Glyoxalase I is a novel nitric-oxide-responsive protein

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To clarify the molecular mechanisms of nitric oxide (NO) signalling, we examined the NO-responsive proteins in cultured human endothelial cells by two-dimensional (2D) PAGE. Levels of two proteins [NO-responsive proteins (NORPs)] with different pI values responded to NO donors. One NORP (pI 5.2) appeared in response to NO, whereas another (pI 5.0) disappeared. These proteins were identified as a native form and a modified form of human glyoxalase I (Glox I; EC 4.4.1.5) by peptide mapping, microsequencing and correlation between the activity and the isoelectric shift. Glox I lost activity in response to NO, and all NO donors tested inhibited its activity in a dose-dependent manner. Activity and normal electrophoretic mobility were restored by dithiothreitol and by the removal of sources of NO

from the culture medium. Glox I was selectively inactivated by NO; compounds that induce oxidative stress (H₂O₂, paraquat and arsenite) failed to inhibit this enzyme. Our results suggest that NO oxidatively modifies Glox I and reversibly inhibits the enzyme's activity. The inactivation of Glox I by NO was more effective than that of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), another NO-sensitive enzyme. Thus Glox I seems to be a novel NO-responsive protein that is more sensitive to NO than G3PDH.

Key words: endothelial cell, nitrosative stress, oxidation, post-translational modification, two-dimensional PAGE.

INTRODUCTION

Nitric oxide (NO) is a signalling molecule that is produced by NO synthases. It influences physiological processes in essentially all organs and tissues [1–3]. The functions of NO cover a remarkably broad spectrum; roles for NO have been identified in neurotransmission and memory formation, the prevention of blood clotting, the regulation of blood pressure and mediation of the bactericidal activity of macrophages. NO is a short-lived molecule that reacts with oxygen molecules, transition-metal ions and thiols [1,4]. Binding of NO to haem in soluble guanylate cyclase activates this enzyme as part of the cGMP-dependent signalling pathways. It has been suggested that the interaction of NO with Fe-S and haem centres in mitochondrial electron transporters and the S-nitrosation of a thiol residue in glyceraldehyde-3-phosphate dehydrogenase (G3PDH) might contribute to the cellular dysfunction mediated by NO. The NO-mediated activation of signal-transducer proteins, such as G-protein [5], kinases and phosphatases [6–8] and transcription factors [9,10], would be expected to modulate a wide variety of responses. However, little is known about the overall cellular response to NO in terms of protein synthesis and the modification of proteins.

Exposure of eukaryotic cells to environmental stresses such as a heat shock [11] and oxidative insult [12] results in the synthesis and modification of a small set of proteins. Although NO is an important participant in several physiological processes, the large amounts of NO produced by the activation of an inducible nitric oxide synthase in an inflammatory situation might result in the exposure of host cells, as well as target cells, to oxidative stress [13]. Under such conditions, complex oxidative species,

formed via reactions of NO with superoxide (O₂^{•−}) and O₂, can modify macromolecules that include proteins and DNA; this phenomenon is known as nitrosative stress [9]. Under such stress conditions, NO must obviously affect the activities of various functional proteins and modulate many cellular functions that act to repair or replace damaged components of the cell. However, little is known about the stress-response mechanism(s) mediated by NO.

Endothelial cells are exposed to large amounts of NO, which is produced by activated macrophages and smooth-muscle cells under inflammatory and infectious conditions [13,14]. In the present study we examined the effects of NO donors on patterns of protein expression in human endothelial cells (ECV304) by two-dimensional (2D) PAGE: we identified a novel NO-responsive protein (NORP26) as glyoxalase I (Glox I). The response of Glox I to NO was reversible oxidative modification with a loss of enzymic activity. We suggest that human Glox I might be a potential target protein under pathophysiological conditions.

