

Nitric Oxide Inactivates Glyoxalase I in Cooperation with Glutathione

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We previously found that glyoxalase I (Glo I) is inactivated upon exposure of human endothelial cells to extracellular nitric oxide (NO), and this event correlates with an increase in its pI on two-dimensional gels. In this study, we demonstrate that NO can modulate Glo I activity in cooperation with cellular glutathione (GSH). Severe depletion of intracellular GSH prevents the inactivation of Glo I in response to NO, although such depletion enhances the inactivation of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a well-known enzyme susceptible to NO-induced oxidation. S-Nitrosoglutathione (GSNO), an adduct of GSH and NO, lowers the activity of purified human Glo I, while S-nitrosocysteine (CysNO) inactivates the enzyme only in the presence of GSH. This indicates that a dysfunction in Glo I would require the formation of GSNO *in situ*. Competitive inhibitors of Glo I, S-(4-bromobenzyl)glutathione and its membrane-permeating form, completely abolish the NO action *in vitro* and inside cells, respectively. Taken together, these results reveal that Glo I can interact directly with GSNO, and that the interaction converts Glo I into an inactive form. Moreover, the data suggest that the substrate recognition site of Glo I might be involved in the interaction with GSNO.

Key words: endothelial cells, glutathione, glyoxalase I, nitrosative stress, S-nitrosoglutathione.

Nitric oxide (NO) is a physiological mediator that is produced from L-arginine and O₂ by NO synthase (NOS) in a wide variety of cells (1). A large amount of NO is produced, in particular, by inducible NOS under inflammatory and infectious conditions. NO is responsible for oxidative injury to host cells and tissues, in addition to its role in the exclusion of cancerous cells from normal tissues and protecting the self-body from external viruses and bacteria. NO reacts with O₂ and O₂⁻ at relatively high rates with the subsequent formation of oxidants such as N₂O₃ and ONOO⁻ (2). These oxidants bring about nitrosative stress (3), which is a form of oxidative stress associated with a change in the redox environment in the direction of oxidation. It has been reported that NO-derived species modify a variety of proteins with subsequent perturbations of biological events. Nitrosylation of heme iron is involved in the inactivation of mitochondrial respiratory enzymes (4, 5), leading to energy

depletion. Nitration of a tyrosine residue in Cu,Zn-superoxide dismutase (6) lowers the ability of the enzyme to scavenge superoxide anion. It has been proposed that S-nitrosation of the β-subunit of hemoglobin might influence the regulation of blood pressure (7, 8). Although it has been suggested that these types of modifications, mediated by NO, might be involved in the regulation of biological functions, it seems that these modified proteins represent, in large part, the results of NO-induced oxidative injury.

Glutathione (GSH) is an anti-oxidant molecule that plays a major role in protecting cells from NO-induced oxidative injury (9, 10). GSH reacts with NO to form S-nitrosoglutathione (GSNO) in the presence of an electron acceptor (11). GSH levels in cells exposed to NO decrease markedly and can be restored by reduction with sodium borohydride (12). Cu(I) degrades GSNO in a relatively specific fashion (13, 14), and the chelation of Cu(I) prevents the NO-mediated activation of guanylate cyclase (15). These observations indicate that GSNO is formed under biological conditions. However, reports of the detection of GSNO are limited (16, 17). GSNO is somewhat stable in pure aqueous solution in darkness, even at ambient temperature (18), but it is too unstable to allow detection under biological conditions. This instability is partly due to the fact that GSNO is degraded by Cu(I) (13, 14), superoxide (19, 20), γ-glutamyltranspeptidase (21) and the thioredoxin/thioredoxin reductase system (22), and to the fact that GSNO transfers its NO moiety to other thiol compounds by transnitrosation to yield unstable S-nitrosothiols (23).

In a previous study (24), we demonstrated that glyoxalase I (Glo I) is a novel NO-responsive protein, and we showed that Glo I is reversibly inactivated by extracellular

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; BSO, L-buthionine-[S,R]-sulfoximine; CysNO, S-nitrosocysteine; DEM, diethyl maleate; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Glo I, glyoxalase I; hGlo I, human glyoxalase I; mGlo I, mammalian glyoxalase I; GSBB, S-(4-bromobenzyl)glutathione; GSBBdiEt, S-(4-bromobenzyl)glutathione diethyl ester; GSNO, S-nitrosoglutathione; MG, methylglyoxal; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium; NO, nitric oxide; PAPANONOate, 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-panamine.

