Biochemical Pharmacology, Vol. 55, pp. 657–665, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00498-X

Cell-Mediated Biotransformation of S-Nitrosoglutathione

Michael P. Gordge, Paolo Addis, Alberto A. Noronha-Dutra and John S. Hothersall*
INSTITUTE OF UROLOGY AND NEPHROLOGY, UNIVERSITY COLLEGE LONDON, UK

ABSTRACT. Spontaneous release of nitric oxide (NO) from S-nitrosothiols cannot explain their bioactivity, suggesting a role for cellular metabolism or receptors. Using immortalised cells and human platelets, we have identified a cell-mediated mechanism for the biotransformation of the physiological S-nitrosothiol compound S-nitrosoglutathione (GSNO) into nitrite. We suggest the name "GSNO lyase" for this activity. GSNO lyase activity varied between cell types, being highest in a fibroblast cell line and lowest in platelets. In NRK 49F fibroblasts, GSNO lyase mediated a saturable, GSNO concentration-dependent accumulation of nitrite in conditioned medium, which was inhibited both by transition metal chelators, and by subjecting cells to oxidative stress using a combination of the thiol oxidant diamide and Zn^{2+}, a glutathione reductase inhibitor. Activity was resistant, however, to both acivicin, an inhibitor of γ-glutamyl transpeptidase (EC 2.3.2.2), and to ethacrynic acid, an inhibitor of P_1 class glutathione-S-transferases (EC 2.5.1.18), thus neither of these enzymes could account for NO release. Although GSNO lyase does not explain the platelet-selective pharmacological properties of GSNO, cellular biotransformation suggests therapeutic avenues for targeted delivery of NO to other tissues. BIOCHEM PHARMACOL 55;5:657–665, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. nitric oxide; S-nitrosothiols; S-nitrosoglutathione; biotransformation; metal chelators; oxidative stress

S-nitrosothiols are bioactive adducts of nitric oxide (NO)† with sulphydryl-containing molecules, for example cysteine residues of proteins and peptides. At physiological pH NO oxidises thiols to generate nitrous oxide and a sulphenic acid [1], however under aerobic conditions S-nitrosothiol formation may arise following reaction of NO with an electron acceptor [2], for example oxygen [3, 4], to yield a nitrosating species such as N_2O_3. In addition, S-nitrosothiol formation during the metabolism of nitrovasodilators may mediate the bioactivity of such drugs [5]. S-nitrosothiols are detectable in both blood and tissues [6–9], and it is speculated that they may be a means to preserve NO from inactivation by superoxide and/or haemoglobin within the biological environment [10].

Glutathione is the most abundant intracellular thiol; its interaction with NO/O_2, and the factors affecting the formation and breakdown of S-nitrosoglutathione (GSNO) are therefore of major importance to an understanding of NO biology [11]. GSNO is a stable molecule, but NO release may be brought about by photolysis [12], catalysis by copper (I) (Cu^+) ions [13], or by transnitrosation to form a less stable species such as S-nitrosocysteine [14]. GSNO shows potent and selective anti-platelet activity [15, 16], and, unlike other NO donors, acts as an arterioselective vasodilator [17]. The basis for this selective behaviour has not yet been defined. The anti-platelet and bronchodilatory activities of GSNO are reported to survive in the presence of the NO scavenging agents oxyhaemoglobin and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolin-1-oxyl 3-oxide (carboxy-PTIO) [18, 8], suggesting that such bioactivity is not mediated by simple release of NO into the surrounding medium. This is consistent with the observed lack of correlation among S-nitrosothiol compounds between their bioactivity and their rate of spontaneous NO release [19, 20], and points to the involvement of mechanisms by which NO is targeted and protected as it passes from the S-nitrosothiol carrier to the responding cell. Evidence for both stereoselective S-nitrosothiol receptors [21], and for metabolising enzymes [19] has been published.

We have previously shown that the anti-platelet aggregatory activity of GSNO is inhibited by the Cu^+ chelating agent, bathocuproine disulphonic acid (BCS) [22], and that NO release from GSNO is accelerated by a BCS-inhibitable enzyme in sonicated platelet preparations [23]. We have now extended these investigations to measure GSNO breakdown by intact cells, and factors involved in its regulation.

* Corresponding author: M. P. Gordge, Institute of Urology and Nephrology, Middlesex Hospital, Mortimer Street, London W1N 8AA, UK; TEL: +44(0)171 636 8333 Ext 3181; FAX: +44(0)1716377006.

† *Abbreviations:* BCS, bathocuproine disulphonic acid; BPS, bathophenanthroline disulphonic acid; BrO, 1-bromooctane; BSO, buthionine sulphoximine; ETA, ethacrynic acid; γ-GT, γ-glutamyl transpeptidase; GSH, reduced glutathione; GSNO, S-nitrosoglutathione; GSSG, oxidised glutathione (glutathione disulphide); NO, nitric oxide.

Received 28 February 1997; accepted 3 September 1997.

MATERIALS AND METHODS

Reagents

All cell culture reagents were from Life Technologies. Bicinchoninic acid reagent for protein measurement was obtained from Pierce. All other reagents were obtained from Sigma Chemicals.

Preparation of S-nitrosoglutathione (GSNO)

GSNO was prepared by the method of Hart [24], dried under vacuum and stored in the dark at −20°. Prior to use, GSNO was dissolved in MilliQ water, and its concentration measured by the absorbance at 334 nm using an extinction coefficient of 0.85 mM^{-1}. Solutions were kept on ice in the dark, and used within 120 min of preparation.

Cell Culture

The following cell lines were obtained from the sources indicated, and cultured for measurement of cell-mediated GSNO biotransformation: (1) NRK 49F rat kidney fibroblasts (ECACC), grown in Dulbecco's MEM F12; (2) AG08473 porcine aortic smooth muscle cells (Coriell), grown in Dulbecco's MEM; (3) AG08472 porcine aortic endothelial cells (Coriell), grown in medium 199; and (4) LLCPK1 porcine kidney epithelial cells (ECACC), grown in Dulbecco's MEM. In all cases medium was supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 10 U/mL penicillin, 10 μg/mL streptomycin and 0.5 μg/mL fungizone. Cells were grown to confluence in 96 well plates at 37° in a humidified atmosphere containing 5% CO_2. Suspensions of washed human platelets from healthy volunteers were obtained as previously described [22], and suspended in Tyrode-HEPES buffer, pH 7.4.