MATERIALS AND METHODS

Materials

S-Nitrosoglutathione (GSNO) and S-nitrosocysteine were synthesized as described by Hart [15]. 3-(2-Hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPANONOate) and 2,2'-(hydroxynitrosohydrazono)bis-ethanamine (DETANONOate) were purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). 3-Morpholino-sydnominine hydrochloride (SIN-1), S-nitroso-N-acetyl-D,L-penicillamine (SNAP), GSH, 8-bromoguanosine 3',5'-cyclic monophosphate, diethyl maleate (DEM),

Abbreviations used: 2D, two-dimensional; DEM, diethyl maleate; DETANONOate, 2,2'-(hydroxynitrosohydrazono)bis-ethanamine; DTT, dithiothreitol; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Glox I, glyoxalase I; GSNO, S-nitrosoglutathione; IEF, isoelectric focusing; MG, methylglyoxal; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; NORP, NO-responsive protein; PAPANONOate, 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine; SIN-1, 3-morpholino-sydnominine hydrochloride; SNAP, S-nitroso-N-acetyl-D,L-penicillamine.

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dithiothreitol (DTT), PMSF, CHAPS and CNBr were obtained from Wako Chemical Co. (Osaka, Japan). A 40% (v/v) solution of methylglyoxal (MG) and 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, U.S.A.). Pharmalytes (preblended pH 5–8 and pH 3–10) were obtained from Pharmacia Biotech (Tokyo, Japan). Medium 199 (M199) was purchased from Gibco BRL (Grand Island, NY, U.S.A.).

Culture of cells and treatment of reagents

Spontaneously immortalized human endothelial cells, ECV304 [16], were cultured in M199 supplemented with 5% (v/v) fetal bovine serum, 100 i.u./ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified air/CO₂ (19:1) atmosphere. Chemicals were dissolved as appropriate solutions, which were filtered before addition to the culture medium. In all experiments described below, cell viability determined by MTT assay [17] remained greater than 90%.

2D PAGE

After treatment, cells were washed with PBS and lysed in a lysis buffer that contained 9.2 M urea, 2% (w/v) CHAPS, 2% (v/v) ampholines [4:1 (v/v) ratio of pH 5–8 to pH 3–10], 2 mM Na₂EDTA and 1 mM PMSF, with or without 70 mM DTT. After centrifugation at 2000 g for 10 min, supernatants were analysed by 2D PAGE. Concentrations of proteins dissolved in the lysis buffer were determined by a modified version of Bradford's assay [18], with ovalbumin as a standard.

2D PAGE was performed by O'Farrell's method [19] with slight modifications. In brief, for the first dimension, isoelectric focusing (IEF) gels (1.7 mm × 105 mm) consisted of 9.2 M urea, 2% CHAPS, 2% pharmalytes [4:1 (v/v) ratio of pH 5–8 to pH 3–10] and 4% (w/v) polyacrylamide [30:0.8 (w/v) ratio of mono to bis]. IEF was performed at a constant 500 V for 15 h; IEF gels were then equilibrated for 10 min in Laemmli's sample buffer [20] containing 62.5 mM Tris/HCl, pH 6.8, 1% (w/v) SDS and 10% (v/v) glycerol and with or without 70 mM 2-mercaptoethanol. Each IEF gel was then fixed with 1% (w/v) agarose on the flat top of a second gel [12.5% (w/v) polyacrylamide]. Electrophoresis in the second dimension was performed as described by Laemmli [20]. Proteins on gels were detected by staining with silver. Dried gels after staining were scanned with a scanner (model GT-8500; Epson, Suwa, Japan) with Photoshop 2.5J software (Adobe System, San Jose, CA, U.S.A.). Intensities of spots were quantified with NIHimage software (National Institutes for Health, Bethesda, MD, U.S.A.).

Preparative 2D PAGE and microsequencing

Preparative 2D PAGE was performed with a slightly modified version of the method described above. In this system, IEF gels (2.0 mm × 95 mm) were prepared in glass tubes; at most 500 µg of protein (80 µl) per gel was applied. After 2D PAGE, gels were stained with Coomassie Brilliant Blue R-250 and the spots of interest were excised and dried completely *in vacuo*. Dried pieces of gel were treated with 1% (v/v) CNBr in 70% (v/v) formic acid for 16 h under nitrogen. Fragmented polypeptides were fractionated electrophoretically [21], transferred to a PVDF membrane (Immobilon P⁸⁰; Millipore, Bedford, MA, U.S.A.), and revealed by staining with Coomassie Brilliant Blue R-250. Amino acid sequences were analysed with a peptide sequencer (model 473A; Applied Biosystems, Foster City, CA, U.S.A.).

