Nitric Oxide-induced S-Glutathionylation and Inactivation of Glyceraldehyde-3-phosphate Dehydrogenase

S-Nitrosylation of protein thiol groups by nitric oxide (NO) is a widely recognized protein modification. In this study we show that nitrosomonium tetrafluoroborate (BF4NO), a NO+ donor, modified the thiol groups of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by S-nitrosylation and caused enzyme inhibition. The resultant protein-S-nitrosothiol was found to be unstable and to decompose spontaneously, thereby restoring enzyme activity. In contrast, the NO-releasing compound S-nitrosothioglutathione (GSNO) promoted S-glutathionylation of a thiol group of GAPDH both in vitro and under cellular conditions. The GSH-mixed protein disulfide formed led to a permanent enzyme inhibition, but upon dithiothreitol addition a functional active GAPDH was recovered. This S-glutathionylation is specific for GSNO because GSH itself was unable to produce protein-mixed disulfides. During cellular nitrosative stress, the production of intracellular GSNO might channel signaling responses to form protein-mixed disulfide that can regulate intracellular function.

Nitric oxide (NO)1 is an important biological messenger that plays a role in physiological and pathophysiological conditions such as endothelium-dependent vasorelaxation, inflammation, and septic shock (1, 2). These multiple effects are based on its redox chemistry. NO can react with oxygen species and transition metals to form NOx, peroxyxynitrite (ONOO−), and metal-NO adducts, respectively (3, 4). The interactions of NO with sulfhydryl-containing molecules and enzymes has gained considerable importance (5, 6). In many biological systems, nitrosation reactions transferring NO to a protein S− group affect protein function. Targets for this type of modification, among others, are bovine serum albumin (7), tissue-plasminogen activator (8), glyceraldehyde-3-phosphate dehydrogenase (9, 10), the N-methyl-D-aspartate receptor (11), oncogenic p21waf1 (12), and transcriptional activators (13).

The S-nitrosothiol of glutathione (GSNO) may be the most relevant biological molecule to carry out nitrosation reactions under physiological conditions (14–16). It has been reestablished that the actions of the endothelium-derived relaxing factor more closely resemble a low molecular weight nitrosothiol rather than the NO− radical itself (17). However, S-nitrosothiols can decompose to form NO− and thyl radicals (18), and the thyl radical can lead to the production of protein-mixed disulfides also known as protein S-glutathionylation.

In this study, we investigated the influence of NO donors on the glycolytic enzyme GAPDH which catalyzes the reversible oxidative phosphorylation of d-glyceraldehyde-3-phosphate by NAD+ and inorganic phosphate. GAPDH is comprised of four identical 37-kDa subunits. Each subunit contains four cysteines; two of them (Cys-149 and Cys-153) are located in the catalytic site of each GAPDH subunit. The catalytically active cysteine 149 interacts with a histidine to form a highly reactive thiolate group (cys-S−) which is required for GAPDH activity. Recently, it has been shown that NO inhibits GAPDH activity (15). In this study, we examined the effect of BF4NO (a NO+ releasing compound) and GSNO on the thiol groups of the enzyme. Our results point to specific differences in thiol modification mechanisms that can be triggered by competing S-nitrosylation and S-glutathionylation.

**EXPERIMENTAL PROCEDURES**

**Materials—**[14C]N-ethylmaleimide (40 mCi/mmol) and [35S]glutathione (83.1 Ci/mmol) were purchased from NEN Life Science Products. [35S]cysteine (1075 Ci/mmol) was from ICN. BF4NO was obtained from Aldrich. Mouse anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody was from Chemikon. Rabbit muscle GAPDH (80 units/mg) was bought from Boehringer Mannheim. Other chemicals were of the highest grade of purity from Sigma.

**Synthesis of GSNO—**GSNO was prepared as described previously (19). GSNO displayed absorption maxima at 335 and 545 nm with extinction coefficients of 922 and 15.9 dl (2) mol−1 cm−1, respectively. GSNO solutions were prepared prior to the experiment.

**Synthesis of [35S]GSNO—**Commercial [35S]GSH solutions (83.1 Ci/mmol) were stabilized with 10 mM DTT. DTT was removed according to a published procedure (20). Briefly, the [35S]GSH solution (89 mCi/mmol) was adjusted to pH 2 with 2 N HCl and then extracted twice with 900 ml of 20% ice-cold triethyl acetate. The [35S]GSH containing phase was used for [35S]GSNO synthesis. An aliquot of 82 μl of [35S]GSH and 25 mg of GSH were dissolved in 0.2 mL HCL in a total volume of 100 μL at 4 °C followed by the addition of 5.67 mg of NaNO2. The following procedure was carried out as described for GSNO synthesis.