Measurement of Cell-Mediated GSNO Breakdown by Cultured Cell Monolayers

Growth medium was removed and cells washed twice with phosphate buffered saline (PBS) pH 7.4, before being incubated for 30 min in 100 μL Basal medium with Earle's salts (BME), containing HEPES (10 mM) and acivicin (100 μM) to inhibit γ-glutamyl transpeptidase (EC 2.3.2.2)(γ-GT) activity. In preliminary experiments using a colorimetric assay to measure γ-GT on NRK 49F cells by hydrolysis of γ-glutamyl *p*-nitroanilide, this concentration of acivicin caused >95% inhibition of γ-GT.

Following pre-incubation with acivicin, GSNO was added in a volume of 50 μL, and cells were incubated for a further 60 min, after which 100 μL Griess reagent (mixture of equal volumes of naphthylethylenediamine (1 g/L) and sulphanilamide (10 g/L in 5% (v/v) phosphoric acid) was added to each well, and nitrite measured at 570 nm using a MR5000 microplate reader (Dynatech). $NaNO_2$, added to control cells, was used to generate a calibration curve. Parallel control measurements of spontaneous breakdown of GSNO were made in the absence of cell monolayers, and cell-mediated breakdown was calculated by subtracting these control values from values obtained in the presence of cells. The time course of cell-mediated GSNO biotransformation and kinetic parameters were determined. The effects of various chelating agents were measured. Compounds used were the Cu^{+} chelator bathocuproine disulphonate (BCS), the Cu^{2+} chelator cuprizone, the Fe^{2+} chelator bathophenanthroline disulphonate (BPS), the Fe^{3+} chelator desferal and the non-specific chelator EDTA. Each was added 30 min before the addition of GSNO. Effects of manipulation of cellular glutathione metabolism on GSNO biotransformation were assessed following: (1) pre-treatment of cells for 18 hr with buthionine sulphoximine (BSO) (200 μM), an inhibitor of γ-glutamyl cysteine synthetase (EC 6.3.2.2), (2) pre-treatment of cells for 30 min with the thiol oxidant diamide (100 μM) plus $ZnSO_4$ (50 μM), an inhibitor of glutathione reductase (EC 1.6.4.2), (3) 1-bromooctane (BrO) (60 μM), a substrate for glutathione-S-transferase (EC 2.5.1.18) or (4) ethacrynic acid (ETA) (30 μM), a substrate for P_i class glutathione-S-transferases with glutathione reductase inhibitory activity. In parallel experiments both reduced and oxidised glutathione (GSH and GSSG) was measured in cell extracts and released GSSG measured in conditioned medium. The effect of GSSG on GSNO biotransformation was determined by pre-incubating cells for 30 min with GSSG (10^{-7}–10^{-4} M).

Release of Nitrite, Nitrate and NO During NRK Cell-Mediated GSNO Breakdown

In order to investigate whether nitrite appearance during GSNO biotransformation could be accounted for by NO release, experiments were performed to compare the accumulation of 1) NO, 2) nitrite and 3) nitrate, during GSNO breakdown. NRK 49F cells were grown to confluence in 24 well plates, washed and pre-incubated with 1 mL BME containing acivicin, as described above. GSNO (25 μM) and oxyhaemoglobin (10 μM) were then added and cell-mediated breakdown to NO measured over a period of 2 to 30 min, by transfer of supernatant medium to a spectrophotometer cuvette and quantification of NO by the oxidation of oxyhaemoglobin to methaemoglobin using the difference-spectrophotometic method described by Kelm *et al.* [25]. A low GSNO concentration and short incubation time were used since the oxyhaemoglobin method is highly sensitive, and the use of higher concentrations of GSNO, or longer incubation periods, resulted in saturation of haemoglobin and loss of sensitivity.

In parallel experiments, nitrite release was measured by incubation of cells with GSNO in 1 ml of BME without haemoglobin, removal at similar time points of 150 μL of supernatant medium and addition of 100 μL Griess reagent. Nitrate was measured after conversion to nitrite by incubation of supernatant for 30 min with nitrate reductase (20 mU/mL^{-1}) and NADPH (40 μM). The method was

calibrated with $NaNO_3$ and nitrate calculated from the measured increase in nitrite concentration. Cell-mediated GSNO breakdown to NO, nitrite and nitrate was assessed by comparing values obtained in the presence and absence of cells, as described above.

Release of Nitrite and NO by Platelet-Mediated GSNO Breakdown

Suspensions of washed human platelets (1 mL) were incubated for 30 min with acivicin (100 μM) to inhibit γ-GT, and then incubated with GSNO (50 μM) for 60 min at 37°. Platelets were then removed by centrifugation for 2 min at 15,600 × *g*, and nitrite measured in 150 μL of supernatant by the addition of 100 μL Griess reagent, as described above, using $NaNO_2$ diluted in Tyrode-HEPES buffer to generate a calibration curve. Platelet-mediated breakdown was determined by subtracting spontaneous breakdown of GSNO (occurring in Tyrode-HEPES buffer in the absence platelets) from breakdown in the presence of platelets. To assess the effect of platelet activation on cell-mediated GSNO breakdown, platelets were treated for 2 min at 37° with 0.1 units mL^{-1} bovine thrombin, or vehicle, before being incubated with GSNO. Platelet-mediated nitrite appearance was then compared in the presence and absence of thrombin stimulation.

In parallel experiments, platelet-mediated NO release from GSNO was measured by inclusion of oxyhaemoglobin (10 μM) in the sample buffer. Following incubation with GSNO and subsequent removal of platelets, supernatant medium was transferred to a spectrophotometer cuvette and NO quantified by the oxidation of oxyhaemoglobin to methaemoglobin, as described above.

