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Inhibition of Protein Tyrosine Phosphatases by Low-Molecular-Weight *S*-Nitrosothiols and S-Nitrosylated Human Serum Albumin

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The homogeneous recombinant mammalian protein tyrosine phosphatase 1B (PTP1B) and *Yersinia* protein tyrosine phosphatase (PTPase) are inactivated by a series of low-molecular-weight *S*-nitrosothiols. These compounds exhibited different inhibitory activities in a time- and concentration-dependent manner with second-order rate constants (k_{inact}/K_I) ranging from 37 to 113 M^{-1} min^{-1} against mammalian PTP1B and from 66 to 613 M^{-1} min^{-1} against *Yersinia* PTPase. Furthermore, the inactivation of *Yersinia* PTPase by S-nitrosylated protein:*S*-nitroso human serum albumin was investigated. Both single-S-nitrosylated and poly-S-nitrosylated human serum albumin show good inhibitory ability to *Yersinia* PTPase. The second-order rate constants are 472 and 1188 M^{-1} min^{-1}, respectively. This result indicates a possibility that S-nitrosylated albumin *in vivo* may function as an inhibitor for a variety of cysteine-dependent enzymes.

***Key Words:* *S*-nitrosothiols; S-nitrosylated human serum albumin; nitric oxide; inhibition; protein tyrosine phosphatases.**

Protein tyrosine phosphatases (PTPase) (EC 2.7.1.112) are immerging as essential regulators of a variety of fundamental cellular processes such as cell growth, mitogenesis, metabolism, gene transcription, cell cycle control and the immune response (1, 2). They are also key participants in kinase-dependent signal-transduction pathways, where they can play both positive and negative regulation roles (3). Maintenance of specific levels of protein tyrosine phosphorylation and dephosphorylation is vital for normal cell proliferation, differentiation and metabolism (4, 5). Furthermore, PTPase activity has also been associated with diabetes (6, 7). Thus, search for specific inhibitors of PTPase has been of distinct interests, because the inhibitors would not only be invaluable tools in assessing their physiological function, but also can lead to artificial control of the signaling pathways regulated by tyrosine kinases and phosphatases.

Due to the diversity of its physiological function and general ubiquity, nitric oxide (NO) has become a species of biological interest (8–12). Nitric oxide and other active nitrogen species have been suggested to modulated thiol-dependent enzymes through the formation of *S*-nitrosothiols (13) and subsequent oxidation to give disulfides (14). All PTPases have the common active site motif consisting of a cysteine and an arginine separated by five residues (CXXXXXR, where X is any amino acid). They form a thiol-phosphate covalent intermediate during the attack of substrate phosphorus by the essential cysteine in the active site (15). The highly reactive sulfhydryl group in PTPase could be a potential target of nitric oxide. Recently, Caselli *et al.* demonstrated that PTPases could be inactivated by nitric oxide both *in vitro* and *in vivo* (16, 17). The inactivation is caused by the reaction of NO with active site cysteine(s) to form a *S*-nitrosothiol, this is followed by the formation of an S-S bridge. The NO donors used in their study included sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1), *S*-nitrosocysteine (SNC), and nitric oxide gas.

S-nitrosothiols (RSNOs) have been identified in a variety of tissues, they are believed to be involved in the reaction of NO (possibly as a store or transport mechanism for NO). Also they have the potential medical use as NO donors, for the treatment of blood circulation problem for example (18–20). Some RSNOs, such as *S*-nitroso glutathione (GSNO), may be the most

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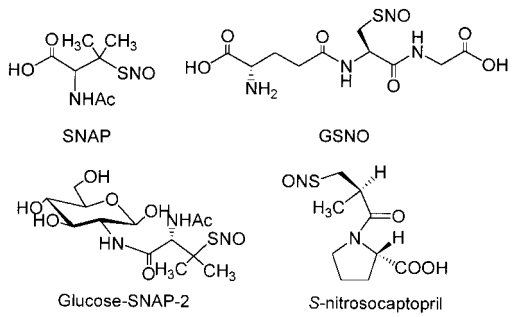

SCHEME 1

relevant biological molecule to carry out nitrosation reactions under physiological conditions (21–23). In this study we investigated the inactivation of recombinant mammalian PTP1B and *Yersinia* PTPase by a series of *S*-nitrosothiols including low molecular weight RSNOs and S-nitrosylated protein (S-nitrosylated human serum albumin).

MATERIALS AND METHODS

Materials and chemicals. *S*-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP), *S*-nitrosoglutathione (GSNO), and *S*-nitroso-captopril were prepared by the methods of Field *et al.* (24), Hart *et al.* (25) and Loscalzo *et al.* (26) respectively. Glucose-SNAP-2 was synthesized according to our previous methods (27). Human serum albumin and other chemicals were purchased from Sigma-Aldrich Co.