Measurement of Glox 1 activity

ECV304 cells, with or without prior treatment with an NO donor, were harvested, disrupted by sonication and centrifuged at 7000 g; supernatants were used for measurements of Glox I activity. Glox I activity was determined by measuring the increase in *A*₂₄₀ resulting from the formation of *S*-esters from hemithioacetal adducts of MG and GSH [22] in a spectrophotometer (model 150-20; Hitachi Ltd., Tokyo, Japan). One unit of Glox I activity was defined as the activity generating 1 µmol of *S*-ester/min at 25 °C under the conditions of the reaction. For the reactivation of inactivated Glox I, proteins were incubated with 50 mM DTT at 37 °C for the indicated durations.

Measurement of G3PDH activity

Cell lysates were prepared as described in the previous section. G3PDH activity was determined by measuring the decrease in *A*₃₄₀ of a mixture that contained 82.3 mM triethanolamine buffer, pH 7.6, 1.1 mM ATP, 6.2 mM 3-phosphoglycerate, 0.2 mM NADH, 0.9 mM EDTA, 2 mM MgSO₄ and 13 units/ml phosphoglycerate kinase [23]. One unit of G3PDH activity was defined as the activity consuming 1 µmol of NADH/min at 25 °C under the conditions of the reaction.

Determination of protein concentrations

Concentrations of protein for the determination of enzymic activities were determined by Lowry's method [24], with BSA as a standard.

Quantification of *S*-nitrosothiols

S-Nitrosothiols in culture supernatants were quantified by Saville's method [25]. In brief, 20 µl of culture medium was acidified by the addition of 200 µl of 0.1 M H₂SO₄. After 2 min, 200 µl of a 0.5% solution of ammonium sulphamate was added and left to react for 3 min. To this mixture, 160 µl of a mixture of 0.25% HgCl₂ and 2.55% sulphanilamide in 0.4 M HCl and 160 µl of a solution of 0.38% *N*-1-naphthylethylenediamine in 0.4 M HCl were added sequentially. After 5 min, *A*₅₄₀ was measured; *S*-nitrosothiols were quantified by extrapolation from a standard curve prepared with GSNO.

RESULTS

Detection of NO-responsive proteins by 2D PAGE

To identify proteins whose levels were affected by exposure to NO, we examined the patterns of expression of proteins in ECV304 cells by a differential protein display method with 2D PAGE. More than 800 protein spots were separated with an apparent range of molecular masses from 10 to 150 kDa and a range of pI values from 4.7 to 7.0, as detected by silver staining. Figure 1 shows the patterns after 2D PAGE for control cells and cells treated with NO radical donors. One newly expressed protein was detected after cells had been treated with PAPAN-ONOate at 1 mM for 2 h. This 26 kDa protein had a pI of 5.2 and was designated NORP26/5.2 (NO-responsive protein of 26 kDa, with a pI of 5.2). At the same time, the intensity of the spot of a 26 kDa protein that had a slightly more acidic pI of 5.0 (NORP26/5.0) seemed to decrease.

Exposure of cells to GSNO, another NO donor, also resulted in the appearance of NORP26/5.2 under non-reducing conditions (Figures 2A and 2B). When the cell lysates were reduced by the addition of DTT to the lysis buffer for 2D PAGE, NORP26/5.2 did not appear and the level of NORP26/5.0 remained unchanged in the analysis of GSNO-treated cells

Figure 1 Patterns of protein expression in control (A) and NO-radical-treated (B) ECV304 cells

Confluent ECV304 cells were incubated with or without 1 mM PAPANONOate for 2 h. After 2D PAGE, proteins were revealed by silver staining. Molecular masses (in kDa) and pI values are shown on the vertical and horizontal axes respectively. The spots corresponding to NORP26/5.0 and NORP26/5.2 are indicated by arrowheads. See the text for details.