Measurement of Cellular Protein

Culture medium was removed and cells washed twice with PBS, before addition to each well of 250 μL sodium dodecyl sulphate (1% w/v) and incubation overnight at room temperature to allow solubilisation of cellular protein. Samples were then mixed, and protein measured using the bicinchoninic acid method, calibrated against a bovine serum albumin control.

Measurement of Glutathione

Total glutathione (GSH/GSSG) was measured by the recycling method of Tietze using glutathione reductase [26] in (1) supernatant conditioned medium, and (2) cellular extracts prepared by freezing and thawing cells in water containing EDTA (1 mM). Oxidised glutathione disulphide (GSSG) in cellular extracts was measured following derivitisation of reduced glutathione (GSH) for 60 min with 2-vinyl pyridine (100 mM). GSH was then derived by subtracting GSSG from the value obtained for total cellular glutathione.

Measurement of Lactate Dehydrogenase Release

As a measure of cellular injury during various treatments, lactate dehydrogenase (LDH) release into conditioned medium was measured spectrophotometrically [27], and expressed as a percentage of the total intracellular concentration of LDH.

Statistics

Statistical comparison of nitrite accumulation from GSNO over the concentration range 0–100 μM in the presence and absence of NRK49F cells was by two way analysis of variance (ANOVA). Comparisons of GSNO biotransformation, and of glutathione concentrations following different treatments to manipulate glutathione homeostasis were performed by one way ANOVA followed by Student's *t*-test using Bonferroni's correction for multiple comparisons. Comparison of platelet-mediated biotransformation in the presence and absence of thrombin stimulation was made using a paired Student's *t*-test.

RESULTS

NRK Cell-Mediated Biotransformation of GSNO

Incubation of GSNO (0–100 μM) in BME-HEPES buffer alone resulted in spontaneous breakdown, shown by a linear, concentration-dependent accumulation of nitrite. In the presence of confluent NRK 49F cells, nitrite concentrations were significantly increased ($P < 0.001$). Pretreatment of cells with acivicin (100 μM) reduced nitrite accumulation at each level of GSNO by an average of 24% (data not shown), indicating that γGT accounted for this component of nitrite release from GSNO. All subsequent results were obtained with acivicin-treated cells, in which γGT activity was >95% inhibited.

Cell-mediated GSNO biotransformation, obtained by subtracting "spontaneous" nitrite accumulation occurring in the absence of cells, from nitrite accumulation in the presence of acivicin-treated cells, was linear over a period of 60 min (data not shown). In the presence of GSNO concentrations ranging from 5 to 100 μM, this activity was substrate concentration-dependent and showed the characteristics of a saturable system (Fig. 1). In both the presence and absence of cells, no nitrite was detectable unless GSNO was added to the system, indicating that nitrite appeared solely as a result of GSNO breakdown. Homogeneity of cell density among wells of a 96 well plate was assessed by measurement of cellular protein concentration, which showed a coefficient of variation of 7%.

In order to account for the possibility that cell-mediated GSNO breakdown was mediated "non-specifically" by reactive groups in proteins, control experiments were performed in which GSNO conversion to nitrite was monitored in the presence of increasing concentrations of foetal calf serum. Protein-mediated acceleration of nitrite accumulation was observed only when the concentration of

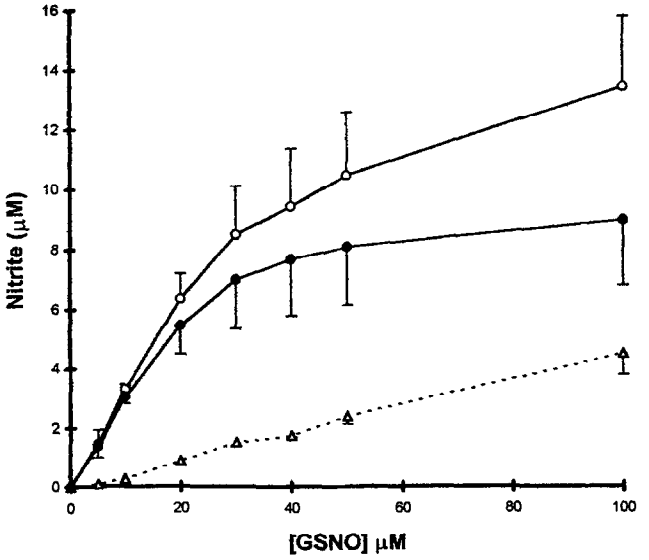

FIG. 1. Nitrite accumulation in conditioned medium following incubation of GSNO (0–100 μM) for 60 min in BME-HEPES medium alone, as a control (open triangles), or BME-HEPES in the presence of a confluent monolayer of acivicin-treated NRK49F cells (open circles). Cell-mediated accumulation of nitrite via biotransformation of GSNO is derived by subtraction of control values from nitrite accumulation obtained in the presence of cells (closed circles). Points shown are mean values from 10 experiments with SD as vertical error bars. Cell-mediated nitrite accumulation is significantly increased compared with control ($P < 0.001$ by two-way ANOVA).

added protein exceeded 1 mg/mL^{-1}, a value approximately 10-fold higher than the total protein concentration obtained after solubilisation of cells. Thus "non-specific" GSNO breakdown did not explain its cell-mediated biotransformation.

Kinetic analysis of NRK 49F cell-mediated GSNO metabolism was performed by the method of Hanes, plotting [S] against [S]/V [28]. Using this transformation, the data failed to follow simple Michaelis–Menton kinetics, indicating the presence of a complex system (Fig. 2). Other transformations of the Michaelis–Menton equation, including Lineweaver–Burk (1/[S] against 1/V) and Eadie–Hofstee (V/[S] against V), failed to provide a clearer kinetic analysis of the data.