Single- and poly-S-nitrosylated HSA were prepared by the methods of Stamler *et al.* (28, 29). Single-S-nitrosylated HSA: HSA was exposed to equimolar $NaNO_2$ in 0.5 M HCl for 30 min at room temperature. Solution was then titrated to pH 7.4 with 1 M NaOH and 10 mM Tris/0.15 M NaCl (TBS), and dilutions were made as necessary in TBS (pH 7.4). Poly-S-nitrosylated HSA: HSA was treated with 100 mM dithiothreitol (DTT) and then dialyzed exhaustively in 0.1 M HCl to remove excess DTT, then the reduced HSA was nitrosylated by exposure to a 25-fold excess concentration of acidified nitrite.

Enzyme preparation. Homogeneous recombinant mammalian PTP1B (30) and *Yersinia* PTPase (31) were purified as described before.

Enzymatic assay. We tested the *S*-nitrosothiols against homogeneous recombinant mammalian PTP1B and *Yersinia* PTPase under comparable conditions (32). PTPase activity was measured spectrophotometrically at 405 nm with an UV/vis spectrophotometer (Hewlett–Packard 8453) using chromogenic substrate *p*-nitrophenyl phosphate (10 mM) in 1 mL of 50 mM tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.4, 1 mM EDTA with an ionic strength of 0.15 M). An *S*-nitrosothiol solution was prepared in the same buffer. To initiate incubation, each *S*-nitrosothiol solution obtained after serial dilution was mixed with PTPase in buffer of equal volume at 25°C. An aliquot was periodically removed from the incubation mixture and diluted into the enzyme assay solution containing the substrate. The residual enzymatic activity was measured. A control preincubation solution, containing all of the ingredients except for the inhibitor itself, was run and assayed in parallel.

RESULTS AND DISCUSSION

S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), *S*-nitrosoglutathione (GSNO), and *S*-nitroso-captopril were used in our bioassay, because these three compounds are the most typical and stable *S*-nitrosothiols and have been wildly used in the biological research of nitric oxide. Glucose-SNAP is a novel class of NO donor compounds synthesized in our group (27). The sugar unit of those compounds enhances water solubility, cell penetration, drug–receptor interaction and influences the dose–response relationships. Moreover, these compounds are more stable than SNAP in aqueous solution. Their molecular structures are shown in Scheme 1.

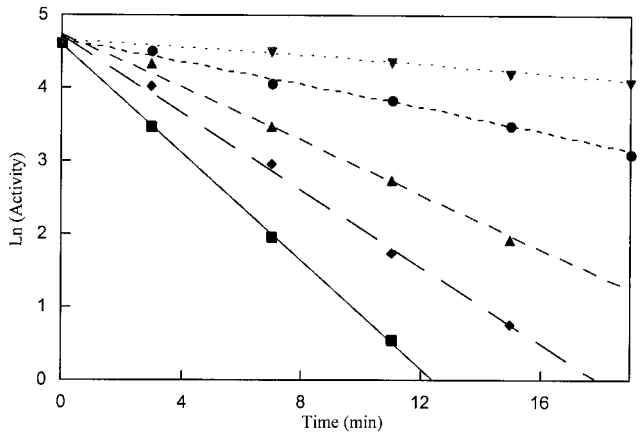

FIG. 1. Time course of inactivation of *Yersinia* PTPase by SNAP, in 50 mM Tris buffer (pH 7.4) at 25°C. SNAP concentration: (■) 2.5 mM; (◆) 0.5 mM; (▲) 0.25 mM; (●) 0.125 mM; (▼) 0.05 mM.

Incubation of both PTPases with each nitrosothiol at pH 7.4 and 25°C resulted in a time- and concentration-dependent loss of enzymatic activity. One typical inactivation process using SNAP to *Yersinia* PTPase is shown in Fig. 1. The figure shows that the inactivation reactions obey apparent first-order kinetics. The apparent first-order inactivation constants (k_{obsd}) can be calculated by plotting the residual activity versus time and fitting a linear equation. The replots of k_{obsd} as a

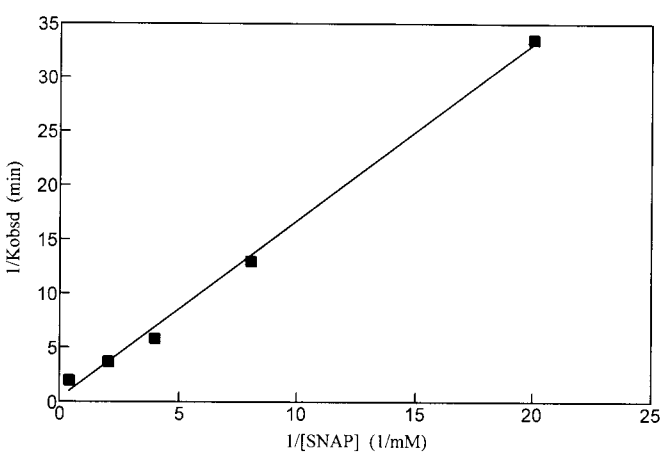

FIG. 2. Kitz–Wilson plot for the inhibition of *Yersinia* PTPase with SNAP in 50 mM Tris buffer (pH 7.4) at 25°C.

