Inactivation of Glutathione Peroxidase by Nitric Oxide

IMPLICATION FOR CYTOTOXICITY*

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S-nitro-N-acetyl-0H-penicillamine (SNAP), a nitric oxide (NO) donor, inactivated bovine glutathione peroxidase (GPx) in a dose- and time-dependent manner. The IC50 of SNAP for GPx was 2 μM at 1 h of incubation and was 20% of the IC50 for another thiol enzyme, glyceraldehyde-3-phosphate dehydrogenase, in which a specific cysteine residue is known to be nitrosylated. Incubation of the inactivated GPx with 5 mM dithiothreitol within 1 h restored about 50% of activity of the start of the SNAP incubation. A longer exposure to NO donors, however, irreversibly inactivated the enzyme. The similarity of the inactivation with SNAP and reactivation with dithiothreitol of GPx to that of glyceraldehyde-3-phosphate dehydrogenase, suggested that NO released from SNAP modified a cysteine-like essential residue on GPx. When U937 cells were incubated with 100 μM SNAP for 1 h, a significant decrease in GPx activity was observed although the change was less dramatic than that with the purified enzyme, and intracellular peroxide levels increased as judged by flow cytometric analysis using a peroxide-sensitive dye. Other major antioxidative enzymes, copper/zinc superoxide dismutase, manganese superoxide dismutase, and catalase, were not affected by SNAP, which suggested that the increased accumulation of peroxides in SNAP-treated cells was due to inhibition of GPx activity by NO. Moreover, stimulation with lipopolysaccharide significantly decreased intracellular GPx activity in RAW 264.7 cells, and this effect was blocked by NO synthase inhibitor Nα-methyl-l-arginine. This indicated that GPx was also inactivated by endogenous NO. This mechanism may at least in part explain the cytotoxic effects of NO on cells and NO-induced apoptotic cell death.

Nitric oxide (NO)† is a messenger molecule with multiple biological functions including smooth muscle relaxation, neurotransmission, and macrophage-mediated cytotoxicity (1). NO is highly reactive with molecular oxygen, superoxide anion, and heme as well as non-heme iron. NO or its derivatives also interacts with the thiol groups of proteins and glutathione to form nitrosothiols (2). By this mechanism, NO can be stabilized and its function prolonged (3). Nitrosoylation of enzymes such as GAPDH and protein kinase C blocks their catalytic activity (4–7).

Glutathione peroxidase (GPx) is an antioxidative enzyme that scavenges various peroxides. Three isozymes, cellular GPx, extracellular GPx, and phospholipid hydroperoxide GPx, are known, and each contains a seleno-cysteine in its catalytic center (8). Cellular GPx, the most characterized form, can react with hydrogen peroxide and organic peroxides but not lipid hydroperoxide. Catalase is found in many types of cells and scavenges hydrogen peroxides as its sole substrate. The content of catalase is lower than the content of GPx in most cells, except for hepatocytes and erythrocytes, and the Km value of catalase for hydrogen peroxide is higher than that of GPx, implying the primary importance of GPx in most tissues. Selenium has been shown to regulate the level of cellular GPx at both the transcriptional and translational stages (9). Selenium deficiency in cells causes a decrease in GPx mRNA and protein, resulting in increased susceptibility of cells to oxidative damage. Moreover, recent reports demonstrated that oxidative stress is one of the direct causes of apoptotic cell death and that GPx as well as bcl2, a proto-oncogene that blocks apoptotic death in multiple contexts (10), can prevent apoptosis (11). Reactive oxygen species also participate in many cellular events including signal transduction and antibacterial defense. Hence, the maintenance of a balance between oxidants and antioxidants is of significance for cellular homeostasis.

We described here the inactivation of GPx by NO in a rather specific manner compared with another NO-sensitive enzyme, GAPDH, and discussed its physiological relevance.