NO with concomitant oxidative modification. The inactivation and chemical modification of Glo I does not occur in response to other inducers of cell stress such as H_2O_2 , sodium arsenite and alkyl radical initiators. Glo I is more sensitive to NO than glyceraldehyde-3-phosphate dehydrogenase (G3PDH), which is known as an enzyme with typical sensitivity to NO. It is known that Glo I is involved in the metabolism of α -oxoaldehydes and catalyzes the isomerization reaction from the adduct of methylglyoxal and GSH to S-D-lactoylglutathione (25). This was the first evidence that Glo I activity is modulated by the oxidation and reduction specifically induced by NO. Glo I recognizes the hemithioacetal adduct of GSH and methylglyoxal as a substrate. Although Glo I has broad specificity for substrates with 2-oxoaldehyde linkages to the sulfhydryl moiety of GSH, Glo I specifically recognizes the GSH moiety. These observations prompted us to consider the possibility that the inactivation of Glo I by NO might result from a specific interaction of Glo I with GSNO at the substrate-recognition site. In this study, we examined the involvement of GSH in the inactivation of Glo I by NO.

MATERIALS AND METHODS

Materials—S-Nitrosoglutathione (GSNO) and S-nitrosocysteine (CysNO) were synthesized as described by Hart (26). S-Octylglutathione (OG) was synthesized as described by Vince *et al.* (27). S-(4-Bromobenzyl)glutathione (GSBB) and S-(4-bromobenzyl)glutathione diethyl ester (GSBB-diEt) were synthesized by a modified version of the method described by Lo and Thornally (28), as described below. PAPANONOate was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). A 40% solution of methylglyoxal (MG), L-buthionine-[S,R]-sulfoximine (BSO), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium (MTT) were obtained from Sigma (St. Louis, MO, USA). Diethyl maleate (DEM) and dithiothreitol (DTT) were obtained from Wako Chemical (Osaka). Other chemicals used were of the highest grade commercially available.

Synthesis of GSBB and GSBB-diEt—GSH (3.07 g; 10 mmol) was dissolved in 1 N NaOH (20 ml) with stirring at room temperature (r.t.). Ethanol was added until the solution turned cloudy. Then, 4-bromobenzyl bromide (2.50 g; 10 mmol) dissolved in ethanol was added to the mixture in aliquots over the course of 10 min. Vigorous stirring was continued for 3 h after the addition had been completed. The pH of the mixture was adjusted to 3.5 by the dropwise addition of 57% HI and the mixture was chilled. Solids were collected by filtration and washed with ice-cold water. The final colorless powder was identified as GSBB.

GSBB (1.0 g; 2.1 mmol) was dissolved in ethanol (50 ml). Then concentrated H_2SO_4 (1 ml) was slowly added on ice and the mixture was stirred at r.t. for 3 d. The pH of the mixture was adjusted to 5 with triethylamine. After the addition of di-*tert*-butyldicarbonate (2 ml) on ice, the mixture was stirred at r.t. for 1 h. A saturated solution of NaHCO_3 (50 ml) was added to raise the pH above 7.0. After ether extraction, the organic layer was washed with 1 N HCl and dried with Na_2SO_4 . After the complete removal of ether, the amorphous residue was dissolved in trifluoroacetic acid and held at r.t. for 1 h. A saturated solution of NaHCO_3 (20 ml) was added and the product was extracted

with ethyl acetate. The organic layer was dried with Na_2SO_4 , and the complete evaporation of this layer yielded a colorless powder, GSBB-diEt, with a yield of 525 mg (47%). The properties of the compound were as follows: melting point, 107–111°C; $^1\text{H-NMR}$ (400 MHz; d_6 -DMSO): δ = 1.15 (t, 3H, $-\text{CH}_3$ of ethyl group at C-terminal, J = 6.4 Hz), 1.17 (t, 3H, $-\text{CH}_3$ of the ethyl group at the N-terminal, J = 6.4 Hz), 1.61 (m, 1H, β - CH_2 - of γ -Glu), 1.83 (m, 1H, β - CH_2 - of γ -Glu), 2.23 (m, 2H, γ - CH_2 - of γ -Glu), 2.48 (m, 1H, β - CH_2 - of Cys), 2.72 (m, 1H, β - CH_2 - of Cys), 3.27 (m, 1H, α -CH- of γ -Glu), 3.71 (s, 2H, benzyl group), 3.81 (d, 2H, $-\text{CH}_2$ - of Gly, J = 5.2 Hz), 4.05 (q, 2H, $-\text{CH}_2$ - of the ethyl group at the C-terminal, J = 6.4 Hz), 4.06 (q, 2H, $-\text{CH}_2$ - of the ethyl group at the N-terminal, J = 6.4 Hz), 4.53 (m, 1H, α -CH- of Cys), 7.27 (d, 2H, *o*-H of the benzyl group, J = 8.0 Hz), 7.47 (d, 2H, *m*-H of the benzyl group, J = 8.0 Hz), 8.15 (d, 1H, $-\text{NH}-$ of Cys, J = 8.8 Hz), 8.49 (t, 1H, $-\text{NH}-$ of Gly, J = 5.2 Hz); fast atom bombard-mass spectrometry: 532:534 = 1:1 (M+H).