Figure 2 Effects of reducing conditions on the expression of NORP26/5.2

Control (A, C) and GSNO-treated (1 mM, 2 h) (B, D) cells were lysed with lysis buffer in the presence or absence of 70 mM DTT. Then 50 μ g of proteins were resolved by 2D PAGE under non-reducing (A, B) or reducing (C, D) conditions. Proteins were revealed by silver staining. Regions of gels around NORP26s are shown. Arrowheads indicate NORP26/5.0 (left) and NORP26/5.2 (right).

(Figures 2C and 2D). Cycloheximide (10 μ g/ml), an inhibitor of protein synthesis, had no effect on the GSNO-mediated expression of NORP26/5.2 (results not shown). When ECV304 cells were pulse-labelled with [35 S]methionine or $H_3^{32}PO_4$ in combination with treatment with GSNO, no radioactivity accumulated in NORP26s, indicating that NORP26/5.2 was formed in response to NO as a consequence of a post-translational event, independently of phosphorylation or dephosphorylation

reactions (results not shown). These results suggested that NORP26/5.2 might be produced by post-translational modification of NORP26/5.0 in a reductant-reversible manner. No other similar proteins were detected in NO-treated cells when we used non-equilibrium pH gradient electrophoresis in the first dimension of 2D PAGE, which resolved proteins with pI values up to approx. 12 (results not shown).

Identification of NORP26s by microsequencing

The NORPs were purified by cutting out the corresponding spots from gels after 2D PAGE. Fragments of purified NORP26/5.0 and NORP26/5.2 were prepared by digestion with CNBr in an attempt to identify these NO-responsive proteins. No differences were found between the peptide maps of NORP26/5.0 and NORP26/5.2, both of which were cleaved into two major fragments of 12 and 14 kDa (results not shown). The amino acid sequences of each 14 kDa fragment were obtained by microsequencing. From the 14 kDa fragments of NORP26/5.0, one serial amino acid sequence, (M)KFSLYFLAYEDKNDI, was obtained. A computer-assisted search of sequence databases revealed that an internal sequence of this fragment was identical with that of an internal peptide [(M)K⁶⁷FSLYFLAYEDKNDI⁸¹] of human Glox I (*S*-D-lactoylglutathione lyase; EC 4.4.1.5), which participates in the glyoxalase system for metabolism of MG, a toxic by-product of glycolysis [26]. These results revealed that human Glox I was a novel NO-responsive protein that undergoes post-translational modification and, moreover, that the pI of Glox I is rendered slightly more basic on modification by NO. Thus NORP26/5.0 and NORP26/5.2 were designated Glox I/5.0 and Glox I/5.2 respectively.

Inhibition of the activity of Glox I by NO and reactivation of the enzyme

Glox I catalyses an isomerization reaction, converting hemithioacetal adducts of MG and GSH to *S*-esters (Scheme 1). We

Scheme 1 Glyoxalase system

The glyoxalase system [26] is involved in the metabolism of toxic α -oxoaldehydes such as MG to α -hydroxycarboxylic acids, with GSH as a cofactor. Hemithioacetal, a non-enzymic adduct of MG and GSH, is converted into S-D-lactoylglutathione by Glox I. The S-ester is hydrolysed by Glox II and a non-toxic metabolite, D-lactate, is formed with the regeneration of GSH.

Figure 3 Correlation between the inactivation of Glox I and modification of the enzyme by GSNO

Confluent ECV304 cells were incubated in the presence or absence of 1 mM GSNO. At the indicated times, cells were harvested and disrupted by sonication. (A) After centrifugation, supernatants were used for assaying Glox I activity. Enzymic activities are shown as means $\pm$ S.D. ($n = 3$). The concentration of S-nitrosothiol in culture supernatants was also determined at the indicated times (A). Symbols: $\circ$, control Glox I activity; $\bullet$, Glox I activity of GSNO-treated cells; $\times$, concentration of GSNO. (B) Intracellular proteins were analysed by 2D PAGE and subsequent silver staining. Arrowheads indicate Glox I/5.0 (left) and Glox I/5.2 (right). The number at the left of each panel is the length of incubation (in h). (C) The intensities of the spots that corresponded to the two forms of Glox I were determined by image analysis. The relative levels of Glox I/5.0 are expressed as percentages.