EXPERIMENTAL PROCEDURES

Materials—Purified bovine GPx was purchased from Toyobo Co., Ltd. and was used without further purification. Glutathione reductase, GSH, rabbit GAPDH, glyceraldehyde 3-phosphate, phosphoglycerate kinase, and xanthine oxidase were purchased from Boehringer Mannheim. SNAP and DTT were purchased from Wako Pure Chemicals, Ltd. Cumeneperoxide was from Nakarai Tesque, Co., Ltd. NADPH and NADH were purchased from Boehringer Mannheim. SNAP and DTT were purchased from Wako Pure Chemicals, Ltd. Cuminaperoxide was from Nakarai Tesque, Co., Ltd. NADPH and NADH were purchased from Oriental Yeast, Co., Ltd. ATP and LPS were obtained from Sigma. Carboxy PTIO was purchased from Dojin Chemicals, Ltd. Copper/zinc superoxide dismutase was a kind gift from Ube Industries, Ltd.

Cell Culture—U937, a human histiocytic lymphoma cell line, and RAW 264.7, a mouse macrophage cell line, were purchased from the American Type Culture Collection and maintained in RPMI 1640 and Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate, respectively. These cells were incubated in a humified atmosphere of 5% CO2 and 95% air at 37°C.
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Purification of Manganese Superoxide Dismutase and Superoxide Dismutase Activity Assay—Manganese superoxide dismutase was purified according to the method involving chromatography on DEAE-cellulose, hydroxylapatite, and butyl-Toyopearl as described previously (12). Manganese superoxide dismutase and copper/zinc superoxide dismutase activities were determined by the method by Beauchamp and Fridovich (13).

GPx and Catalase Activity Assays—GPx activity was determined according to the method of Lawrence and Burk (14). One unit was defined as the amount of enzyme oxidizing 0.5 μmol of NADPH (corresponding to 1 μmol of GSH) per min. U-937 cells in 10-cm dishes were incubated in the presence of 100 μM SNAP for various lengths of time. After washing with phosphate-buffered saline twice, the cells were harvested and sonicated. The resulting homogenates were used for the GPx assay. RAW 264.7 cells were incubated with 10 ng/ml LPS for 18 h in the presence or absence of 1 mM N’-methyl-L-arginine. After homogenization, GPx activities were determined. Catalase activity was determined by the method of Aebi (15) using hydrogen peroxide as a substrate.

GAPDH Activity Assay—Enzyme activity was measured according to the manufacturer’s instructions. The assay mixture contained 82.3 mM triethanolamine buffer, pH 7.6, 1.1 mM ATP, 6.2 mM glycylate 3-phosphate, 0.2 mM NADH, 0.9 mM EDTA, 2 mM MgSO₄, and phosphoglycerate kinase (13 units/ml). Changes in absorbance at 340 nm were continuously monitored. One unit was defined as the amount of enzyme oxidizing 1 μmol of NADH/min.

Nitrite Production Assay—NO was measured as its stable oxidative metabolite, nitrite, as described previously (16). At the end of the incubation, 100 μl of the culture medium was mixed with an equal volume of Griess reagent (1 part 0.1% naphthylethylene diamine dihydrochloride and 1 part 1% sulfanylamide in 5% phosphoric acid) and allowed to stand at room temperature for 10 min. The absorbance at 550 nm was measured, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

Flow Cytometry—To assess levels of intracellular peroxide, flow cytometric analysis was carried out using an oxidation-sensitive fluorescent probe, DCFH-DA. U937 cells were incubated with DCFH-DA (Molecular Probes, Inc.), which was deacylated to the nonfluorescent probe, DCFH-DA. U937 cells were incubated with DCFH-DA and then subjected to peroxidase assay using hydrogen peroxide or cumene hydroperoxide as a substrate.