Cell Culture—Human endothelial cells (ECV304) were cultured in M199 medium (Gibco; Grand Island, N.Y., USA), supplemented with 5% heat-inactivated fetal bovine serum (FBS) and containing 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin, at 37°C in a humidified atmosphere of 5% CO_2 in air. When the cells were treated with chemicals, the medium was replaced by M199 plus 5% FBS. Chemicals were dissolved as appropriate solutions that were filtered before being added to the culture medium. Cell viability was assessed by MTT assay (29).

Depletion of Intracellular GSH—Confluent ECV304 cells were incubated in culture medium with or without 1 mM BSO for 20 h. Then the medium was changed to freshly prepared culture medium containing BSO (1 mM) and/or DEM (0.5 mM). After 2 h, individual NO donors were added to the medium and the cells were incubated for a further 2 h.

Estimation of Intracellular Levels of GSH—Approximately 5×10^6 confluent ECV304 cells treated with BSO and/or DEM were lysed in 400 μl of 15% trichloroacetic acid, and the lysate was centrifuged at 10,000 $\times g$ for 2 min. Then 350 μl of the supernatant was removed and brought to pH 7 with 300 μl of 4 M K_2HPO_4 . GSH levels were determined by measuring the increase in absorbance at 412 nm after mixing 960 μl of reaction mixture (100 μM NADPH, 5 mM DTNB, 1 U/ml glutathione reductase, 1 mM EDTA, and 50 mM potassium phosphate, pH 7.0) with 40 μl of the sample or a standard solution of GSH (30).

Purification of Glyoxalase I from Human Erythrocytes—Human Glo I was purified as described by Mannervik *et al.* (31). In brief, hemoglobin was first removed from erythrocytes by sedimentation with an organic solvent. The supernatant was loaded onto a column of ethylamino-Sepharose 4B, prepared by conjugation of aminoethane with cyanogen bromide-activated Sepharose 4B (Pharmacia, Tokyo), and the column was eluted with a linear gradient of potassium phosphate. The active fraction was then applied to a column of S-OG-Sepharose 4B, prepared by conjugation of OG with cyanogen bromide-activated Sepharose 4B. The column was washed with 2 mM GSH and then purified Glo I was obtained by elution with 2 mM OG. Before the *in vitro* inactivation assay, the Glo I preparation was passed through a size-exclusion column (G-25) to separate OG from the protein. A solution of 50 mM Tris-HCl buffer (pH

8.0) containing 1 mM DTT and 20% glycerol was used as the elution buffer. One unit of Glo I activity was defined as the activity that generates 1 μ mol of thioester per minute at 25°C (32).

In vitro Inactivation Assay—Purified Glo I was not so stable at 25°C and the basal level of activity was not maintained. An inactivation assay was performed at 4°C in assay buffer containing 10 units/ml purified Glo I, 1 mM DTT, and 20% glycerol in 50 mM Tris-HCl buffer (pH 8.0). The solution was supplemented with 1 mM S-nitrosothiol (final concentration) in a total volume was 100 μ l. Small aliquots were removed at the indicated times and Glo I activity was determined by measuring the increase in absorbance at 240 nm (A_{240}) (32). None of the additives had any effect on the absorbance change at 240 nm. The effects of GSH and GSBB were examined by mixing these compounds with the Glo I solution prior to the addition of S-nitrosothiol.

Quantification of S-Nitrosothiols—S-Nitrosothiols (RSNOs) were quantified by the method of Saville (33). In brief, 20 μ l of sample was acidified by adding 200 μ l of 0.2 N H_2SO_4 . After 2 min, 200 μ l of a 0.5% solution of ammonium sulfamate was added and the mixture was allowed to react for 3 min. The mixture was then supplemented with 160 μ l of a mixture of 0.25% $HgCl_2$ and 2.55% sulfanilamide in 0.4 N HCl, and then with 160 μ l of a solution of 0.38% N-1-naphthylethylenediamine in 0.4 N HCl. After 5 min, the absorbance at 540 nm was measured and the RSNOs were quantified by extrapolation from a standard curve generated with GSNO.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)—Two-dimensional PAGE was performed as described previously (24). Proteins on the gels were visualized by silver staining.

Measurements of Enzyme Activities—Cells were harvested with a rubber policeman and disrupted by sonication. Homogenates were centrifuged at 7,000 $\times g$ for 10 min at 4°C and the enzymatic activities in the supernatants

were determined. Glo I activity was determined by measuring the increase of A_{240} due to the formation of S-D-lactoylglutathione (32). G3PDH activity was determined as described by Vedia *et al.* (34). Protein concentrations in the cell lysates were determined by Lowry's method with bovine serum albumin as the standard (35).

