (used as a measure of intracellular NO entry). cGMP accumulation in response to GSNO (1 μM) was inhibited by MEG-01 treatment with bacitracin or DTNB, suggesting a role for PDI and surface thiols in NO delivery. PDI activity was present in MEG-01 conditioned medium, and was inhibited by high concentrations of GSNO (500 μM). A number of cell surface thiol-containing proteins were labelled using the impermeable thiol specific probe 3-(N-maleimido-propionyl) biocytin (MPB). Pretreatment of cells with GSNO resulted in a loss of thiol reactivity on some but not all proteins, suggesting selective cell surface thiol modification. Immunoprecipitation experiments showed that GSNO caused a concentration-dependent loss of thiol reactivity of PDI. Our data indicate that PDI is involved in both rapid metabolism of GSNO and intracellular NO delivery and that during this process PDI is itself altered by thiol modification. In contrast, the relevance of PDI-mediated albSNO metabolism to NO signalling is uncertain.

Keywords: Nitric oxide; S-nitrosoglutathione; protein disulphide isomerase; cell surface thiol; megakaryocytes

Abbreviations

4-DP	4, 4'-dithiodipyridine
ACD	acid citrate dextrose
albSNO	S-nitrosoalbumin
BCA	bicinchoninic acid
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)
GSH	glutathione
GSNO	S-nitrosoglutathione
HBS	HEPES buffered saline
MPB	3-(N-maleimido-propionyl) biocytin
PDI	protein disulphide isomerase
PMSF	phenylmethylsulfonyl fluoride
RSNO	S-nitrosothiol
γ-GT	γ-glutamyl transpeptidase

Introduction

S-nitrosothiols (RSNOs) may serve as a reservoir of nitric oxide (NO) in biological systems and represent a class of NO donor with many potential clinical uses [1]. The potent and selective anti-platelet activity of some of these compounds has suggested that they may be particularly useful as anti-thrombotic agents [2].

RSNOs produce antiplatelet activity either by releasing NO and stimulating guanylyl cyclase, or via cyclic GMP-independent mechanisms [3]. S-nitrosoglutathione (GSNO) releases NO only slowly, nevertheless it has the ability rapidly to inhibit platelet aggregation, in part through cyclic GMP-independent mechanisms [4]. Several studies have reported delivery of NO into cells via transnitrosation reactions, which involve transfer of NO^+ from one thiol to another [5-7]. For protein RSNOs (such as S-nitrosoalbumin) to mediate platelet inhibition, prior exchange of NO onto a low molecular weight thiol appears necessary [8, 9]. Free thiols are available on the platelet surface itself [10, 11] and S-nitrosation of these thiols may be a further possible pathway of anti-platelet action. However the relatively unfavourable kinetics of transnitrosation reactions [12] suggest that some enzyme mediated system is required for the process to occur sufficiently rapidly to mediate signalling events.

A number of studies have documented the existence of enzyme-catalysed systems that facilitate delivery of NO from RSNO into cells, either by cell mediated metabolism or via amino acid transport systems [13]. These may be important both for mediating RSNO bioactivity and for enabling cells to deal with nitrosative stress during periods of

long-term exposure [14, 15]. For membrane-impermeable molecules such as GSNO, a cell surface enzyme such as γ-glutamyl transpeptidase (γ-GT) or protein disulphide isomerase (PDI) may act as a receptor or entry point for the delivery of the nitric oxide signal [16-18]. Both γ-GT [19] and PDI [20] have been shown to metabolise GSNO and mediate NO release. These metabolism studies, however, have generally been carried out using relatively high GSNO concentrations (high μM to mM range) and long time periods (minutes – hours) [21, 22]. The fact that GSNO inhibits platelet aggregation at very low concentrations (nM to low μM), and within seconds, suggests that its metabolism should be assessed over more relevant concentrations and time periods in order to understand its relevance to anti-platelet activity.

We have previously shown rapid metabolism of GSNO by platelets and megakaryocytes and the involvement of extracellular thiols in this process [23]. Here we extend these studies to report the role of PDI in rapid metabolism of GSNO and S-nitroso human serum albumin (albSNO) at concentrations relevant to most published pharmacological studies and the relevance of this metabolism to NO delivery. We also report the effects of these interactions on PDI activity and its thiol redox status.

Experimental Procedures

Materials and reagents

Cell culture medium (RPMI-1640), phosphate buffered saline (PBS), penicillin, streptomycin and L-glutamine were obtained from Biowhittaker (Wokingham**,** UK). Foetal calf serum (FCS) was purchased from Biowest (Ringmer, UK), MPB from

Molecular Probes (Eugene, OR), BCA protein assay kit from Perbio Science (Cheshire, UK) and RL 90 antibody against PDI from Alexis Biochemicals (Nottingham, UK). Reagents for cGMP measurement by enzyme immunoassay, Western blot chemiluminescence detection, immunoprecipitation, and streptavidin horseradish peroxidase (HRP) were obtained from Amersham Biosciences (Chalfont St. Giles, UK). All other chemicals were purchased from Sigma (Poole, UK).