**[14C]N-ethylmaleimide (NEM) Labeling of GAPDH—**GAPDH (50 μg/assay) was incubated in 100 mM Tris buffer (pH 8.0) and different NO donors in a total volume of 100 μL at 30 °C for 5 min, or as otherwise indicated. Proteins were precipitated with 200 μl of 20% ice-cold trichloroacetic acid (TCA), left on ice for 15 min, and centrifuged for 10 min at 10,000 × g and 4 °C. The pellets were washed twice with 1 ml of...
ice-cold water-saturated ether and resuspended in 100 mM Tris buffer (pH 8.0) containing 0.1% Triton X-100 and 50 μM [35S]C(INE)M (0.17 μCi). Samples were incubated for 1 h at 30 °C. The reaction was stopped with 200 μl of 20% ice-cold TCA. Proteins were left on ice for 15 min and then centrifuged for 10 min at 10,000 × g and 4 °C. The pellets were washed twice with 1 ml of ice-cold water-saturated ether and separated in a nonreducing 11% sodium dodecyl sulfate-polyacrylamide gel. Radioactivity was quantified using the PhosphorImager system (Molecular Dynamics). NO donor solutions were freshly prepared prior to the experiments. A stock solution of BF₄NO was prepared under acidic conditions (pH 2.0; 0.2 M HCl) as described previously (9).

**DTT Reversibility of NO-induced GAPDH Modification**—GAPDH (50 μg/assay) was incubated with 100 mM Tris buffer (pH 8.0) and different NO donors in a total volume of 100 μl at 30 °C. After 5-min incubation, proteins were precipitated with 200 μl of 20% ice-cold TCA, left on ice for 15 min, and then centrifuged for 10 min at 10,000 × g and 4 °C. The pellets were washed twice with 1 ml of ice-cold water-saturated ether and resuspended in 100 mM Tris buffer (pH 8.0) containing 10 mM DTT. Incubations were carried out for 5 min at 30 °C. Proteins were precipitated again with 200 μl of 20% ice-cold TCA, left on ice for 15 min, and then centrifuged for 10 min at 10,000 × g and 4 °C. The pellets were washed twice with 1 ml of ice-cold water-saturated ether and resuspended in 100 mM Tris buffer (pH 8.0) containing 10 mM DTT. The reaction was stopped with 200 μl of ice-cold water-saturated ether and separated in a nonreducing 11% sodium dodecyl sulfate-polyacrylamide gel. GAPDH Activity—GAPDH (1 μg/assay) and up to 200 μg individual NO donor were incubated in 50 mM triethyloxonium buffer (pH 7.5) in a total volume of 50 μl at 37 °C for 5 min. Samples were diluted into 950 μl of 50 mM triethyloxonium buffer (pH 7.5) containing 50 μM arsenate, 2.4 mM glutathione, and 100 μg/ml glycolaldehyde-3-phosphate, at 37 °C. The enzymatic reduction of NAD⁺ to NADH was started with the addition of 250 μM NAD⁺. GAPDH activity was monitored by recording the fluorescence emission above 430 nm after excitation at 313 and 366 nm, respectively. Samples without NO donors were served as controls.

**Modification of GAPDH by [35S]GSNO—GAPDH (10 μg/assay) was incubated in 100 mM Hepes buffer (pH 7.5) and 0.5, 1, or 2 mM [35S]GSNO (5000 cpm/assay), respectively.** Incubations contained GAPDH (10 μg/assay), 100 mM Hepes buffer (pH 7.5) and 0.5, 1, or 2 mM [35S]GSNO (45000 cpm/assay) served as controls. After 5 or 10 min at 37 °C, proteins were precipitated with 200 μl of 20% ice-cold TCA, left on ice for 15 min, followed by centrifugation for 10 min at 10,000 × g and 4 °C. The pellets were washed twice with 1 ml of ice-cold water-saturated ether and separated in a nonreducing 11% sodium dodecyl sulf-polyacrylamide gel. For the detection of radioactivity, gels were exposed (PhosphorImager exposing cassettes) for 8 up to 30 days.