RESULTS

The NO-Dependent Inactivation and Modification of Glo I Require Intracellular GSH—Both PAPANONOate and GSNO, compounds that release NO, markedly inhibited the

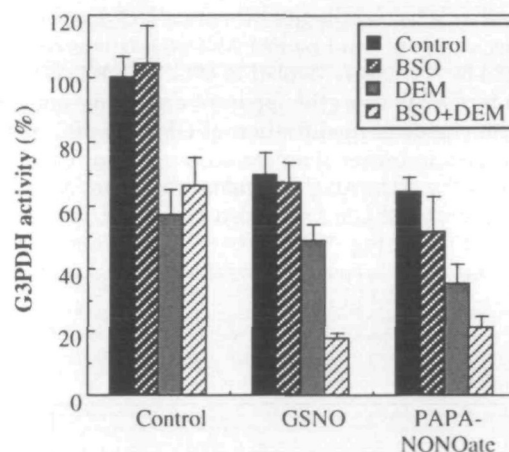


Fig. 2. Effects of GSH depletion on the NO-mediated inactivation of G3PDH. The levels of GSH were reduced and samples were prepared as described in the legend to Fig. 1, and then G3PDH activity was measured. Activities are expressed as mean \pm SD ($n = 3$). The 100% activity (0.56 unit/mg protein) corresponds to the activity in control cells without pretreatment with GSH depletor. Black bars, no pretreatment; thickly hatched bars, pretreatment with BSO; gray bars, pretreatment with DEM; and finely hatched bars, pretreatment with both BSO and DEM.

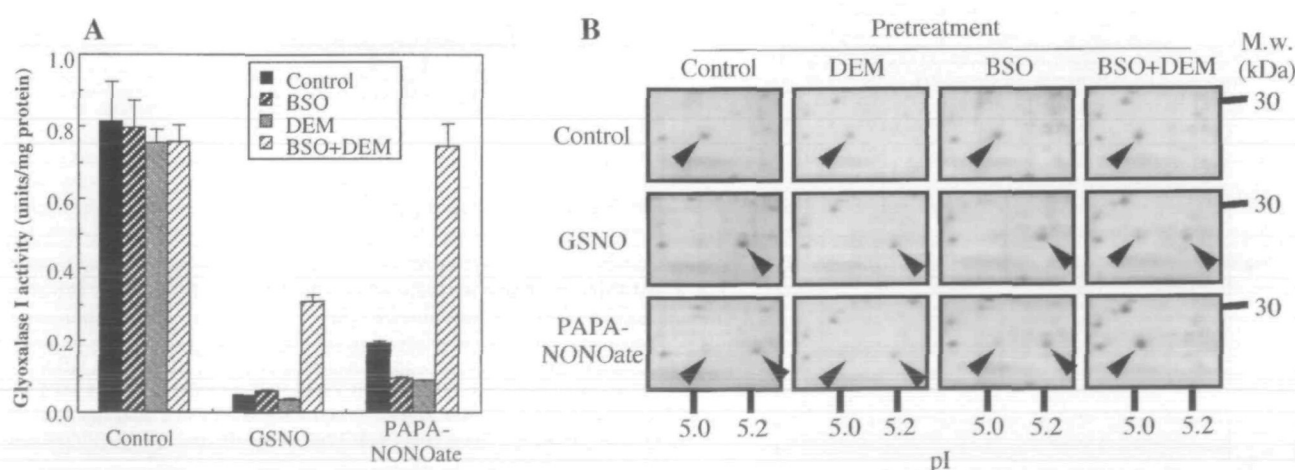


Fig. 1. Effects of GSH depletion on the response of Glo I to NO. Confluent ECV304 cells were incubated with and without BSO (1 mM, 20 h), an inhibitor of the biosynthesis of GSH. The medium was changed to medium containing BSO (1 mM) and/or DEM (0.5 mM), which alkylate the sulfhydryl group of GSH. After a 2-h incubation, NO donors (1 mM) were added to the medium and the cells were incubated for an additional 2 h. Cell lysates were prepared for the mea-

surement of Glo I activity (A) and analysis by 2D-PAGE (B). (A) Solid bars, no pretreatment; thickly hatched bars, pretreatment with BSO; gray bars, pretreatment with DEM; finely hatched bars, pretreatment with both BSO and DEM. (B) Proteins were visualized by silver staining. The areas corresponding to pI 4.7 to pI 5.3 and 20 kDa to 30 kDa are shown. Arrowheads indicate Glo I with a pI of 5.0 (left) and Glo I with a pI of 5.2 (right).