Preparation of RSNO compounds

GSNO and S-nitrosocysteine (cysNO) were prepared as previously described [4] by nitrosation under acid conditions. Briefly, equal volumes of either glutathione or cysteine (20 mM) and sodium nitrite (20 mM) were incubated in the presence of 100 mM HCl on ice for 30 minutes in the presence of 1 mM EDTA. GSNO and cysNO were freshly prepared and stored in the dark on ice until used. Concentrations of GSNO and cysNO were estimated by absorbance at 334 nm using extinction coefficients of 0.85 and 0.75 mM^{-1} cm^{-1}, respectively.

AlbSNO was prepared by nitrosation of free thiols present on albumin. CysNO (20 mM) was incubated with albumin (1 mg/ml) in the dark for 1 hr. AlbSNO was then separated from low-molecular weight CysNO by passing it through Sephadex G-25 gel filtration column and collecting albumin fractions. The protein concentration of each fraction was measured by BCA protein kit and albSNO in each fraction was quantified using an NO electrode with reference to GSNO calibration curve. AlbSNO fractions were then pooled, quantified and stored at -20ºC until used.

Preparation of MEG-01 cell suspensions

The megakaryocyte cell line MEG-01 [24] was obtained from ATCC (Manassas, VA) and grown in RPMI-1640 medium supplemented with 10% v/v foetal calf serum (FCS), 50 IU/ml aqueous penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine and incubated at 37ºC with a humidified atmosphere of 5% CO_2. For experiments cells were suspended in HBS at a count of 3×10^9 l^{-1}.

Preparation of washed platelets

Washed platelets were prepared from informed healthy volunteers who had not received any medication known to alter platelet function for at least 2 weeks before study. Blood was anticoagulated with ACD and processed within 1 hour of collection. Washed platelets were separated from platelet rich plasma as previously described [25] and platelet count was adjusted to 200×10^9 l^{-1} by suspending in HEPES buffered saline (HBS), containing NaCl (140 mM), KCl (2.7 mM), Glucose (5mM), BSA (1mg/ml), $CaCl_2$ (1mM), $MgCl_2$ (1 mM) and HEPES **(**10 mM) (pH 7.3).

Cell mediated RSNO metabolism and effects of enzyme inhibitors

Cell mediated RSNO metabolism was analysed by treatment of MEG-01 cells with 1 μM GSNO or albSNO at 37ºC for 2 minutes, following which cells were centrifuged at 8300g for 30 seconds and residual RSNO in the supernatant was measured either using the fluorescent agent diaminonaphthaline [26] or by an electrochemical assay using an NO electrode (WPI, Stevenage**,** UK) [27], calibrated against GSNO (0-2000 nM). Control experiments were performed in the absence of MEG-01 cells.

The effect of different enzyme inhibitors and surface thiol blocking agents on RSNO metabolism was further investigated by pre-treatment of MEG-01 cells for 30 minutes at 37ºC. Bacitracin (5 mM) and acivicin (100 μM) were used to block PDI and γ-GT respectively, and an impermeable thiol blocking agent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (100 μM) was used to modify extracellular thiol groups. RSNO decay, occurring after two minutes incubation with MEG-01 cells, was then measured.

Inhibition of platelet aggregation by RSNO

Platelet aggregation was performed using washed platelets suspended in HBS. Platelets were incubated with GSNO and albSNO (both 1 μM) at 37ºC for 5 or 30 seconds prior to addition of thrombin (0.1 unit ml^{-1}) to induce aggregation. Aggregation was then monitored turbidometrically for 2 minutes. Effects of RSNO compounds were assessed by comparison with control experiments carried out in the vehicle.

Cyclic GMP accumulation in response to RSNO

GSNO or albSNO (1 μM) was incubated with MEG-01 cells at 37ºC for 2 minutes, after which an equal volume of 20% v/v perchloric acid was added. Samples were then stored at –20ºC. Prior to measurement of cGMP, pH of the supernatants was corrected to 7.4 by addition of K_3PO_4 (0.54 M), after which samples were centrifuged to remove precipitate and cGMP measured by enzyme immunoassay. Experiments were performed following treatment of cells with acivicin (100 μM), bacitracin (5 mM), DTNB (100 μM) or a vehicle control for 30 minutes prior to addition of NO donors.

PDI activity in cell supernatant

PDI has been shown to be released from platelets into the surrounding medium [28]. To measure PDI activity in MEG-01 supernatant, PDI-catalysed reduction of insulin was measured using a turbidometric assay [20]. MEG-01 cells were centrifuged at 8300g for 30 seconds to obtain supernatant for use in the insulin assay. Cell supernatant was added to insulin (250 μM) in the presence of glutathione (GSH) (500 μM) in HBS containing 2 mM EDTA, pH 7.0. The increase in turbidity was monitored at 650 nm over a period of 40 hours. PDI activity in the supernatant was also measured following the addition of GSNO (1 - 500 μM).