RESULTS

Inactivation of Purified Bovine Cytosolic GPx by SNAP—Because NO modifies the activities of several enzymes in which thiol groups are essential for catalytic function (4–6), it was of interest to determine whether NO could also affect the activity of GPx, which contains a seleno-cysteine in its catalytic center. Purified bovine liver cytosolic GPx was initially treated with SNAP, an NO-generating reagent, and then subjected to peroxidase assay using hydrogen peroxide or cumene hydroperoxide as a substrate. The enzyme used was >95% pure on SDS-polyacrylamide gel electrophoresis (data not shown). Although we employed a conventional GPx assay system that used glutathione reductase as a coupling enzyme, neither NO nor its donor affected the assay system (data not shown). GPx activity decreased markedly after incubation with SNAP in a dose- and time-dependent manner (Fig. 1). When the effect of SNAP on GPx was compared with that on GAPDH, GPx activity decreased at lower concentrations of SNAP (IC₅₀ of 2 μM) than GAPDH activity (IC₅₀ of 10 μM) with a similar time course. When carboxy PTIO, an NO scavenging reagent that accelerates nitrate formation from NO (20), was present together with SNAP, the decrease in GPx activity was suppressed (Fig. 2). These results indicated that released NO and not a donor molecule affected GPx activity.

Inactivation of GPx by NO Was Reversed by DTT—To characterize the modification of GPx by NO, the effects of DTT on the inactivated GPx were examined. The enzyme was first converted to a reduced form by incubation with 5 mM DTT at 37 °C for 1 h, and DTT was rapidly removed by a PD-10 column. The reduced enzyme was immediately treated with 100 μM SNAP for 1 h. Then, 5 mM DTT was added to the inactivated enzyme. As shown in Fig. 3, GPx activity rapidly recovered to 52% of the control level. The potential to recover activity was lost after prolonged incubation with SNAP (data not shown), probably due to NO-mediated oxidation of other residues. The same reversibility was found for GAPDH (data not shown), which suggests that the modification of GPx by NO was similar.

**Fig. 1.** Dose dependence (A) and time course (B) of inactivation of bovine GPx and bovine GAPDH. Purified bovine GPx (●, ○) or GAPDH (●, ●) at a concentration of 1 mg/ml was preincubated with various concentrations of SNAP for 1 h (●, ○) or 2 h (●, ●) (A) or with 10 μM SNAP for various times (B) at 37 °C. Activities are given as the percentage of the control value.

**Fig. 2.** Reduction of the SNAP-induced inactivation of GPx by Carboxy PTIO. GPx (1 mg/ml) was incubated with 10 μM SNAP in the presence (●) or absence (○) of 1 mM carboxy PTIO, and GPx activities were measured at each time point.
to that of GAPDH.

Inactivation of GPx in Intact Cells Treated with SNAP—Because NO reacts readily with thiol groups and various molecules have free thiol groups that would scavenge NO and abolish its effects in cells, we examined GPx activity in U937, a histiocytic lymphoma cell line, after treatment with 10 μM SNAP. After 1 h, GPx activity in SNAP-treated cells was maximally reduced to 75.9% of the activity in control cells and gradually recovered without any treatment (Fig. 4). To eliminate the possibility that SNAP might decrease GPx activity by suppressing the protein level of GPx, we performed Western blot analysis of the proteins from SNAP-treated cells by anti-human GPx monoclonal antibody was unchanged (data not shown), suggesting that the decreased GPx activity was due to post-translational modification of the protein.

Inactivation of GPx in Intact Cells Treated with LPS—To study the effect of endogenous NO on GPx activity in cells, we examined the GPx activity in RAW 264.7, a mouse macrophage cell line, after treatment with 10 ng/ml LPS for 18 h (Fig. 5). LPS-treated cells showed a marked increase in nitrite accumulation, and GPx activities were significantly lower than those of control cells (p < 0.05). In the presence of 1 mM N′-methyl-l-arginine, an NO synthase inhibitor, nitrite accumulation and GPx activities remained at near control levels, suggesting the participation of NO produced by LPS-treated NO synthase in the cells.

NO Increases Intracellular Peroxides—Because GPx is an enzyme that removes intracellular peroxides, the inactivation of GPx was expected to cause an increase in the accumulation of peroxides. As shown in Fig. 6, the levels of intracellular peroxides in U937 cells treated with SNAP increased as evaluated by fluorescence-activated cell sorting scan analysis using a peroxide-sensitive dye, DCFH-DA. Because other antioxidants, enzymes, copper/zinc superoxide dismutase, manganese superoxide dismutase, and catalase, were not affected by NO (Table I), this increase in peroxides would be explained by a decrease in peroxide scavenging capacity due to inactivation of GPx by NO.