activity of Glo I in ECV304 cells (Fig. 1), consistent the previous result (24). To assess the involvement of GSH in the inactivation of Glo I by NO, ECV304 cells were treated with GSH depletors, as described in "MATERIALS AND METHODS." The levels of intracellular GSH (reduced form) in cells treated with BSO or DEM fell to 14 or 36% of the control level (3.01 ± 0.54 nmol/ 10^6 cells), respectively. Treatment with both BSO and DEM reduced the intracellular level of GSH to about 1% of the control level. Cell viability remained above 90% during a 30-h incubation with BSO and/or DEM (data not shown). The inactivation of Glo I by GSNO (1 mM) or PAPANONOate (1 mM) was prevented to a considerable extent by the severe depletion of intracellular GSH, while treatment with either BSO or DEM had no effect in the absence of an NO donor (Fig. 1A). The inactivation of Glo I by PAPANONOate was completely prevented by the severe depletion of GSH, while some inactivation by GSNO was still apparent under the same conditions. The chemical modification of Glo I by NO has been characterized in terms of an increase in the pI value of Glo I. Analysis by 2D-PAGE revealed that the NO-induced change in the pI of Glo I was also prevented by the severe depletion of GSH (Fig. 1B). These results demonstrate that intracellular GSH is essential for the chemical modification

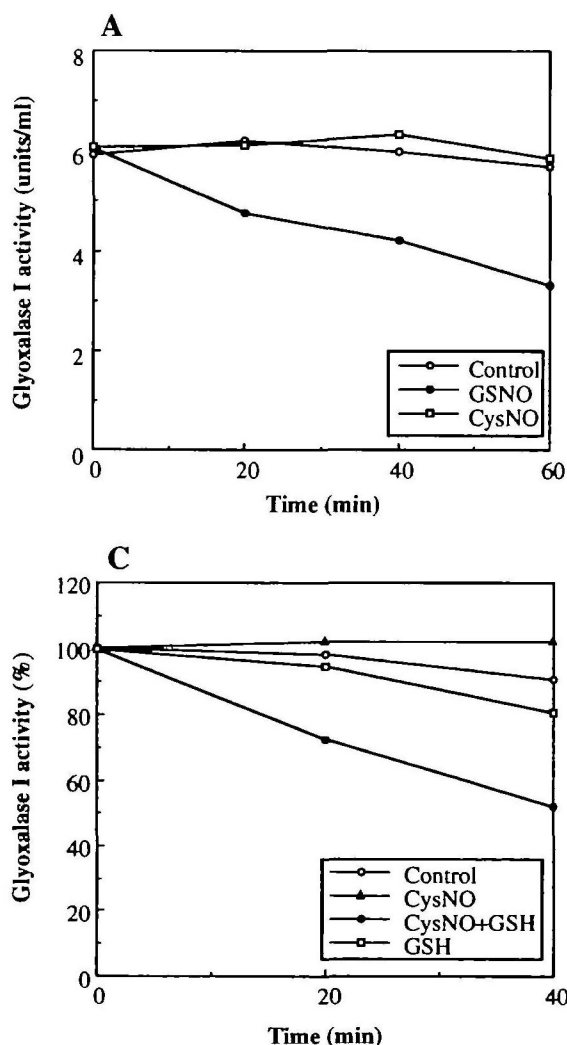


Fig. 3. Inhibition of the activity of purified hGlo I by GSNO. The effect of NO donors on Glo I was examined *in vitro*. Purified Glo I from human erythrocytes was used after the complete removal of S-octylglutathione as described in the text. (A) Purified hGlo I (3 units) was mixed with 1 mM GSNO or CysNO in assay buffer (50 mM Tris-HCl (pH 8.0) containing 1 mM DTT and 20% glycerol). At the indicated times, aliquots were removed and residual Glo I activity was measured. Data represent typical results from four independent experiments. (B) An aliquot of the reaction mixture described in (A), removed after 60 min, was analyzed by 2D-PAGE. The areas corresponding to pI 4.7 to pI 5.3 and 20 to 30 kDa are shown. The native form (left; pI 5.0) and the modified form (right; pI 5.2) of hGlo I were visualized by silver staining. (C) Purified hGlo I was mixed with 1 mM CysNO in the presence or absence of 1 mM GSH in the assay buffer. At the indicated times, aliquots were removed and the residual Glo I activity was measured. A 100% activity (0.52 unit/ml) corresponds to the initial activity in the control sample.