**Isolation of Endothelial Cells (EC)—Bovine nortic EC were isolated as described (21) and were passaged and maintained in Dulbecco’s modified Eagle’s and F12 media, supplemented with 5% fetal bovine serum in a humidified atmosphere containing 5% CO2. Confluent cultures in 10-cm dishes were made quiescent by replacing medium with serum-free medium containing 1% gelatin for 24 h before use.

**Metabolic Labeling of EC—For protein S-thiolation studies, EC were prelabeled by incubation with [35S]S-cysteine (50 μCi/ml) in the presence of 200 μM cycloheximide to block protein synthesis. Cells were then treated with GSNO (500 μM) or hydrogen peroxide H₂O₂ (500 μM), respectively, for times indicated.**

**Immunoprecipitation of GAPDH—**Total cell lysate of EC was denatured by incubation with 0.5% SDS, 50 mM sodium phosphate, pH 8.0, and 2 mM EDTA at 90 °C, to allow immunoprecipitation. The samples were supplemented to obtain a composition of 50 mM sodium phosphate, pH 7.2, 1% sodium deoxycholate, 1% Triton X-100, 0.5% SDS, 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 2 mM Na₃PO₄, 2 mM NaVO₄, 1% aprotinin, and 200 μg/ml leupeptin at 4 °C. Mouse anti-GAPDH monoclonal antibody (usually at 1:100 dilution) was added, followed 18 h later by protein A-Sepharose. Washed immunoprecipitates were analyzed by autoradiography (for [35S]S) after separation in a nonreducing or reducing 11% sodium dodecyl sulfate-polyacrylamide gel.

**RESULTS**

We have examined the effect of nitric oxide-releasing compounds on sulfhydryl group modifications of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To detect the thiol modifications of the GAPDH subunits, we used NEM which binds to the free, unmodified sulfhydryl groups. Nitrosodimethylformamide (BF₄NO) is an exclusively NO⁺ releasing NO donor that forces protein-S-nitrosothiol generation via a transnitrosation reaction. In the first set of experiments we incubated GAPDH with BF₄NO for 5 min. Following protein precipitation, the enzyme was resuspended, exposed to [14C]NEM, and electrophoretically separated on a nonreducing 11% SDS gel (Fig. 1). A concentration of 200 μM BF₄NO completely modified all accessible cysteines of GAPDH subunits as shown by the lack of subsequent [14C]NEM binding to GAPDH thiol groups. GAPDH that was not exposed to BF₄NO showed the highest amount of [14C]NEM labeling (Fig. 1).

Next, we investigated the effect of the NO donor S-nitroso-glutathione (GSNO) that may act as a NO⁺-transferring agent on GAPDH modification (Fig. 2). However, GSNO can also be degraded homolytically to produce NO⁺ and GS⁻ radicals. In contrast, the effect of GSNO on GAPDH activity was much different. GSNO promoted enzyme inhibition that was not spontaneous. Only a period of 60 min with GSNO were all accessible thios modified as shown by the disappearance of [14C]NEM binding (Fig. 2). These data show a clear difference between the actions of BF₄NO and GSNO. It might indicate that the action of GSNO is not related to NO⁺.

It has been established that nitrosylation of Cys-149 in the active site of GAPDH attenuates enzymatic activity. To further characterize the effect of NO donors on this reactive cysteine, we assessed enzyme activity. A concentration of 200 μM BF₄NO decreased GAPDH activity by about 60% within 1 min (Fig. 3). However, attenuation of enzyme activity was only transient, and GAPDH activity was recovered over a time period of 15 min (Fig. 3). This effect was paralleled by [14C]NEM labeling of the cysteines, although with a slower progression (Fig. 3). In contrast to BF₄NO, a concentration of 500 μM GSNO was not able to block GAPDH cysteine residues within a 5-min incubation. Only after a period of 60 min with GSNO were all accessible thios modified as shown by the disappearance of [14C]NEM binding (Fig. 2). These data show a clear difference between the actions of BF₄NO and GSNO. It might indicate that the action of GSNO is not related to NO⁺.
nitrosothiol by nitric oxide facilitates the GAPDH. [³⁵S]labeling of GAPDH was reversed by DTT, which protein synthase inhibitor cycloheximide to generate intracellular conditions. Therefore, freshly isolated bovine EC were preincubated with [³⁵S]-cysteine in the presence of the protein synthase inhibitor cycloheximide to generate intracellular [³⁵S]GSH. After treatment of EC with GSH, GAPDH was immunoprecipitated and separated on an SDS-gel under nonreducing conditions (Fig. 5A). GSH (500 μM) evoked significant [³⁵S]labeling of GAPDH within 15 min, indicating S-glutathionylation of the enzyme similar to our *in vitro* studies. The [³⁵S]labeling of GAPDH was stable for at least 30 min (data not shown). Hydrogen peroxide H₂O₂ (500 μM), known to produce intracellular protein-mixed disulfides, also caused S-glutathionylation of GAPDH, albeit to a lesser extent (Fig. 5A). The addition of DTT to the sample buffer (reducing SDS gel) removed the [³⁵S]label from GAPDH caused by GSH or H₂O₂, respectively (Fig. 5B). In contrast, alcohol dehydrogenase that contains a non-histidine-activated cysteine could not be modified by GSHO via S-glutathionylation under cellular conditions (data not shown).