Sulfhydryl labelling of intact MEG-01 cells

Cell surface thiol-containing proteins were labelled using the impermeable thiol specific probe, 3-N-maleimidylpropionyl-biotin (MPB) as described by Essex *et al.* [11]. MEG-01 cells were incubated with MPB (100 μM) for 30 minutes on a rotating wheel at room temperature. The labelling reaction was stopped by addition of GSH (200 μM) for 30 minutes and excess GSH was quenched using iodoacetamide (400 μM) for 10 minutes. Cells were washed twice with HBS and resuspended in 0.5 ml of ice-cold 50 mM Tris/HCl buffer (pH 7.3) containing sucrose (250 mM), leupeptin (10 μM), PMSF (2 mM) and EDTA (2 mM). Cells were lysed with three freeze/thaw cycles and centrifuged at 900g for 10 minutes to remove debris. The protein concentration of each sample was measured using BCA protein assay kit. Non-reducing SDS sample buffer was added to the cell lysate and the sample analysed using 10% SDS PAGE. Samples were transferred to a nitrocellulose membrane and biotinylated proteins were detected

using streptavidin-horseradish peroxidase and a chemiluminescent substrate. Negative control experiments were performed with MEG-01 cells by (1) omitting MPB, and (2) incubating cells with the thiol blocking agent 4,4'-dithiodipyridine (4-DP) (4 mM) for 30 minutes at 37ºC prior to addition of MPB. In both cases, there was a complete abolition of thiol labelling. To assess the effect of GSNO on cell surface thiols MEG-01 cells were incubated with GSNO (3.5 mM) prior to addition of MPB.

Immunoprecipitation of MEG-01 surface PDI

MEG-01 cells (3×10^9 l^{-1}) were incubated for 30 minutes at 37ºC with GSNO (0 - 5 mM) and then washed once in HBS prior to labelling with MPB as described above. In experiments to investigate the reversibility of thiol modification, cells were exposed to glutathione (4mM) for 30 minutes after GSNO treatment. Cells were then washed twice prior to MPB labelling. The labelled MEG-01 cells were then treated with lysis buffer (0.15 M NaCl, 0.5% v/v Triton X-100, 0.05% v/v Tween 20, 10 µM leupeptin, 10 µM aprotinin, 2 mM phenylmethylsulfonyl fluoride, 5 mM EDTA and 10 mM Tris-HCl, pH 8.0). Samples were pre-cleared by incubation for 60 minutes at 4ºC with a mixture of protein G plus protein A beads, followed by centrifugation to remove beads. Supernatant was incubated for 60 min at 4ºC on a rotating wheel with RL 90 mouse monoclonal anti-PDI antibody (4.2 µg ml^{-1}) or isotype control antibody. Samples were then centrifuged at 12,000g for 30 min at 4ºC to remove debris, after which a mixture of protein G plus protein A beads was added and samples incubated for 1 hr at 4ºC on a rotator. Beads were then washed once in lysis buffer and twice in 50 mM Tris-HCl, pH 8.0, resuspended in loading buffer and boiled for 2 minutes. Protein concentration of

each sample was measured and samples were then run on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane and visualized using streptavidin-horseradish peroxidase and chemiluminescent substrate.

Statistical Analysis

Effects of various treatments on cyclic GMP accumulation and RSNO metabolism were analysed on log-transformed data by one-way ANOVA following by Student t test, using Bonferroni correction for multiple comparisons.

Results

Cell mediated RSNO metabolism and effects of inhibitors

Cell-mediated RSNO decay was analysed by measuring the amount of residual RSNO in cell supernatant after incubation with MEG-01 cells. Addition of GSNO or albSNO (1 μM) to suspensions of MEG-01 cells resulted in a loss of approximately 84 or 93 nM per 10^9 cells respectively after two minutes. Similar results were obtained whether RSNO quantification was performed by either fluorescence assay or NO electrode methods. Prior incubation of cells with DTNB (100 μM) (an impermeable thiol blocking agent) or bacitracin (5 mM) (an impermeable PDI blocking agent) significantly blocked cell-mediated removal of both GSNO (figure 1a) and albSNO (figure 1b). However, inhibition of γ-GT with acivicin (100 μM) failed to influence RSNO removal.

In separate experiments bacitracin and acivicin, at the stated concentrations, were shown to completely inhibit the activity of authentic PDI, measured by turbidometric assay using insulin [20] and γ-GT, measured by colorimetric assay using γ-glutamyl paranitroaniline (data not shown). Cell viability, measured by trypan blue exclusion, was unaffected by treatment of cells with bacitracin, acivicin or DTNB at the stated concentrations (data not shown).

Inhibition of platelet aggregation by RSNO

The rapidity of GSNO as an antiplatelet agent was examined by platelet aggregation studies. Pre-incubation of platelets with 1 μM GSNO for as little as 5 seconds was sufficient to almost completely inhibit thrombin induced platelet aggregation compared with control (figure 2). In contrast, albSNO inhibited platelet aggregation only minimally even after 30 seconds pre-incubation.

Mechanism of intracellular NO delivery from GSNO

Intracellular accumulation of cyclic GMP was analysed as a measure of NO entry from GSNO and albSNO. GSNO incubation with MEG-01 for two minutes resulted in a significant cGMP accumulation (figure 3a), whereas albSNO failed to stimulate cGMP generation. Both bacitracin and DTNB, but not acivicin, significantly inhibited cGMP accumulation in response to GSNO ($p < 0.05$) (figure 3b). Control experiments omitting NO donors showed no effect of the various inhibitors on cGMP levels in their own right.