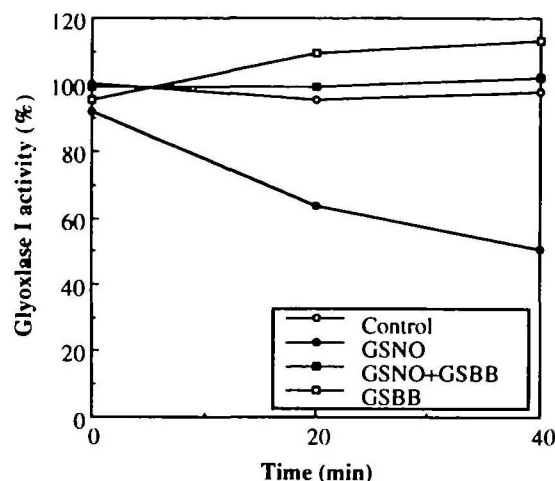


Fig. 4. Effect of GSBB on the GSNO-induced inactivation of hGlo I. Purified hGlo I was mixed with 1 mM GSNO in the presence or absence of 100 μ M GSBB in the assay buffer. At the indicated times, aliquots were removed and residual Glo I activity was measured. Data represent typical results from two similar experiments.

and inactivation of Glo I by NO.

We also examined the effects of GSH depletion on the activity of G3PDH in cells treated with GSNO or PAPANONOate (Fig. 2). PAPANONOate and GSNO each reduced the activity of G3PDH to 60% of the control level. Treatment with BSO and/or DEM partially inhibited the activity of G3PDH in control cells, but the inactivation of G3PDH was potentiated upon exposure of the cells to GSNO or PAPANONOate. In contrast to the inactivation of Glo I by NO, the inactivation of G3PDH by NO was not prevented, but rather, was stimulated by GSH depletion.

Glo I Interacts Directly with GSNO—We postulated that the inactivation of Glo I might be caused by interaction of the enzyme with GSNO in a direct fashion, since the effect of extracellular NO on Glo I required intracellular GSH, and Glo I ordinarily uses GSH as a cofactor. To examine this hypothesis, we monitored the effects of NO donating compounds on the activity of purified human Glo I *in vitro* (Fig. 3). GSNO at a concentration of 1 mM decreased the activity of Glo I to 60% of the control level within 40 min (Fig. 3A). However, neither CysNO (Fig. 3A) nor PAPANONOate (data not shown), both of which are considered to donate NO radicals, at the same concentration as GSNO, had any effect on the enzymatic activity. We analyzed the

isoelectric point of Glo I by 2D-PAGE after exposure of Glo I to GSNO and CysNO (Fig. 3B). A spot of Glo I with a pI of 5.2 appeared upon the exposure of Glo I to GSNO, while no protein spot with that pI was observed after treatment with CysNO. GSNO was so stable under the conditions of the inactivation assay that RSNOs could be detected at 90% of the level of the initial GSNO concentration after 60 min incubation. On the other hand, CysNO decomposed spontaneously and less than 10% of the CysNO could be detected 60 min after its addition to the mixture. The failure of CysNO and PAPANONOate to inactivate Glo I indicates that the inactivation of Glo I expresses a relative specificity to GSNO, an adduct of NO and GSH. Since Glo I is clearly responsive to the extracellular addition of these compounds (24), it seems that the formation of GSNO is required for Glo I modulation by NO donors. Upon the addition of GSH to the reaction mixture, Glo I activity was inhibited by CysNO (Fig. 3C). The requirement for GSH for the inactivation of Glo I by CysNO indicates that CysNO can generate to GSNO *in situ*. Taken together, these results confirm that Glo I can interact directly with GSNO, and that the interaction leads to a decline in enzymatic function.