examined the effects of NO on the activity of Glox I by exposing cells to GSNO for increasing periods (Figure 3A). The activity decreased rapidly within 0.5 h and reached 10% of the control level within 2 h. The activity remained low for up to 20 h when GSNO was present in the culture medium, even though the amount of GSNO gradually decreased during the incubation (Figure 3A). The activity was partly restored after the complete disappearance of GSNO. GSNO-treated cells were analysed by

2D PAGE for changes in the mobility of Glox I during the incubation, to estimate the relationship between the shift in mobility and the activity (Figure 3B). The spot of Glox I/5.2 was clearly detected after the exposure of cells to GSNO for 0.5 h. The level of Glox I/5.2 reached a maximum at 2 h; that of Glox I/5.0 was very much lower at 2 h than in control cells. Figure 3(C) shows the relative levels of Glox I/5.0 and Glox I/5.2 as determined by the semi-quantification of the intensities of the

Figure 4 Restoration of Glox I activity after the removal of GSNO

Confluent ECV304 cells were incubated with 1 mM GSNO for 1 h, washed with PBS and incubated in fresh medium without GSNO. At the indicated times, cells were harvested, disrupted and centrifuged. Glox I activity was measured in the supernatants. The activities are shown as means $\pm$ S.D. ($n = 3$).

spots of Glox I by image analysis. The proportion of Glox I/5.0 decreased rapidly to its lowest level within 2 h and remained low until 20 h; then the relative level of Glox I/5.0 began to increase again. These results indicated that the modification of Glox I was correlated with the loss of enzymic activity.

We investigated the reversibility of the NO-mediated inactivation of Glox I. Cells were incubated with GSNO for 1 h to inhibit Glox I and rinsed to remove extracellular GSNO. Washed

cells were reincubated with fresh medium without GSNO for 4 h; Glox I activity was monitored (Figure 4). The activity returned to approx. 80 % of the control level. We also examined whether the inactivation and modification of Glox I by NO could be reversed by exposure of the enzyme to the reducing agent DTT. After the inhibition of Glox I activity in intact cells, lysates were incubated in the absence and in the presence of 50 mM DTT for 4 h (Figure 5A). The activity of Glox I that had been inhibited by NO increased gradually during the first 2 h of incubation, returning to 70 % of the control level in the presence of DTT. In contrast, GSH (10 mM) and cysteine (10 mM) had no restorative effects (results not shown). We monitored the pI of Glox I in DTT-treated lysates by 2D PAGE (Figure 5B). Glox I/5.2 was found predominantly in GSNO-treated lysates before the addition of DTT, whereas Glox I/5.0, which had disappeared as a result of the treatment of cells with GSNO, reappeared on treatment of the lysate with DTT. These results suggested that NO inhibited Glox I activity by reversible modification at a critical thiol residue of the active site and that the chemical modification of Glox I with NO was reversed by reducing systems.

Modulation of the activity of Glox I by various oxidative reagents

The enzymic activity and mobility on 2D gels of Glox I were monitored after cells had been treated with the following: generators of NO radicals (PAPANONOate and DETANONOate); a co-generator of NO radicals and $O_2^{\cdot-}$ (SIN-1); S-nitrosothiols (SNAP, GSNO and S-nitrosocysteine); generators of reactive oxygen species (H_2O_2 and paraquat); and thiol-reacting compounds (NaAsO₂ and DEM) (Figure 6). The activity of Glox I was decreased significantly and in a dose-dependent manner when the cells were treated with each of the NO donors. In contrast, reactive oxygen species and thiol-reacting compounds had no effects on the activity (Figure 6A), in spite of the fact that the inactivation of Glox I had been reversed by reducing systems, as described above. A membrane-permeable