Detection of PDI activity in cell supernatant

PDI activity, detected using a turbidometric assay of insulin disulphide reduction was evident in MEG-01 cell supernatant. GSNO inhibited this activity, however very high concentrations were required to show this effect (figure 4).

Cell surface thiol modification by GSNO

Our experiments suggested that GSNO, but not albSNO, was capable of delivering NO into MEG-01 cells to provoke cGMP accumulation. This intracellular transfer of NO depended upon the availability of cell surface sulphydryls and active PDI. We therefore further examined the action of GSNO by measuring its effects on the redox state of MEG-01 membrane thiol proteins. Treatment of cells with MPB resulted in labelling of a number of thiol-containing proteins on the MEG-01 cell surface. Prior incubation of MEG-01 cells with GSNO (3.5 mM) resulted in a loss of thiol reactivity on several, but not all proteins (figure 5).

Identification of PDI as a target for redox modification by GSNO

To determine whether PDI is a one of the target proteins modified by GSNO, MEG-01 cells were labelled with MPB and RL 90 anti-PDI antibody was then used to immunoprecipitate PDI. Pretreatment of MEG-01 cells with GSNO (0.5 – 5 mM) resulted in a loss of thiol labelling of PDI in a concentration dependent manner (figure 6). Subsequent treatment with GSH resulted in a restoration of thiol labelling, indicating that the modification was reversible (figure 7).

Discussion

Our study shows that cell surface thiols and protein disulphide isomerase are required for NO delivery from GSNO into MEG-01 cells. Both GSNO and albSNO were rapidly metabolized by MEG-01 cells, however there was a discrepancy between albSNO metabolism and its NO signalling function. We have demonstrated cell surface thiol modification by GSNO and identified PDI as one of the target proteins. This is consistent with previous suggestions that PDI-catalysed S-nitrosation of cell surface thiols is a mechanism of nitric oxide delivery from GSNO [5, 18].

GSNO, an endogenous molecule which may act as a storage form of NO, is a potent inhibitor of platelet aggregation despite its slow release of free NO, and this paradox led us to analyse its rapid metabolism at therapeutically relevant concentrations. The human megakaryocyte cell line MEG-01 displays platelet-like properties such as surface expression of PDI and $\alpha_{IIb}\beta_3$ as well as NO-induced activation of guanylyl cyclase and these properties, together with its ready availability, led us to employ this cell line in our experiments.

In most published studies of cell mediated GSNO metabolism, relatively high concentrations and long time periods have been employed, yet even at concentrations as low as 1 μM, inhibition of platelet aggregation is evident within seconds. It is therefore difficult to assess the relevance of such studies to the anti-platelet actions of GSNO. Here, we found that cell mediated decay of 1 μM GSNO was evident within two minutes. This rapid GSNO removal was significantly inhibited both by modification of

cell surface thiols with DTNB, and by inhibition of cell surface PDI with bacitracin. Exposure of cells to acivicin to block γGT failed to influence GSNO removal, indicating that cell surface thiols and PDI, but not γGT, are required for rapid metabolism of GSNO. This is consistent with recent literature demonstrating PDI mediated GSNO consumption by platelets [20] and suggests that GSNO consumption may be relevant to its anti-platelet function. Ramachandran *et al.* [18] have provided evidence that NO released from impermeable RSNO molecules by cell surface PDI reacts with oxygen within the hydrophobic environment of the lipid membrane to produce N_2O_3, which then nitrosates intracellular thiol-containing molecules. Our data indicate that this, or a similar, pathway may be involved in the delivery of NO from GSNO onto the haem group of soluble guanylyl cyclase so as to stimulate generation of cyclic GMP. PDI inhibitors blocked this cyclic GMP accumulation significantly but not completely, implying the existence of alternative pathways for NO delivery.

In contrast, the fate of the removed fraction of albSNO, and its relevance to NO signalling, are not clear. We observed that albSNO was metabolized in a similar way to GSNO, yet failed to inhibit platelet aggregation or stimulate intracellular cGMP accumulation. This suggests a discrepancy between the metabolism phenomenon, and subsequent signalling events. It has been documented that NO delivery into cells from protein RSNO molecules, such as albSNO, requires a prior transfer of NO to a low molecular weight thiol, via transnitrosation [8, 9]. Our experiments were performed in the absence of added thiol and this may explain why metabolism of albSNO was not accompanied by cGMP accumulation or inhibition of platelet aggregation.

The involvement of PDI in transferring NO across the plasma membrane raises the question of how this process affects the function of PDI itself. PDI activity is dependent on its vicinal free thiol groups at the active site [29] which themselves may act as a target for nitrosation. We studied changes brought about by GSNO in thiol redox state of MEG-01 cell surface proteins. Consistent with earlier reports [10, 11] a number of such proteins were detectable by western blotting using MPB, an impermeable thiol specific probe. Cell treatment with GSNO caused a loss of thiol reactivity on most, but not all, proteins suggesting a selective process of GSNO-mediated thiol redox modification. Thiol modification of PDI itself was demonstrated using immunoprecipitation with a specific antibody, and this was GSNO concentration-dependent. Subsequent addition of GSH led to restoration of thiol labelling, indicating that the GSNO-mediated thiol modification was reversible. It has been discussed that in the presence of sulphydryl compounds S-nitrosated proteins can undergo either transnitrosation, to restore the reduced thiol state, or S-thiolation reactions, yielding disulphide and nitroxyl [30, 31]. Our results indicate that, under the conditions used, the former reaction was predominant.