GSNO Metabolism by Different Cell Types

GSNO metabolism was detectable for each of the five cell types studied. After 60 min incubation, cell-mediated nitrite production was detectable at concentrations in the low micromolar range. Comparison of the different cell types was made after normalisation of results to cellular protein concentration, to account for differences in cell density. NRK 49F fibroblasts showed the highest activity, and human platelets the lowest. Thrombin-induced platelet activation induced a small, but statistically significant increase in platelet-mediated GSNO breakdown ($P < 0.05$, $n = 9$), although values remained lower than those obtained with other cells (Fig. 3).

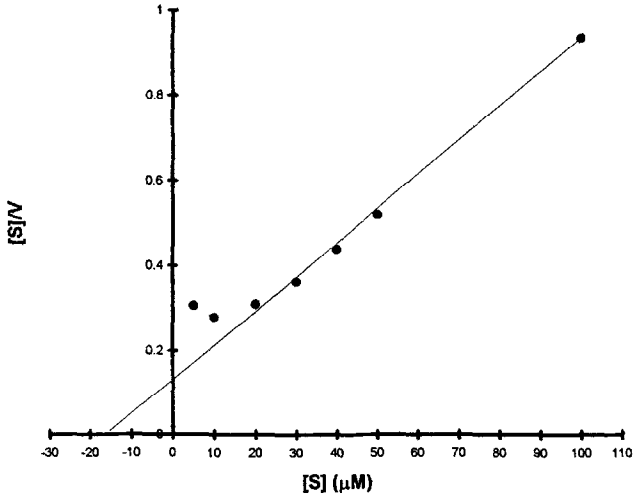

FIG. 2. Hanes plot ([S] vs [S]/V) of cell-mediated nitrite accumulation following incubation of NRK49F cells with GSNO (5–100 μM). Points represent mean values from 5 experiments. K_m value obtained by extrapolation of the linear portion of the curve is 16.2 μM.

Release of Nitrite, Nitrate and NO During Cell-Mediated GSNO Breakdown

NRK cell-mediated biotransformation of GSNO resulted in a parallel, progressive rise of both NO and nitrite, reaching a concentration in the medium of approximately 2 μM after 30 min. In contrast, no nitrate accumulation was observed following cell-mediated GSNO breakdown (Fig. 4). Platelet-mediated biotransformation of GSNO resulted

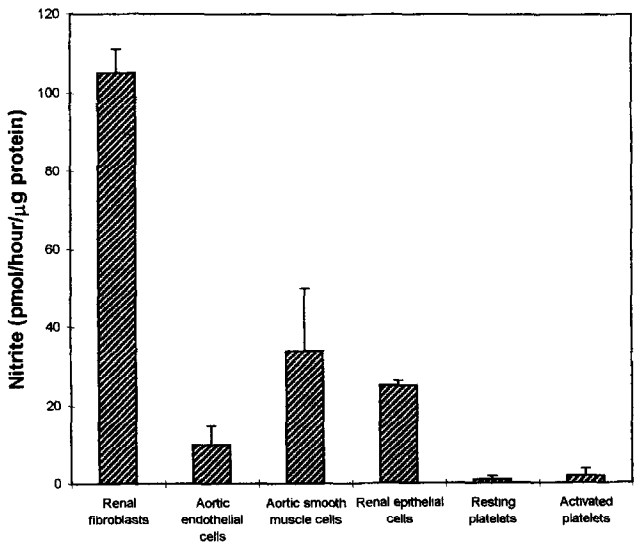

FIG. 3. Biotransformation of GSNO (100 μM), expressed as pmol nitrite/h/μg cellular protein, by NRK 49F rat kidney fibroblasts, AG08473 porcine aortic smooth muscle cells, AG08472 porcine aortic endothelial cells, LLCPK1 porcine kidney epithelial cells and both resting and thrombin-activated human platelets. Biotransformation by activated platelets was significantly increased compared with resting platelets ($P < 0.05$, $n = 6$). Mean values from 3 to 6 experiments are shown, with SD as vertical error bars.

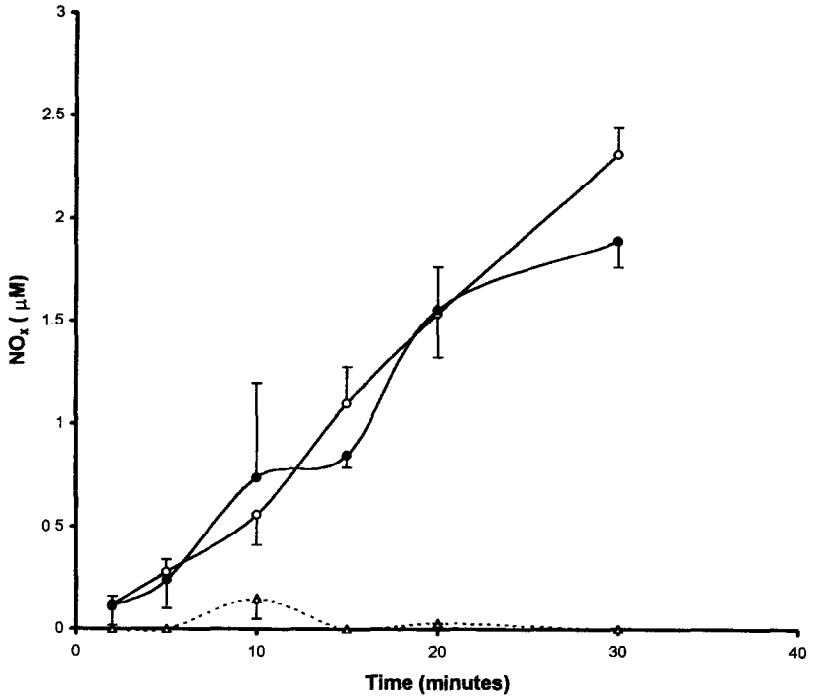

FIG. 4. Accumulation of NO (open circles), nitrite (closed circles) and nitrate (open diamonds) during biotransformation of GSNO. Values shown represent the cell-mediated accumulation of NO_x, derived by subtraction of control values, obtained in the absence of cells, from those obtained in the presence of cells. Mean values from 4 experiments are shown, with SD as vertical error bars.

in accumulation of nitrite to concentrations of 1.16 ± 0.88 μM and 2.16 ± 1.81 μM (mean ± SD) in supernatant medium from resting and activated platelets, respectively. The corresponding values for NO were 0.62 ± 0.76 μM and 1.67 ± 0.69 μM, respectively. In the absence of added GSNO there was no appearance of either nitrate or nitrite, nor any oxidation of oxyhaemoglobin to methaemoglobin.