**FIG. 3. Effect of BF₄NO on GAPDH activity and NEM-labeling.** GAPDH (50 μg/assay) was incubated at 30 °C in the presence of 100 μM BF₄NO. GAPDH activity was measured as described under "Experimental Procedures." Results represent the mean (±S.D.) of three different experiments.

| NO donor       | GAPDH activity inhibition (Ci/assay) | 1 min | 5 min | 30 min |
|----------------|--------------------------------------|-------|-------|--------|
| BF₄NO (200 μM) |                                      | 60 ± 4.4 | 45 ± 5.6 | 10 ± 3.8 |
| GSHO (200 μM)  |                                      | 60 ± 5.3 | 59 ± 8.9 | 51 ± 4.2 |

10-min treatment of GAPDH with 500 μM [³⁵S]GSH was sufficient to label the enzyme, thus indicating S-glutathionylation of GAPDH (Fig. 4A). This effect was absent when we used [³⁵S]GSH. These data indicate that the formation of a nitrosothiol by nitric oxide facilitates the S-glutathionylation of GAPDH. [³⁵S]labeling of GAPDH was reversed by DTT, which is consistent with the formation of a protein-mixed disulfide (Fig. 4A). S-Glutathionylation of GAPDH by [³⁵S]GSH occurred within 5 min and only slightly increased after 10 min (Fig. 4B).

To verify a homolytic breakdown of GSHO under our experimental conditions, we measured the NO' release from GSHO incubated in buffer. Under these specific conditions, GSHO was not homolytically degraded as determined by an NO' electrode (9), which excludes the formation of GS' radicals and, furthermore, the possibility of GSSG generation as a likely S-glutathionylation agent. The results indicate more a direct attack of the reactive cysteine of GAPDH at the S–N bond leading to a homolytic breakdown of GSHO.

After we have shown that GSHO promoted S-glutathionylation of GAPDH *in vitro*, we were interested to see if GSHO was able to induce formation of GAPDH/GSH-mixed disulfides under cellular conditions. Therefore, freshly isolated bovine EC were preincubated with [³⁵S]-cysteine in the presence of the protein synthase inhibitor cycloheximide to generate intracellular [³⁵S]GSH. After treatment of EC with GSHO, GAPDH was immunoprecipitated and separated on an SDS-gel under nonreducing conditions (Fig. 5A). GSHO (500 μM) evoked significant [³⁵S]labeling of GAPDH within 15 min, indicating S-glutathionylation of the enzyme similar to our *in vitro* studies. The [³⁵S]labeling of GAPDH was stable for at least 30 min (data not shown). Hydrogen peroxide H₂O₂ (500 μM), known to produce intracellular protein-mixed disulfides, also caused S-glutathionylation of GAPDH, albeit to a lesser extent (Fig. 5A). The addition of DTT to the sample buffer (reducing SDS gel) removed the [³⁵S]label from GAPDH caused by GSH or H₂O₂, respectively (Fig. 5B). In contrast, alcohol dehydrogenase that contains a non-histidine-activated cysteine could not be modified by GSHO via S-glutathionylation under cellular conditions (data not shown).