Involvement of the Substrate Recognition Site in the

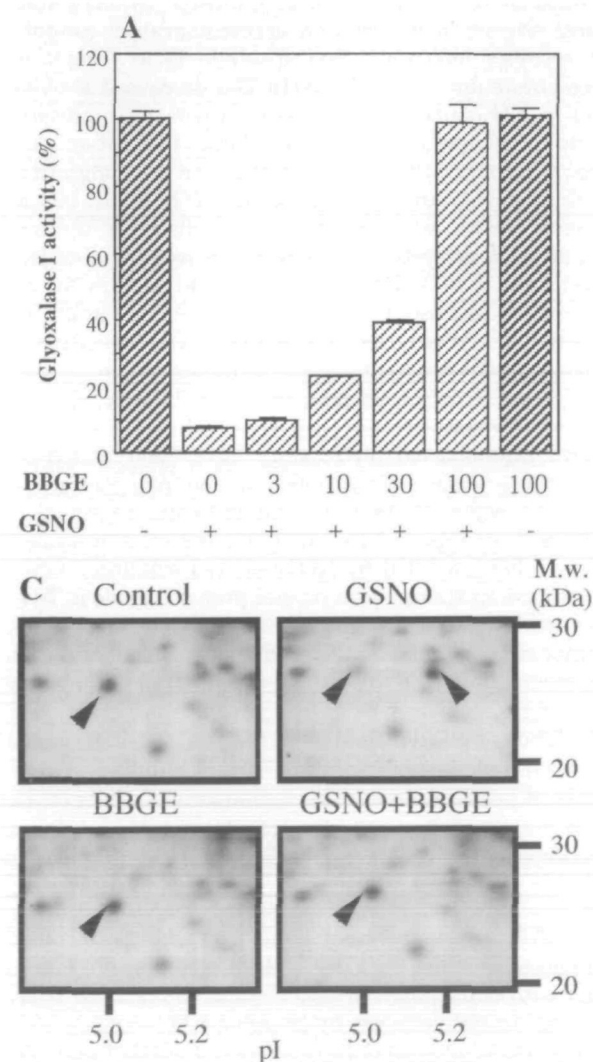


Fig. 5. Effects of GSBBdiEt on the response of Glo I to GSNO in ECV304 cells. Confluent ECV304 cells were treated with GSBBdiEt at the indicated concentrations for 30 min prior to the addition of GSNO. After a 2-h incubation with 1 mM GSNO, the cells were harvested and lysed. Glo I activity in the supernatants was measured by monitoring the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione. Data are represented as mean \pm SD ($n = 3$). The 100% activity (0.9 units/mg protein) corresponds to the activity in control cells without treatment with GSBBdiEt. (B) Confluent ECV304 cells were treated with 100 μ M GSBBdiEt for 30 min prior to the addition of GSNO. At the indicated times, the cells were harvested and lysate supernatants were prepared. Glo I activity was measured as described above. Data are represented as mean \pm SD ($n = 3$). The 100% activity (1.0 units/mg protein) corresponds to the activity in control cells prior to the addition of GSNO. (C) Proteins (50 μ g) in the supernatants were analyzed by 2D-PAGE and visualized by silver staining. The areas corresponding to pI 4.7 to pI 5.3 and 20 to 30 kDa are shown. Arrowheads indicate the native form (left; pI 5.0) and the modified form (right; pI 5.2) of Glo I.

Interaction of Glo I with GSNO—Glo I can recognize *S*-substituted GSH derivatives as substrates or inhibitors. So, can Glo I interact with GSNO at the substrate recognition site? To answer this question, we examined the effects of *S*-(4-bromobenzyl)glutathione (GSBB) on the GSNO-induced inactivation of Glo I *in vitro*. We chose GSBB since it has been well characterized as a competitive inhibitor of Glo I with highest affinity found so far (36). GSBB at 100 μ M completely blocked the GSNO-induced inactivation (Fig. 4). GSBB also prevented the GSNO-induced change in the pI of Glo I (data not shown).

Moreover, we studied the effects of *S*-4-(bromobenzyl)glutathione diethyl ester (GSBBdiEt), a membrane-permeable form of GSBB (37), on the inactivation of Glo I in GSNO-treated cells. As shown in Fig. 5A, GSBBdiEt prevented the inactivation of Glo I activity by GSNO in a dose-dependent manner. No inactivation of Glo I occurred in the presence of 100 μ M GSBBdiEt during 2 h of incubation of the cells with 1 mM of GSNO (Fig. 5B). Furthermore, GSBBdiEt also blocked the GSNO-induced change in the pI of Glo I (Fig. 5C). Taken together, these results strongly suggest that the substrate-recognition site is involved in the interaction of Glo I with GSNO.

DISCUSSION

In the present study, we demonstrate that the inactivation of Glo I by NO requires cellular GSH, and that Glo I interacts directly with GSNO but not with CysNO or PAPANONOate. The inactivation of Glo I by PAPANONOate depends on the level of intracellular GSH to a greater extent than does inactivation by GSNO (Fig. 1), indicating that a higher level of GSH is required for the formation of GSNO within cells from the NO radical and GSH. Hogg *et al.* (38) detected the formation of GSNO when NO radicals reacted with GSH in the presence of O_2 , suggesting that NO might diffuse through cells in the presence of an intracellular pool of GSH at concentrations of 5 to 10 mM. We revealed that Glo I interacts with GSNO even within cells. Thus, NO-derived species might be converted to GSNO within cells and the resultant GSNO might then interact with target proteins such as Glo I.