Figure 5 Reversibility of the inactivation of Glox I by DTT

Lysates from control and GSNO-treated (1 mM, 2 h) ECV304 cells were incubated at 37 °C in the presence or absence of 50 mM DTT. (A) At the indicated times, aliquots were removed and Glox I activity was measured. Glox I activities are shown as means $\pm$ S.D. ($n = 3$). Symbols: ○, control cell lysates without DTT; ●, control cell lysates with DTT; □, GSNO-treated cell lysates without DTT; ■, GSNO-treated cell lysates with DTT. (B) The mobility of Glox I was analysed by 2D PAGE under non-reducing conditions. Arrowheads indicate Glox I/5.0 (left) and Glox I/5.2 (right).

Figure 6 Effects of various compounds on the activity and electrophoretic mobility of Glox I

Confluent ECV304 cells were treated with various NO donors, inducers of oxidative stress and other chemicals at the indicated concentrations for 2 h. **(A)** Cells were harvested, disrupted and centrifuged; Glox I activity was determined in the supernatants. Glox I activities are shown as means $\pm$ S.D. ($n = 3$). Abbreviations: 8BrcGMP, 8-bromoguanosine 3',5'-cyclic monophosphate; CysNO, S-nitrosocysteine; SNAP, S-nitroso-N-acetyl-D,L-penicillamine. **(B)** The proteins were analysed by 2D PAGE and revealed by silver staining. In **(B)** the panels are as follows: **(a)** control; **(b)** PAPANONOate at 1 mM; **(c)** SIN-1 at 5 mM; **(d)** GSNO at 1 mM; **(e)** H₂O₂ at 0.3 mM; **(f)** DEM at 1 mM. Arrowheads indicate Glox I/5.0 (left) and Glox I/5.2 (right).

Figure 7 Inactivation of G3PDH in ECV304 cells treated with NO donors

Confluent ECV304 cells were incubated in the presence or absence of 1 mM PAPANONOate or 1 mM GSNO. At the indicated times, cells were harvested, disrupted and centrifuged; G3PDH activity in the supernatants was determined. Enzymic activities are shown as means $\pm$ S.D. ($n = 3$). Symbols: ○, control cells; ●, GSNO-treated cells; □, PAPANONOate-treated cells.

analogue of cGMP, 8-bromoguanosine 3',5'-cyclic monophosphate, had no effect, indicating that the effect of NO on Glox I was independent of cGMP. All the NO donors increased the pI

of Glox I, whereas neither H₂O₂ nor DEM did so (Figure 6B). The shift in mobility on gels was closely correlated with the Glox I-inactivating ability of each compound tested. These results demonstrated that the modification and inactivation of Glox I were specific to NO-mediated oxidation and indicated that the modified thiol residue was probably located in a site that was inaccessible to DEM.

Sensitivity of enzymes to NO

The effects of NO on G3PDH activity were examined under the same conditions as those described in the legend to Figure 5, to compare Glox I with G3PDH in terms of sensitivity to NO. G3PDH is an enzyme that is modified at a critical thiol residue by NO with concomitant loss of activity [27]. As shown in Figure 7, the activity of G3PDH decreased transiently to 60% and 80% of the control level after treatment of cells with PAPANONOate and GSNO respectively; it returned to the control level after 4 h. The extent of inactivation of G3PDH was more moderate than that of Glox I, demonstrating that Glox I was more sensitive than G3PDH to NO.

DISCUSSION

We separated proteins in ECV304 cells into approx. 800 species by 2D PAGE and showed that the levels of two proteins (NORPs) were affected by treating cells with NO generators. The NORPs were proteins of 26 kDa with pI values of 5.0 (NORP26/5.0) and 5.2 (NORP26/5.2). The level of NORP26/5.0 decreased

in response to NO, with the concomitant appearance of NORP26/5.2 (Figure 3). The NORPs were identified as human Glox I by microsequencing. Glox I seemed to be a novel and unique enzyme in ECV304 cells that was selectively responsive to NO (Figure 6). The activity of Glox I was always lost when the pI of the enzyme shifted to 5.2. The response of Glox I to NO was the oxidative modification of Glox I, leading to a loss of the enzymic activity.