**DISCUSSION**

Our study shows that GSHO causes the S-glutathionylation of GAPDH. This effect is not seen when GSH is used. Therefore, the homolytic degradation of GSHO is essential for S-glutathionylation to occur. Because we excluded the possibility of NO' formation as a result of homolytic GSHO cleavage with resultant GSSG formation as a potential S-glutathionylating agent, we conclude that the breakage of GSHO is directly induced by a histidine-activated thiol group such as cysteine 149 in the active site of GAPDH. A strong nucleophile like the histidine-activated thiolate 149 can attack the S–NO bond of GSHO, leading to the formation of GSH-mixed protein disulfides. Thiols that are not activated by a histidine have a lower nucleophilicity and are not strong enough to break the S–NO bond of GSHO. These thiol is more likely to become nitrosylated by GSHO. This is exemplified for alcohol dehydrogenase that contains a non-histidine-activated cysteine in its active site that does not become S-glutathionylated by GSHO in comparison to GAPDH. Therefore, GSHO can induce S-nitrosylation or S-glutathionylation according to the nucleophilicity of the cysteine residues. On the contrary, BF₄NO can only produce S-nitrosylation of cysteines by spontaneous release of NO'. This also explains why BF₄NO reduces NEM labeling of GAPDH fast, whereas GSHO affects the labeling at a much slower rate, despite rapid enzyme inactivation which results from S-glutathionylation.

The formation of GSH-mixed protein disulfides is mostly a consequence of cellular oxidative stress. A recent report demonstrated that a small pool of glutathione (less than 15% of the total) is able to produce a significant increase in protein-mixed disulfides (22). GAPDH has been identified as a major S-glutathionylated protein in endothelial cells that were exposed to hydrogen peroxide (23) and in activated monocytes (24). Other reports described inactivation of GAPDH in the ischemic myocardium, most likely via S-glutathionylation (25). Besides GAPDH, several other proteins such as actin, creatine kinase, glycogen phosphorylase b, and the homodimeric HIV-1 protease are targets for S-glutathionylation in intact cells following exposure to oxidative stress (for review, see Ref. 26). In line, it recently was shown that treatment of endothelial cells with the combination of GSHO and cysteine, a system that generates GSSG and nitric oxide, evoked loss of intracellular glutathione probably because of the formation of protein-mixed disulfides (27).

Our study shows that GSHO induces S-glutathionylation of GAPDH in endothelial cells. In comparison with hydrogen peroxide, the formation of the GAPDH/GSH-protein-mixed disulfide caused by GSHO is more efficient. Hydrogen peroxide probably oxidizes GSH to GSSG. In turn, the reactive cysteine of GAPDH might be nucleophilic enough to break the disulfide bond in GSSG, thus leading to the formation of a protein-mixed disulfide. The S–N bond in GSHO is much weaker than the
Fig. 4. S-Glutathionylation of GAPDH. A. GAPDH (10 μg/assay) was incubated with different concentrations of [35S]GSNO (45,000 cpm/assay) at 37 °C for 10 min in the presence or absence of DTT (10 mM). Incubations containing GAPDH (10 μg/assay) and [35S]GSH (45,000 cpm/assay) were performed in parallel. B. GAPDH (10 μg/assay) was incubated with 2 mM [35S]GSNO (45,000 cpm/assay) at 37 °C for the indicated times. Incubations with GAPDH (10 μg/assay) and 2 mM [35S]GSH (45,000 cpm/assay) were also performed in parallel. The incorporated radioactivity was measured as described under “Experimental Procedures.” Results are representative of four similar experiments.

Fig. 5. GSNO-induced S-glutathionylation of GAPDH in bovine endothelial cells. Freshly isolated bovine EC were prelabeled with [35S]L-cysteine (50 μCi/ml) for 2 h in the presence of cycloheximide (200 ng/ml) to allow formation of [35S]GSH. EC were treated with GSNO (500 μM) or H2O2 (500 μM), for times indicated. Following immunoprecipitation of GAPDH with a mouse anti-GAPDH monoclonal antibody, protein was separated in an 11% SDS-gel under nonreducing (A) or reducing (B) conditions. Radioactivity was measured as described under “Experimental Procedures.” Results are representative of three independent experiments.

S–S bond in GSSG. Therefore, S-glutathionylation induced by GSNO is facilitated because it needs less energy to break a S–N rather than a S–S bond.

The concentration of glutathione in cells could be as high as 10 mM. This glutathione can be S-nitrosylated, and the S-nitrosothiol of glutathione has been implicated in the storage and delivery of cellular NO. Our present study points to a new cellular function of GSNO which is the covalent modification of proteins by S-glutathionylation. This function of GSNO is strengthened by the fact that it is GSNO, and not GSH, that facilitates mixed disulfide formation. Therefore, cells under conditions of nitrosative stress (formation of NO) could produce GSNO which in turn promotes oxidative stress by formation of GSH-mixed-disulfides. The homolytic degradation of GSNO facilitated by histidine-activated thiol groups represents a unique mechanism of protein glutathionylation. It would be important in the future to differentiate the effects of NO donors on signal transduction events that could be mediated by S-glutathionylation or S-nitrosylation.

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