Effect of Transition Metal Chelating Agents on Cell-Mediated GSNO Metabolism

Concentration-dependent inhibition of NRK cell-mediated GSNO breakdown was shown by both the Cu^+ chelator BCS, and by the iron chelators BPS and desferal (Fig. 5). The Cu^{2+} chelator cuprizone was only partially effective at concentrations above 100 μM. EDTA was ineffective. Cellular injury was not detectable following incubation of cells with any of the chelating agents except desferal. LDH release in the presence of desferal (1 mM) showed a slight increase from control values of 0.5 ± 0.1% to 1.7 ± 0.3% (mean ± SD).

Effect of Manipulation of Cellular Glutathione Homeostasis on GSNO Biotransformation

Intracellular GSH was significantly depleted, compared with control cells, by each of the treatments used ($P < 0.05$, $n = 5$) (Fig. 6). Total glutathione (GSH/GSSG) in cells and conditioned medium was significantly depleted

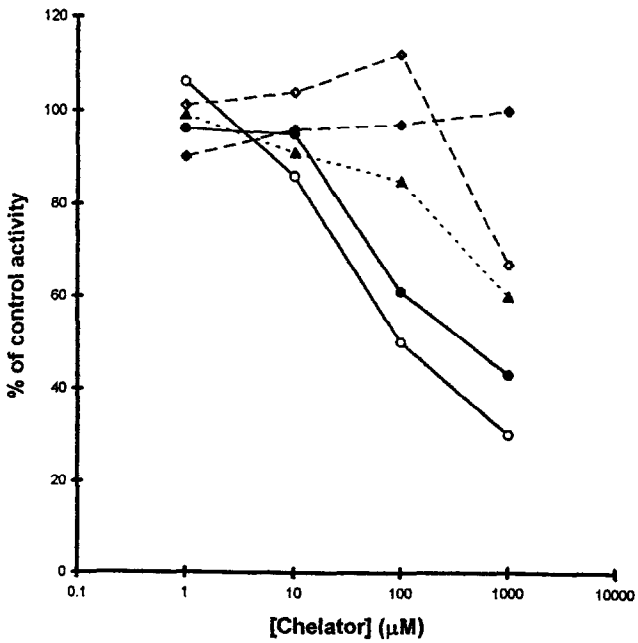

FIG. 5. Effect of metal chelators on biotransformation of GSNO by NRK 49F cells, expressed as a percentage of a control value obtained in the absence of any chelator. Agents used were the Cu^+ chelator BCS (open circles), the Cu^{2+} chelator cuprizone (open diamond), the Fe^{2+} chelator BPS (closed circles), the Fe^{3+} chelator desferal (closed triangles), and the non-specific chelator EDTA (closed diamonds). Points shown are mean values from 8 to 10 experiments. SD error bars have been omitted for clarity.

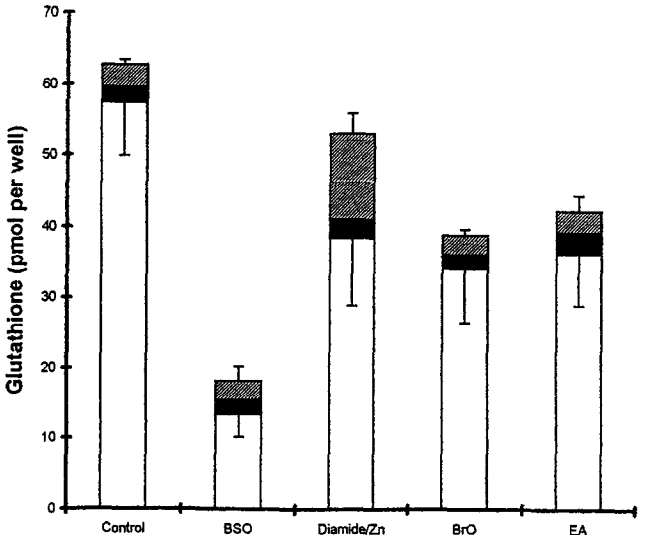

FIG. 6. Intracellular GSH (open bars), GSSG (closed bars) and extracellular GSSG in conditioned medium (hatched bars) following manipulation of glutathione homeostasis in NRK 49F cells. Values shown are means from 5 experiments. Upward vertical error bars are SD for extracellular GSSG. Downward vertical error bars are SD for intracellular GSH. SD values for intracellular GSSG have been omitted for clarity. Intracellular GSH is significantly lower than control following all treatments ($P < 0.05$); total GSH/GSSG in cells and conditioned medium together is significantly lower than control following treatment with BSO, BrO and EA ($P < 0.05$), but not following diamide/Zn.