PDI is known to be present on the surface of platelets and to be released during platelet activation [28, 32]. We also found PDI activity to be released from MEG-01 cells, and inhibited by GSNO, again consistent with an earlier report [20]. Thus the involvement of PDI in mediating NO transfer results in thiol modification and enzyme inactivation. The mechanisms employed to restore the redox state of active site thiols in PDI

following interaction with GSNO are not yet clear. Burgess *et al.* [10] showed that the redox state of platelet surface PDI was refractory to extracellular reducing agents, but discussed the possibility that it might be controlled by a plasma membrane electron transfer system such as NAD(P)H oxidase.

PDI activity was inhibited by interaction with GSNO, although high concentrations were required to show this effect. Our findings are consistent with those of Root *et al.* [20] who showed full inhibition of PDI was not achieved by GSNO at concentrations lower than 500 μM. We also found that very high concentrations of GSNO were needed to demonstrate PDI thiol modification. In view of the fact that GSNO suppresses platelet activation in the nanomolar to micromolar range, it is clear that further investigations will be required to establish the physiological relevance of these interactions.

The integrin family of cell surface adhesive receptors mediates platelet aggregation by up-regulating their affinity state for ligand binding. Free thiols become available on the platelet surface upon activation [10] and those present on integrin $\alpha_{IIb}\beta_3$ provide a direct target for redox modification [33]. Thiol-disulphide exchange within $\alpha_{IIb}\beta_3$, catalysed by an endogenous isomerase activity [34], by PDI [11, 35], or by ERP5 [36] is necessary for transition to its active conformation. Thus the thiol redox state of $\alpha_{IIb}\beta_3$ influences platelet adhesive function and any agent that modifies this redox state may interfere with platelet aggregation. Our data are consistent with a model in which GSNO mediates its actions by both cGMP-dependent and independent pathways.

Targeting of NO to cell surface free thiols facilitates its entry into cells and its interaction with soluble guanylyl cyclase to promote cGMP-dependent pathways. cGMP-independent actions may be mediated by thiol modification of surface targets such as PDI, thus inhibiting activation of $\alpha_{IIb}\beta_3$. This does not exclude other mechanisms of cGMP-independent action such as inhibition of intracellular calcium entry [37] or direct nitrosation of $\alpha_{IIb}\beta_3$ [33, 38].

Overall, our data show that PDI is involved in the rapid metabolism of RSNOs and in NO delivery from GSNO to soluble guanylyl cyclase. Redox modification of thiols by GSNO on PDI and other thiol containing proteins may be significant in mediating both cGMP-dependent and cGMP-independent antiplatelet actions of GSNO. In view of emerging evidence that integrin function and platelet aggregation are controlled by redox transitions occurring at the cell surface and catalysed by both PDI and other thiol isomerases, the interaction of GSNO with PDI in bringing about platelet inhibition takes on a new significance.

Acknowledgements

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References

1. Richardson G, Benjamin N: Potential therapeutic uses for S-nitrosothiols. *Clin Sci (Lond)* 2002, 102(1):99-105.

2. de Belder AJ, MacAllister R, Radomski MW, Moncada S, Vallance PJ: Effects of S-nitroso-glutathione in the human forearm circulation: evidence for selective inhibition of platelet activation. *Cardiovasc Res* 1994, 28(5):691-694.

3. Sogo N, Magid KS, Shaw CA, Webb DJ, Megson IL: Inhibition of human platelet aggregation by nitric oxide donor drugs: relative contribution of cGMP-independent mechanisms. *Biochem Biophys Res Commun* 2000, 279(2):412-419.

4. Gordge MP, Hothersall JS, Noronha-Dutra AA: Evidence for a cyclic GMP-independent mechanism in the anti-platelet action of S-nitrosoglutathione. *Br J Pharmacol* 1998, 124(1):141-148.

5. Zai A, Rudd MA, Scribner AW, Loscalzo J: Cell-surface protein disulfide isomerase catalyzes transnitrosation and regulates intracellular transfer of nitric oxide. *J Clin Invest* 1999, 103(3):393-399.

6. Park JW, Billman GE, Means GE: Transnitrosation as a predominant mechanism in the hypotensive effect of S-nitrosoglutathione. *Biochem Mol Biol Int* 1993, 30(5):885-891.

7. Liu Z, Rudd MA, Freedman JE, Loscalzo J: S-Transnitrosation reactions are involved in the metabolic fate and biological actions of nitric oxide. *J Pharmacol Exp Ther* 1998, 284(2):526-534.

8. Simon DI, Stamler JS, Jaraki O, Keaney JF, Osborne JA, Francis SA, Singel DJ, Loscalzo J: Antiplatelet properties of protein S-nitrosothiols derived from nitric oxide and endothelium-derived relaxing factor. *Arterioscler Thromb* 1993, 13(6):791-799.