**DISCUSSION**

The molecular basis of the cytocidal and cytostatic effects of NO is unclear and could be rooted in any of the numerous functions of this small molecule. Several enzymes that catalyze reactions essential to metabolism are known to be inactivated or modified by NO (22). The modifications of these enzymes are classified into two groups. (i) In proteins containing heme or non-heme irons as their cofactors, such as soluble guanylate cyclase (1), aconitase (22), cytochrome c oxidase (23), and cytochrome c oxidase (24), NO binds to the iron molecule of these cofactors. (ii) In enzymes that contain a catalytically essential sulphydryl group, such as GAPDH (4–6), the reactivity or absence of 1 mM N′-methyl-l-arginine for 18 h. Nitrite levels in the medium were measured (15). The GPx activities of these cells were assayed as described in the legend to Fig. 4. Data are presented as means ± S.D. of triplicate experiments.

**Fig. 3.** Reversal of the SNAP-induced inactivation of GPx and GAPDH with DTT. GPx (1 mg/ml) was reduced by preincubation with 5 mM DTT for 1 h. After incubation of the reduced GPx with (●, ●) or without (○) 100 μM SNAP for 1 h, DTT was added (arrows) to 5 mM (●, ●), and GPx activities were measured at each time point.

**Fig. 4.** Inactivation of GPx in U937 cells by incubation with SNAP. U937 cells were incubated with 100 μM SNAP at each time and disrupted by sonication for 10 min. After centrifugation at 10,000 × g for 10 min, the GPx activity of the supernatant was measured. Data are presented as means ± S.D. of triplicate experiments.

**Fig. 5.** Inactivation of GPx in RAW 264.7 cells treated with LPS. RAW 264.7 cells were incubated with 10 ng/ml LPS in the presence or absence of 1 mM N′-methyl-l-arginine for 18 h. Nitrite levels in the medium were measured (15). The GPx activities of these cells were assayed as described in the legend to Fig. 4. Data are presented as means ± S.D. of triplicate experiments.
nitroso compounds (27). If inactivation is due to S- or Se-nitrosylation, it is likely that SNAP is acting as an NO donor. In spite of extensive trials to identify a specific residue bound NO using 15N NMR by essentially the same method as used for bovine serum albumin (28), we failed to detect any definite signals characteristic to a chemical shift in GPx incubated with acidified Na15NO2 (data not shown). The following could be explanations for this. (i) Because GPx was not soluble at pH 1.0, which is the pH necessary for efficient production of [15N]NO from [15N]NaNO2, we had to incubate GPx with NaNO2 at pH 4.0. Although we prolonged the incubation time, this might not have supplied enough [15N]NO for modification of GPx. (ii) As is well known, GPx is easily autooxidized in the absence of reducing compounds. GPx–NO complex would therefore be unstable and undergo further oxidation, resulting in release of [15N]NO, as hypothesized for protein kinase C (7) and N-methyl-D-aspartic acid subtype of glutamate receptor (25).

NO can bind spontaneously to thiol groups in various compounds such as glutathione that are rich in cells. Nevertheless, treatment of cells with SNAP decreased GPx activity without changing the enzyme level, although to a lesser extent than in the experiment using the purified enzyme (Figs. 1 and 4). A significant decrease in intracellular GPx activity was also observed in LPS-treated RAW 264.7 cells (Fig. 5), presumably due to induction of NO synthase by LPS because GPx activity was protected in the presence of Nω-methyl-L-arginine. Inactivation of GPx may, therefore, occur in certain cells that produce NO or in surrounding cells. Because other antioxidative enzymes were not affected by NO (Table I), the increased accumulation of peroxides within cells after treatment with an NO donor (Fig. 4) or induction of NO synthase (Fig. 5) was likely a consequence of inactivation of GPx by NO. However, we can’t neglect the possibility that production of peroxides in the cells was increased by SNAP treatment. Recent reports showed that, in addition to having a cytostatic effect, NO induced apoptotic cell death in several types of cells (29, 30). Because redox regulation of cells and GPx activity are closely tied to apoptosis (31, 32), inactivation of GPx by NO maybe one of the causes of apoptotic cell death in these cells.

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