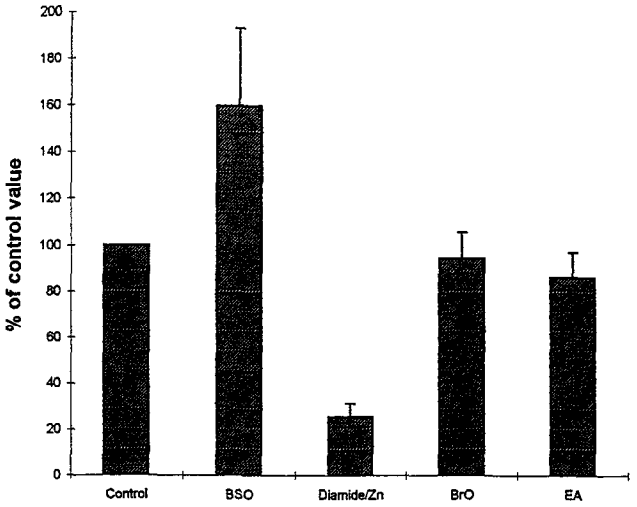

FIG. 7. Biotransformation of GSNO (50 μM) following manipulation of glutathione homeostasis in NRK 49F cells, expressed as a percentage of control value obtained in the absence of any pre-treatment. Values are mean from 5 experiments, with SD shown as vertical error bars. Biotransformation is significantly higher than control following treatment with BSO ($P < 0.05$), and significantly lower than control following treatment with diamide/Zn ($P < 0.001$).

following inhibition of glutathione synthesis with BSO, and conjugation of GSH with either BrO or ETA. Treatment with the thiol oxidant diamide together with the glutathione reductase inhibitor Zn^{2+}, led to an efflux of GSSG into conditioned medium, to a concentration of 12 nM. (Fig. 6).

Cell-mediated GSNO biotransformation was significantly inhibited only following treatment with diamide/Zn^{2+} ($P < 0.001$, $n = 5$). Zn^{2+} alone had no effect (data not shown). BrO and ETA failed to produce any significant alteration, whilst BSO treatment significantly increased GSNO biotransformation ($P < 0.05$, $n = 5$) (Fig. 7). LDH release, as a measure of cellular injury, showed no change from control values following treatments to manipulate glutathione metabolism (data not shown). Alterations in GSNO biotransformation could not, therefore, be explained by cell injury. Pre-treatment of cells with GSSG (0.1–100 μM) failed to produce any inhibition of cell-mediated GSNO breakdown (data not shown).

DISCUSSION

We have demonstrated a saturable activity associated with intact cells which accelerates the biotransformation of the S-nitrosothiol compound GSNO, with consequent release of NO and accumulation of nitrite in the conditioned medium. We suggest the name "GSNO lyase" for this activity.

GSNO lyase was measured in five cell types: fibroblasts, endothelial cells, smooth muscle cells, epithelial cells and platelets. In order to compare activity, results were normalised to the amount of cellular protein. Of the five cell types studied, four were immortalised cell lines, in contrast to platelets, which were freshly prepared. GSNO lyase was present in each of the cell lines studied, but there was variation in the level of activity. It is not yet known whether this reflects differences in expression of enzyme protein, or in regulation of activity between cell types. GSNO lyase in platelets was much lower than that observed in the immortalised cells. We have previously reported a GSNO metabolising enzyme in sonicated platelet preparations, [23], in which NO release proceeded approximately tenfold faster than in the present study, using intact platelets. A similar pattern of accelerated NO release from GSNO by lysed, but not by intact, platelets was reported by Radomski *et al.* [15], who suggested that platelet activation might be required for GSNO metabolism to occur. Following thrombin-induced platelet activation, we found a statistically significant increase in GSNO breakdown, but values remained lower than those obtained with other cell types. These findings are not compatible with a role for GSNO lyase in mediating the platelet selective action of GSNO, however it is possible that measurement of extra-cellular NO release, with subsequent accumulation of nitrite in supernatant medium, is inappropriate to reflect the physiological function of GSNO and GSNO lyase. To address this further we are currently developing methods to detect intra-platelet uptake of NO from GSNO.

NRK 49F fibroblasts showed the highest activity of

GSNO lyase, and were therefore used for investigation of regulatory factors. This activity may be relevant to the physiological control of fibroblast function, since NO regulates fibroblast collagen synthesis, contractility and proliferation [29, 30]. Analysis of GSNO lyase activity failed to show simple Michaelis–Menton kinetics, and suggested the presence of a complex system. This might be due to the fact that enzyme activity was measured using intact cells, where other factors such as substrate accessibility, intracellular redox regulation and intracellular product transfer may act as confounding variables. Extrapolation of the linear portion of this plot predicts an apparent K_m of 16.2 μM, which is greater than the reported concentration of endogenous GSNO [7]. It is possible that the activity at high concentrations of GSNO might arise as a consequence of long term cell culture, but this will require investigation using primary cultures of freshly obtained fibroblast, endothelial, smooth muscle and epithelial cells.

GSNO lyase was inhibited by the Cu^+ chelator BCS, but the Cu^{2+} chelator cuprizone was only weakly inhibitory at mM concentration. These results support our earlier conclusion that Cu^+ must be available for optimum enzymatic release of NO from GSNO. The well-recognised catalytic effect of Cu^+ on spontaneous GSNO breakdown in solution [13] cannot explain this, since it is accounted for in our experiments by subtraction of control GSNO breakdown. We found NRK 49F cell-mediated GSNO metabolism also to be inhibited by chelation of iron using BPS and, more weakly, using desferal. This may be explained either by the small cross-reactivity (2–3%) for copper shown by BPS [31], or, in experiments using intact cells, by a requirement for iron acting in a redox capacity to maintain enzyme-associated copper in a reduced (Cu^+) state. This function can be performed by glutathione in the broken platelet preparations studied previously. Cu^+ redox maintenance would also explain why EDTA was ineffective as an inhibitor, since EDTA-chelated transition metals retain their redox activity. Alternatively, the high capacity system we now describe on NRK 49F and the previously-described platelet-associated GSNO metabolising activity may be distinct, with different metal requirements, and hence different sensitivities to chelators. It is also possible that BCS and BPS inhibit GSNO lyase by virtue of some common mechanism unrelated to metal chelation, although it is unlikely that the sulphonic acid groups on BCS and BPS are responsible, since all experiments were performed in the presence of 10 mM HEPES, itself a sulphonated compound. Inhibition by chelators of metal-dependent enzymes is well described, for example copper-zinc superoxide dismutase (SOD) is inhibited by removal of copper using diethyldithiocarbamate [32]. BPS and BCS are able to bind to protein-bound iron and copper, respectively, and inhibit enzymes such as phenylalanine hydroxylase [33] and dopamine β-monoxygenase [34]. The mechanism of inhibition appears to involve both formation of enzyme-metal-chelator ternary complexes and removal of metal from enzyme protein. BCS and BPS are membrane-impermeable compounds [35], unable to inhibit intracellular metal-dependent enzymes such as SOD [36]. Susceptibility of GSNO lyase to these chelating compounds therefore implies that the activity must reside on the extracellular plasma membrane.