9. Crane MS, Ollosson R, Moore KP, Rossi AG, Megson IL: Novel role for low molecular weight plasma thiols in nitric oxide-mediated control of platelet function. *J Biol Chem* 2002, 277(49):46858-46863.

10. Burgess JK, Hotchkiss KA, Suter C, Dudman NP, Szollosi J, Chesterman CN, Chong BH, Hogg PJ: Physical proximity and functional association of glycoprotein 1balpha and protein-disulfide isomerase on the platelet plasma membrane. *J Biol Chem* 2000, 275(13):9758-9766.

11. Essex DW, Li M, Miller A, Feinman RD: Protein disulfide isomerase and sulfhydryl-dependent pathways in platelet activation. *Biochemistry* 2001, 40(20):6070-6075.

12. Hogg N: The kinetics of S-transnitrosation--a reversible second-order reaction. *Anal Biochem* 1999, 272(2):257-262.

13. Zhang Y, Hogg N: S-Nitrosothiols: cellular formation and transport. *Free Radic Biol Med* 2005, 38(7):831-838.

14. Zhang Y, Hogg N: The mechanism of transmembrane S-nitrosothiol transport. *Proc Natl Acad Sci U S A* 2004, 101(21):7891-7896.

15. Liu L, Yan Y, Zeng M, Zhang J, Hanes MA, Ahearn G, McMahon TJ, Dickfeld T, Marshall HE, Que LG *et al*: Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell* 2004, 116(4):617-628.

16. Lipton AJ, Johnson MA, Macdonald T, Lieberman MW, Gozal D, Gaston B: S-nitrosothiols signal the ventilatory response to hypoxia. *Nature* 2001, 413(6852):171-174.

17. Askew SC, Butler AR, Flitney FW, Kemp GD, Megson IL: Chemical mechanisms underlying the vasodilator and platelet anti-aggregating properties of S-nitroso-N-acetyl-DL-penicillamine and S-nitrosoglutathione. *Bioorg Med Chem* 1995, 3(1):1-9.

18. Ramachandran N, Root P, Jiang XM, Hogg PJ, Mutus B: Mechanism of transfer of NO from extracellular S-nitrosothiols into the cytosol by cell-surface protein disulfide isomerase. *Proc Natl Acad Sci U S A* 2001, 98(17):9539-9544.

19. Hogg N, Singh RJ, Konorev E, Joseph J, Kalyanaraman B: S-Nitrosoglutathione as a substrate for gamma-glutamyl transpeptidase. *Biochem J* 1997, 323 (Pt 2):477-481.

20. Root P, Sliskovic I, Mutus B: Platelet cell surface protein disulfide isomerase mediated S-nitrosoglutathione consumption. *Biochem J* 2004, Pt.

21. Zeng H, Spencer NY, Hogg N: Metabolism of S-nitrosoglutathione by endothelial cells. *Am J Physiol Heart Circ Physiol* 2001, 281(1):H432-439.

22. Gordge MP, Addis P, Noronha-Dutra AA, Hothersall JS: Cell-mediated biotransformation of S-nitrosoglutathione. *Biochem Pharmacol* 1998, 55(5):657-665.

23. Shah CM, Locke IC, Chowdrey HS, Gordge MP: Rapid S-nitrosothiol metabolism by platelets and megakaryocytes. *Biochem Soc Trans* 2003, 31(Pt 6):1450-1452.

24. Ogura M, Morishima Y, Ohno R, Kato Y, Hirabayashi N, Nagura H, Saito H: Establishment of a novel human megakaryoblastic leukemia cell line, MEG-01, with positive Philadelphia chromosome. *Blood* 1985, 66(6):1384-1392.

25. Cooper MB, Tan KC, Betteridge DJ: Platelet transmembrane signalling responses to collagen in familial hypercholesterolaemia. *Eur J Clin Invest* 1994, 24(11):737-743.

26. Park JK, Kostka P: Fluorometric detection of biological S-nitrosothiols. *Anal Biochem* 1997, 249(1):61-66.

27. Pfeiffer S, Schrammel A, Schmidt K, Mayer B: Electrochemical determination of S-nitrosothiols with a Clark-type nitric oxide electrode. *Anal Biochem* 1998, 258(1):68-73.

28. Chen K, Lin Y, Detwiler TC: Protein disulfide isomerase activity is released by activated platelets. *Blood* 1992, 79(9):2226-2228.

29. Freedman RB, Hirst TR, Tuite MF: Protein disulphide isomerase: building bridges in protein folding. *Trends Biochem Sci* 1994, 19(8):331-336.

30. Stamler JS: Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* 1994, 78(6):931-936.

31. Hogg N: Biological chemistry and clinical potential of S-nitrosothiols. *Free Radic Biol Med* 2000, 28(10):1478-1486.

32. Essex DW, Chen K, Swiatkowska M: Localization of protein disulfide isomerase to the external surface of the platelet plasma membrane. *Blood* 1995, 86(6):2168-2173.

33. Yan B, Smith JW: A redox site involved in integrin activation. *J Biol Chem* 2000, 275(51):39964-39972.

34. O'Neill S, Robinson A, Deering A, Ryan M, Fitzgerald DJ, Moran N: The platelet integrin alpha IIbbeta 3 has an endogenous thiol isomerase activity. *J Biol Chem* 2000, 275(47):36984-36990.