Both the inactivation and the modification of Glox I were largely reversed by DTT and by the removal of the source of NO (Figures 4 and 5). Inactivation by NO has also been demonstrated in enzymes that contain thiol groups essential for catalysis, such as protein kinase C [6], G3PDH [27] and caspases [28]. Inactivation of these enzymes by NO can be reversed by treatment with reducing thiols such as DTT or GSH. The NO-mediated inactivation of these enzymes is probably due to the interaction of NO with essential thiols to form *S*-nitrosothiols. The reversibility of the NO-induced response of Glox I suggests that the modification of a critical thiol residue in human Glox I is responsible for the inhibition of Glox I activity.

Human Glox I has four Cys residues in the entire amino acid sequence [29]. One of these, Cys¹³⁹, is conserved from bacteria and yeast to mammals. However, no interest has been taken in Cys residues for the catalytic activity of Glox I, partly because chemical modification studies revealed that mammalian Glox I was insensitive to thiol-modifying reagents [30,31], in contrast with yeast Glox I [32]. In our experiments, DEM had no effects on the activity of Glox I in human endothelial cells. The crystal structure of Glox I [33] indicates that the active site of human Glox I is situated at the dimer interface and the Cys residue at position 61 is located in the hydrophobic cavity of the active site. The distance between sulphur atoms of Cys⁶¹ and a substrate is 8.65 Å, as calculated by a RasMol software [34], indicating that Cys⁶¹ could interact with substrates. Thus Cys⁶¹ might be involved in the catalysis in the hydrophobic cavity of human Glox I that interacts with NO.

The mobility shift on gels of Glox I in response to NO indicates structural modification of the protein by NO. The change in pI of Glox I was reversed by the addition of DTT to a cell extract or the removal of the source of NO. It therefore seems likely that the structural modification of Glox I by NO is disulphide formation, either protein–protein or protein–GSH, as well as nitrosothiol formation described above. Because 2D PAGE indicates that the molecular mass is unchanged after the modification, intermolecular disulphide formation between Glox I subunits is negligible. In addition, the basic shift in the pI value indicates that the net charge is increased after the modification. Because free thiol groups have neutral charges, the modification must lead to the formation of positive charges in the whole subunit. Disulphide bond formation with GSH and nitrosothiol formation would cause a decrease and no alteration in the net charge of parent proteins respectively. Therefore all of these types of modification are unlikely. One possible candidate for the modification is disulphide bond formation with basic thiols such as cysteamine. Although the stable isoform of Glox I modified by NO remains to be characterized, it is likely that the response of Glox I is induced via *S*-nitrosation.

Glox I is involved in the metabolism of MG, which is a toxic by-product of glycolysis. The accumulation of MG in cells causes the formation of advanced glycation end-products ('AGEs') [35], with inhibitory effects on DNA synthesis and cell proliferation [26]. Abnormal overexpression of Glox I has been linked with both diabetes mellitus and cellular proliferative disorders, including cancer [26]. Inactivation of Glox I by NO would induce the accumulation of MG, affecting various bio-

logical functions and pathophysiological conditions. Inhibitors of Glox I have been prepared as potent anti-tumour [36] and anti-malarial drugs [37]. In this context it is interesting to note that NO also has anti-tumour activity [38] and that GSNO is an effective anti-malarial drug that is 1000-fold more potent than NO [39]. GSNO might inhibit Glox I activity more than donors of NO radicals (Figure 6). Glox I might be a more sensitive target for NO than other NO-sensitive enzymes such as G3PDH because the extent of inactivation of Glox I was much greater than that of G3PDH (Figures 3 and 7). These results suggest that biological effects of NO might result, in part, from the potent inhibition of Glox I.

The inhibitors of Glox I that were synthesized as drugs, as described above, were all competitive inhibitors. Our study indicates that NO might convert Glox I into an inactive isoform. Because extracellularly added NO donors can potentially lead to Glox I inactivation, we suggest that all types of NO donor should be considered as potential effective therapeutic agents for the treatment of diseases in which the expression of Glox I is responsible for initiation and/or progression.

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