In view of the existence of trans-plasma membrane electron transport systems involving transition metals [35], and of the importance of redox conditions upon the reactivity of S-nitrosothiols [37], the cellular redox environment might play an important regulatory role in GSNO metabolism. In order to investigate this GSNO biotransformation was measured following manipulation of glutathione homeostasis. The treatments used were designed not only to deplete intracellular GSH, but also to alter the state of the GSH/GSSG redox couple. Depletion of GSH, either by inhibition of synthesis with BSO, or by conjugation with either BrO or ETA, failed to inhibit GSNO biotransformation, indeed nitrite accumulation was significantly increased following BSO. In contrast, alteration of the glutathione redox couple using a combination of diamide and Zn^{2+}, resulted both in an accumulation of GSSG in the conditioned medium, and a significant inhibition of GSNO metabolism. It was notable in these experiments that the depletion of intracellular GSH was no more severe than following either BrO or ETA, thus inhibition of GSNO biotransformation resulted not from a loss of glutathione, but from an increase in oxidative stress. Oxidation of extracellular protein thiol groups by diamide, leading to formation of mixed disulphides, might underlie the observed inhibition. GSNO lyase was unaffected by pretreatment of cells with GSSG up to 100 μM (~1,000-fold high than the concentration observed in cell supernatants), indicating that inhibition by diamide/Zn^{2+} was not mediated by competition between GSNO and extracellular GSSG, but rather on oxidative mechanisms involving mixed disulphide formation.

GSNO lyase cannot yet be identified, however a number of extra- and intracellular enzymes could be responsible for GSNO lyase-like activity. For example, extracellular γ-GT cleaves the glutamyl-cysteine bond to convert GSNO to the much less stable compound S-nitrosocystylglycine, with subsequent release of NO [38]. γ-GT is present on NRK 49F cells and is capable of accelerating NO release from GSNO, as demonstrated by inhibition of nitrite accumulation following treatment with acivicin. There remained, however, a substantial cell-associated capacity to release NO from GSNO, as shown in our study, where all results were obtained following inhibition of γ-GT. The failure of ethacrynic acid to inhibit GSNO biotransformation also excludes P_i class glutathione-S-transferase as the metabolising enzyme. Recent published data have shown catalysis of S-nitrosothiol breakdown by glutathione peroxidase [39], thioredoxin [40], and an unidentified enzyme present in *E. coli* [41]. Further studies will be required to determine their relationship to GSNO lyase.

Surveys of the biological activity of S-nitrosothiols clearly indicate that their mechanism of action cannot rely

solely upon spontaneous decomposition to release NO into solution [19, 20]. The cellular biotransformation mechanism we have described may help to explain some of their actions, and to provide possible targets for tissue-selective delivery of NO.

We thank Dr. David Meyer for helpful discussions, and the St. Peter's Trust for supporting this project.