35. Lahav J, Jurk K, Hess O, Barnes MJ, Farndale RW, Luboshitz J, Kehrel BE: Sustained integrin ligation involves extracellular free sulfhydryls and enzymatically catalyzed disulfide exchange. *Blood* 2002, 100(7):2472-2478.

36. Jordan PA, Stevens JM, Hubbard GP, Barrett NE, Sage T, Authi KS, Gibbins JM: A role for the thiol isomerase protein ERP5 in platelet function. *Blood* 2005, 105(4):1500-1507.

37. Crane MS, Rossi AG, Megson IL: A potential role for extracellular nitric oxide generation in cGMP-independent inhibition of human platelet aggregation: biochemical and pharmacological considerations. *Br J Pharmacol* 2005, 144(6):849-859.

38. Walsh GM, Sheehan D, Kinsella A, Moran N, O'Neill S: Redox modulation of integrin [correction of integin] alpha IIb beta 3 involves a novel allosteric regulation of its thiol isomerase activity. *Biochemistry* 2004, 43(2):473-480.

Legends

Figure 1. MEG-01 cell mediated metabolism of RSNO. MEG-01 cells were incubated with 1 μM GSNO (figure 1a) or albSNO (figure 1b) at 37°C for 2 minutes following which residual RSNO was measured in supernatant (control sample). Effects of enzyme inhibitors were studied by incubating cells with acivicin (100 μM), DTNB (100 μM) or bacitracin (5 mM) for 30 minutes prior to addition of RSNO. Results shown are mean (SEM) from 3-6 separate experiments. *P < 0.05, compared with control.

Figure 2. Inhibition of platelet aggregation by RSNO. Washed platelets were pre-incubated with 1 μM GSNO or albSNO for 5 or 30 seconds prior to addition of thrombin (0.1 units ml^{-1}) to induce platelet aggregation. Aggregation was monitored turbidometrically over 2 minutes. GSNO was able to almost completely inhibit thrombin induced platelet aggregation within 5 seconds. However, albSNO only partially inhibited platelet aggregation. Values are expressed as mean (SEM) (n = 4).

Figure 3. cGMP accumulation in response to RSNO. MEG-01 cells were incubated with GSNO or albSNO (both 1 μM) for 2 minutes, following which cGMP levels were analysed using enzyme immunoassay (figure 3a). Effects of enzymes or surface thiols were analysed by treatment of MEG-01 cells with inhibitors for 30 minutes at 37°C prior to addition of GSNO (1 μM) (figure 3b). Results shown are mean (SEM) from 6 separate experiments *P < 0.05 compared with control.

Figure 4. Effect of GSNO on PDI activity in MEG-01 cell supernatant. GSNO (0 – 500 μM) was incubated with supernatant for 30 minutes prior to addition of insulin substrate. PDI activity was then monitored by measuring the increase in turbidity (at 650 nm) over a period of 40 hours. Results shown are mean values for 4 - 6 experiments.

Figure 5. Redox modification of cell surface thiol-containing proteins by GSNO.

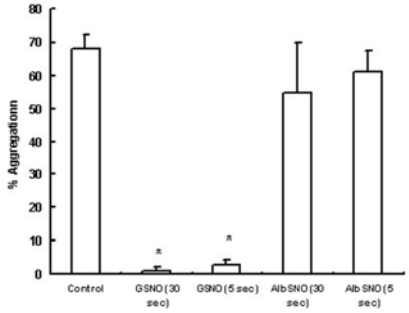
MEG-01 cells were labelled with impermeable thiol specific probe, MPB (100 μM), cells were lysed and samples were run using 10 % SDS polyacrylamide gel under non-reducing conditions. Samples were then transferred to a nitrocellulose membrane and protein thiols were detected with streptavidin-HRP. A number of thiol containing proteins were labelled on the MEG-01 cell surface (*lane 1*). Pre-incubation of cells with GSNO (3.5 mM) resulted in loss of thiol reactivity on several proteins, shown by arrows (*lane 2*). Results shown are representative of 3 similar experiments**.**

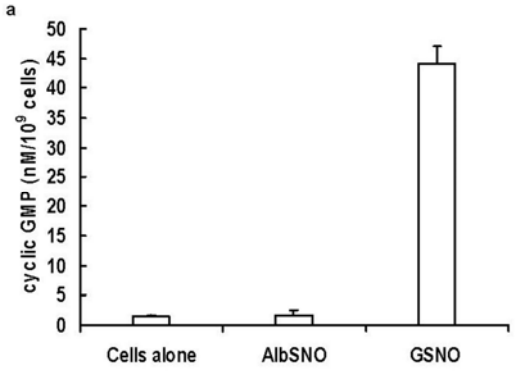
Figure 6. Thiol modification of cell surface PDI by GSNO.