Evidence for the presence of GSNO under biological conditions is, however, strictly limited at present (16, 17, 39). In the present study, we used whole cells and the purified enzyme to demonstrate that Glo I is inactivated and its pI is changed by GSNO in a specific manner. Our results suggest that measuring changes in the pI of Glo I by 2D-PAGE may be used as an indicator of the formation of GSNO in cells exposed to NO. The lower limit of Glo I for a response is about 10 μ M of GSNO in the extracellular medium (unpublished data). In human and mouse serum under inflammatory situations, *S*-nitrosothiols are detected at 7 μ M (40) and 18 μ M (41), respectively. And Glo I in ECV304 cells clearly responds to the bolus addition of PAPANONOate at 100 μ M (24). The half life of PAPANONOate in 0.1 M phosphate buffer, pH 7.6, at 22°C is 76.6 min (42). This indicates that the rate of NO radical formation is about 10 nM/sec and is physiologically relevant (43). These considerations raise the possibility that Glo I might be an indicator of GSNO under physiological conditions.

Inhibitor analysis suggests the involvement of the substrate-recognition site in the interaction of Glo I with

GSNO both *in vitro* and inside cells. Mammalian Glo I (mGlo I) (25) is a dimeric protein consisting of two identical or similar subunits. The active site is located at the dimer interface and includes binding sites for a Zn^{2+} ion and the substrate (44). Although Glo I has broad substrate-specificity with respect to 2-oxoaldehydes, intact GSH is essential as a cofactor for the recognition of the substrate by the enzyme (25). In GSBB, a large hydrophobic substituted group is linked to the sulfhydryl moiety of GSH, and GSBB is the most powerful competitive inhibitor of human Glo I (hGlo I) identified to date (36). In this study, GSBB and its membrane-permeating derivative GSBBdiEt, completely prevented both the inactivation and the modification of hGlo I by GSNO *in vitro* and within cells. These results suggest that GSBB might compete with GSNO at the substrate-binding pocket of Glo I.

There are four Cys residues in the entire amino acid sequence of hGlo I and the crystal structure of hGlo I indicates that the Cys residue at position 60 is located in the hydrophobic cavity of the active site (44, 45). This residue also makes contact with the side residue of the co-crystallized ligand. GSNO can transfer an NO moiety to the sulfhydryl group of a protein by a transnitrosation reaction that yields the *S*-nitrosoprotein with concomitant regeneration of GSH (46). The molecular mass of hGlo I remains unchanged in response to NO, as indicated by 2D-PAGE (24) and size exclusion column chromatography (unpublished results), indicating that disulfide bond formation between intersubunits is not likely. The decreased activity of Glo I by NO is restored by treatment with dithiothreitol (24) and a competitive inhibitor blocks the interaction between Glo I and GSNO. This suggests the possibility that a Cys sulfhydryl group in the active site of Glo I may be the most likely target of NO, raising the possibility of the formation of an *S*-nitrosothiol. However, it is unlikely that the modified form of hGlo I detected after 2D-PAGE is the *S*-nitrosoprotein because it is unlikely that *S*-nitrosation increases the pI of the parent protein (47). The structure of the modified hGlo I remains to be characterized, but *S*-nitrosation, even if only transient, might be involved in the modification process.

Several physiological phenomena have been linked to NO-induced oxidative stress, such as neurodegenerative diseases (5), septic shock (48), and ischemia reperfusion (49). It has been reported that G3PDH and other enzymes are irreversibly modified by NO-mediated oxidation. GSH is an antioxidant that plays a pivotal protective role in NO-induced oxidative stress (50). Cellular GSH effectively protects thiol enzymes, such as G3PDH, from inactivation by oxidative insult, and it has been demonstrated that the NO-mediated inactivation of these enzymes is potentiated by a depletion of cellular thiol pools (9, 10, 50). Lander *et al.* (51) showed that the G-protein p21^{ras} is a common target of a variety of redox stresses. The exchange of GDP/GTP in p21^{ras} is enhanced by oxidants such as NO, H_2O_2 , and a GSH depletor, indicating that p21^{ras} is oxidized non-specifically. In our story, the response of Glo I is specific to NO-mediated oxidation (24) and is observed only in the presence of GSH (Fig. 1). The modified form of Glo I is so stable that it can be detected on 2D-gels. It is possible that Glo I, together with GSH, might protect cellular components from NO-induced oxidative injury by trapping NO.

The glyoxalase system (GS) is composed of Glo I and Glo

II. The latter enzyme hydrolyzes S-D-lactoylglutathione, a product of the reaction catalyzed by Glo I, to D-lactic acid with regeneration of GSH. Although the GS is strongly conserved in all organisms from bacteria to mammals (25), the physiological roles of the GS remain to be fully defined. One of the major roles of the GS is believed to be the detoxification of 2-oxoaldehydes, such as methylglyoxal (MG), which appears primarily to be a byproduct of the metabolism of carbohydrates and lipids (52). Glo I is a ubiquitous protein present in almost all mammalian cells. Further analysis of the interaction of NO with mGlo I will provide novel insights into the biological roles of mGlo I.

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