References

1. Demaster EG, Quast BJ, Redfern B and Nagasawa HT, Reaction of nitric oxide with the free sulphydryl group of human serum albumin yields a sulfenic acid and nitrous oxide. *Biochemistry* **34:** 11494–11499, 1995.
2. Gow AJ, Buerk DG and Ischiropoulos H, A novel reaction mechanism for the formation of S-nitrosothiol *in vivo*. *J Biol Chem* **272:** 2841–2845, 1997.
3. Wink DA, Nims RW, Darbyshire JF, Christodoulou D, Hanbauer I, Cox GW, Laval F, Laval J, Cook JA, Krishna MC, Degraff WG and Mitchell JB, Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solutions at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O_2 reaction. *Chem Res Toxicol* **7:** 519–525, 1994.
4. Kharitonov VG, Sundquist AR and Sharma VS, Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. *J Biol Chem* **270:** 28158–28164, 1995.
5. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ and Gruetter CA: Mechanism of vascular smooth muscle relaxation by organic nitrates, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* **218:** 739–749, 1981.
6. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR and Loscalzo J, Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc Natl Acad Sci USA* **89:** 7674–7677, 1992.
7. Gaston B, Reilly J, Drazen JM, Fackler J, Ramdev P, Arnelle D, Mullins ME, Sugarbaker DJ, Chee C, Singel DJ, Loscalzo J and Stamler JS, Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. *Proc Natl Acad Sci USA* **90:** 10957–10961, 1993.
8. Naseem KM, Chirico S, Mohammadi B and Bruckdorfer KR, The synergism of hydrogen peroxide with plasma S-nitrosothiols in the inhibition of platelet activation. *Biochem J* **318:** 759–766, 1996.
9. Do KQ, Benz B, Grima G, Gutteck-Amsler U, Kluge I and Salt TE, Nitric oxide precoursor arginine and S-nitrosoglutathione in synaptic and glial function. *Neurochem Int* **29:** 213–224, 1996.
10. Stamler JS, A radical vascular connection. *Nature* **380:** 108–111, 1996.
11. Hogg N, Singh RJ and Kalyanaraman B, The role of glutathione in the transport and catabolism of nitric oxide. *FEBS Lett* **382:** 223–228, 1996.
12. Sexton DJ, Muruganandam A, McKenney DJ and Mutus B, Visible light photochemical release of nitric oxide from S-nitrosoglutathione: potential photochemotherapeutic applications. *Photochem Photobiol* **59:** 463–467, 1994.
13. Dicks AP, Swift HR, Williams DLH, Butler AR, Al-Sa'Doni HH and Cox BG, Identification of Cu^+ as the effective reagent in nitric oxide formation from S-nitrosothiols (RSNO). *J Chem Soc Perkin Trans* **2:** 481–487, 1996.
14. Singh RJ, Hogg N, Joseph J and Kalyanaraman B, Mechanism of nitric oxide release from S-nitrosothiols. *J Biol Chem* **271:** 18596–18603, 1996.
15. Radomski MW, Rees DD, Noronha-Dutra A and Moncada S, S-nitroso-glutathione inhibits platelet activation *in vitro* and *in vivo*. *Br J Pharmacol* **107:** 745–749, 1992.
16. De Belder AJ, Macallister R, Radomski M, Moncada S and Vallance PJT, Effects of S-nitrosoglutathione in the human forearm circulation: evidence for selective inhibition of platelet activation. *Cardiovasc Res* **28:** 691–694, 1994.
17. Macallister RJ, Calver AL, Riezebos J, Collier J and Vallance P, Relative potency and arteriovenous selectivity of nitrovasodilators on human blood vessels: an insight into the targeting of nitric oxide delivery. *J Pharmacol Exp Ther* **273:** 154–160, 1995.
18. Bannenberg G, Xue J, Engman L, Cotgreave I, Moldeus P and Ryrfeldt A: Characterization of bronchodilator effects and fate of S-nitrosothiols in the isolated perfused and ventilated guinea pig lung. *J Pharmacol Exp Ther* **272:** 1238–1245, 1995.
19. Kowaluk EA and Fung H, Spontaneous liberation of nitric oxide cannot account for in vitro vascular relaxation by S-nitrosothiols. *J Pharmacol Exp Ther* **256:** 1256–1264, 1990.
20. Mathews WR and Kerr SW, Biological activity of S-nitrosothiols: the role of nitric oxide. *J Pharmacol Exp Ther* **267:** 1529–1537, 1993.
21. Davisson RL, Travis MD, Bates JN and Lewis SJ: Hemodynamic effects of L- and D-S-nitrosocysteine in the rat. Stereoselective S-nitrosothiol recognition sites. *Circ Res* **79:** 256–262, 1996.
22. Gordge MP, Meyer D, Hothersall JS, Neild GH, Payne NN and Noronha-Dutra AA, Copper chelation-induced reduction of the biological activity of S-nitrosothiol. *Br J Pharmacol* **114:** 1083–1089, 1995.
23. Gordge MP, Hothersall JS, Neild GH and Noronha-Dutra AA, Role of a copper (I)-dependent enzyme in the antiplatelet action of S-nitrosoglutathione. *Br J Pharmacol* **119:** 533–538, 1996.
24. Hart TW, Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. *Tetrahed Lett* **26:** 2013–2016, 1995.
25. Kelm M, Feelisch M, Spahr R, Piper H, Noack E and Schrader J, Quantitative and kinetic characterization of nitric oxide and EDRF released from cultured endothelial cells. *Biochem Biophys Res Comm* **154:** 236–244, 1988.
26. Tietze F, Enzymic method for quantitative determination of nanogram amounts of total and oxidised glutathione: applications to mammalian blood and other tissues. *Anal Biochem* **27:** 502–522, 1969.
27. Bergmeyer HU, *Methods of Enzymatic Analysis*. (2nd ed). Academic Press, New York, London, 1965.
28. Dowd JE and Riggs DS, A comparison of estimates of Michaelis–Menton kinetic constants from various linear transformations. *J Biol Chem* **240:** 863–869, 1965.
29. Schaffer MR, Efron PA, Thornton FJ, Klingel K, Gross SS and Barbul A, Nitric oxide, an autocrine regulator of wound fibroblast synthetic function. *J Immunol* **158:** 2375–2381, 1997.
30. Garg UC and Hassid A, Nitric oxide-generating vasodilators inhibit mitogenesis and proliferation of BALB/C 3T3 fibroblasts by a cyclic GMP-independent mechanism. *Biochem Biophys Res Comm* **171:** 474–479, 1990.
31. Blair D and Diehl H, Bathophenanthrolinedisulphonic acid and bathocuproinedisulphonic acid, water soluble reagents for iron and copper. *Talanta* **7:** 163–174, 1961.
32. Cocco D, Calabrese L, Rigo A, Argese E and Rotilio G, Re-examination of the reaction of diethyldithiocarbamate with the copper of superoxide dismutase. *J Biol Chem* **256:** 8983–8986, 1981.
33. Shiman R, Gray D and Hill MA, Regulation of rat liver

phenylalanine hydroxylase. 1. Kinetic properties of the enzyme's iron and enzyme reduction site. *J Biol Chem* **269:** 24637–24646, 1994.
34. Skotland T and Ljones T, Enzyme-bound copper of dopamine beta-monooxygenase. Activation of the holoenzyme by added copper and uncoupling of electron transfer from hydroxylation by copper salicylate. *J Inorg Biochem* **18:** 11–18, 1983.
35. Alcain FJ, Low H and Crane FL, Iron at the cell surface controls DNA synthesis in CC1 39 cells. *Biochem Biophys Res Comm* **203:** 16–21, 1994.
36. Kelner MJ, Bagnell R, Hale B and Alexander NM, Inactivation of intracellular copper-zinc superoxide dismutase by copper chelating agents without glutathione depletion and methemoglobin formation. *Free Radica Biol Med* **6:** 355–360, 1989.
37. Arnelle DR and Stamler JS, NO^+, NO^- and NO^- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch Biochem Biophys* **318:** 279–285, 1995.
38. Hogg N, Singh RJ, Konorev E, Joseph J and Kalyanaraman B, S-nitrosoglutathione as a substrate for γ-glutamyl transpeptidase. *Biochem J* **323:** 477–481, 1997.
39. Hou Y, Guo Z, Li J and Wang PG, Seleno compounds and glutathione peroxidase catalysed decomposition of S-nitrosothiols. *Biochem Biophys Res Comm* **228:** 88–93, 1996.
40. Nikitovic D and Holmgren A, S-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J Biol Chem* **271:** 19180–19185, 1996.
41. Hausladen A, Privalle CT, Keng T, Deangelo J and Stamler JS, Nitrosative stress: activation of the transcription factor OxyR. *Cell* **86:** 719–729, 1996.