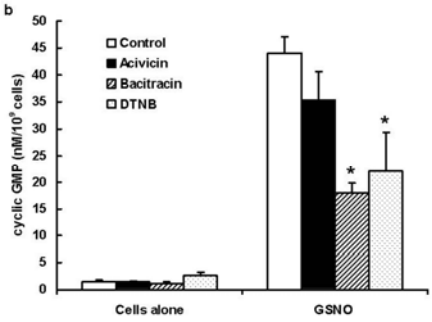
Cell surface thiol labelling with MPB was performed as previously described. Cells were treated with graded GSNO concentrations (0 – 5 mM) prior to labelling with MPB. Cells were then lysed and PDI was immunoprecipitated using a monoclonal antibody (RL 90) (4.2 μg ml^{-1}). Control experiments were performed using an isotype control antibody. Results shown are representative of 3 similar experiments.

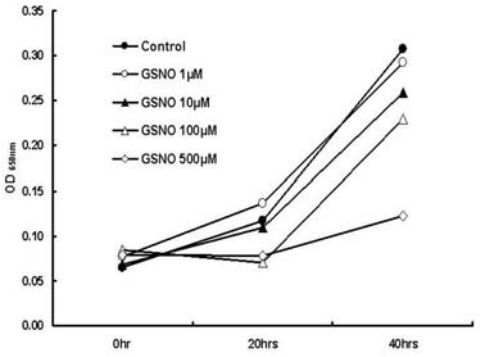
Figure 7. Reversal of GSNO-mediated thiol modification by GSH.

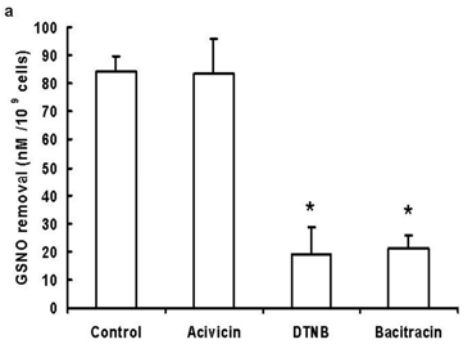
MEG-01 cells were treated with GSNO (4 mM) to modify surface thiols, after which they were washed and exposed to GSH (4 mM). MPB labelling was carried out as previously described and PDI immunoprecipitated using RL 90 monoclonal antibody. Lane 1 - Untreated cells; Lane 2 - Cells treated with GSNO; Lane 3 - Cells treated with GSH; Lane 4 - Cells treated with GSNO followed by GSH.

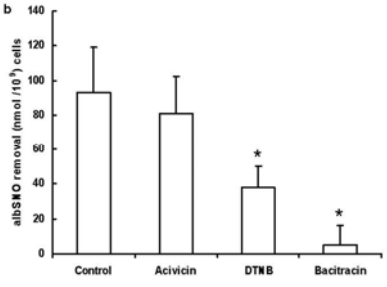

% Aggregation
Control
GSNO (30 sec)
GSNO (5 sec)
AlbSNO (30 sec)
AlbSNO (5 sec)

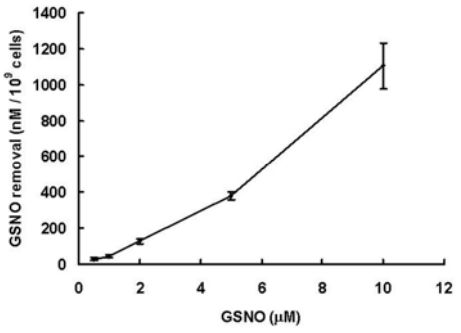

a
cyclic GMP (nM/10^9 cells)
Cells alone
AlbSNO
GSNO

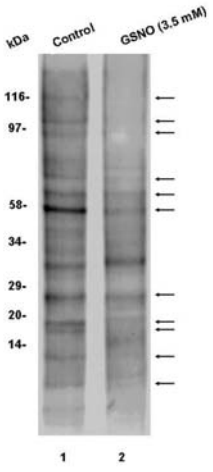

b
Control
Acivicin
Bacitracin
DTNB
cyclic GMP (nM/10^9 cells)
Cells alone
GSNO

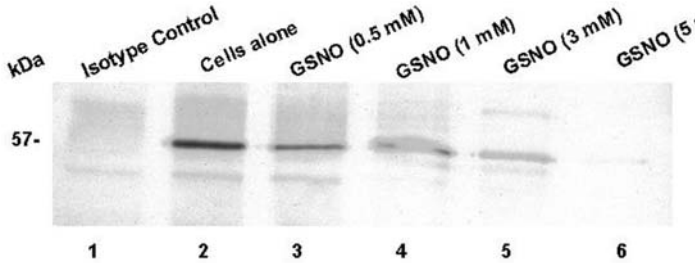

Control
GSNO 1μM
GSNO 10μM
GSNO 100μM
GSNO 500μM
OD 650nm
0hr
20hrs
40hrs


a
GSNO removal (nM /10^9 cells)
Control
Acivicin
DTNB
Bacitracin


b
albSNO removal (nmol /10^9) cells
Control
Acivicin
DTNB
Bacitracin


GSNO removal (nM /10^9 cells)
GSNO (μM)


kDa
Control
GSNO (3.5 mM)
116-
97-
58-
34-
29-
20-
14-
1
2


kDa
Isotype Control
Cells alone
GSNO (0.5 mM)
GSNO (1 mM)
GSNO (3 mM)
GSNO (5 mM)
57-
1
2
3
4
5
6