TABLE 1

Kinetic Parameters for the Inactivation of Mammalian PTP1B by *S*-nitrosothiols

Inhibitor	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I (M^{-1} min^{-1})
SNAP	0.109	0.969	113
GSNO	0.046	0.466	99
Glucose-SNAP-2	0.111	3.014	37
S-NO captopril	0.108	1.040	104

function of *S*-nitrosothiol concentration (Fig. 2) gave straight lines, indicating that the inactivation of PTPases by *S*-nitrosothiols is a bimolecular process. The calculated second-order rate constants (33) for each *S*-nitrosothiol inactivation of mammalian PTP1B and *Yersinia* PTPase are shown in Table 1 and Table 2, respectively.

The kinetic results presented in Tables 1 and 2 show that these four low molecular weight *S*-nitrosothiols are good inactivators of PTPases, while SNAP is the most active inhibitor of them. The second-order rate constant for inactivation of PTP1B by SNAP is 113 M^{-1} min^{-1} and of *Yersinia* PTPase is 613 M^{-1} min^{-1}. Both of the two values are much higher than that exhibited by dephostatin, a natural PTPase inhibitor with a functional *N*-NO residue (34). The inactivation of other three *S*-nitrosothiols is also much better than *N*-nitrosoaniline derivatives of dephostatin (34). By comparing the kinetic parameters of SNAP and Glucose-SNAP-2, it appeared that the introduction of sugar fragment decreased the inhibitory potency of SNAP.

Next, the recovery of activity of inactivated PTPases was studied. After inactivation with 0.5 mM SNAP, the inactivated *Yersinia* PTPase was treated with 20 mM dithiothreitol. About 65% of the initial enzymatic activity was recovered in 35 min (Fig. 3). Similar results were obtained using other *S*-nitrosothiol-inactivated PTPase (data not shown). These findings demonstrate that inactivation of PTPases by *S*-nitrosothiols is a reversible process.

Furthermore, the inactivation of PTPase by S-nitrosothiol was partially prevented in the presence

TABLE 2

Kinetic Parameters for the Inactivation of *Yersinia* PTPase by *S*-nitrosothiols

Inhibitor	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I (M^{-1} min^{-1})
SNAP	2.09	3.41	613
GSNO	0.038	0.573	66
Glucose-SNAP-2	0.084	1.152	73
S-NO captopril	0.124	0.848	146

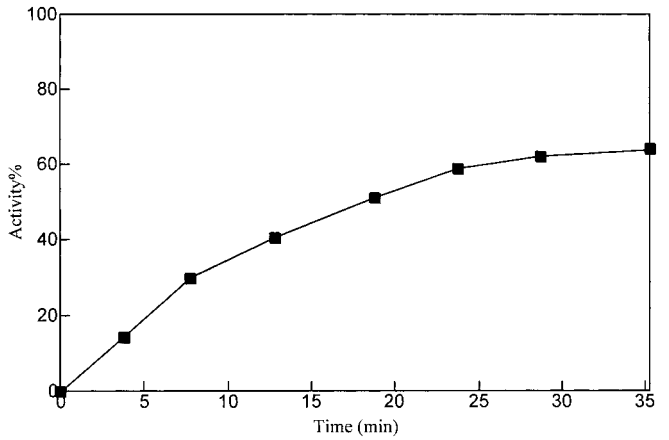

FIG. 3. Reactivation of *Yersinia* PTPase with dithiothreitol (20 mM).

of inorganic phosphate, a competitive inhibitor of PTPase (Fig. 4). At 40 mM phosphate concentration, the inactivation of *Yersinia* PTPase by SNAP was slowed down by 33%. These observations strongly suggest that the inactivation with these RSNOs takes place within the active site of PTPases, and is through a S-nitrosylation process as other NO donors like SNP, SIN-1 did (16, 17).

We next investigated the influence of S-nitrosylated human serum albumin (HSA) on *Yersinia* PTPase. In human plasma, the concentration of *S*-nitrosothiols is about 7 μM, while 96% of them are *S*-nitrosoproteins and 82% of them is accounted for S-nitrosylated serum albumin (35). HSA is the most abundant protein in plasma. Its physiological roles include the maintenance of colloid osmotic pressure and the transport of different ligands. In addition, HSA may have important roles as an extracellular antioxidant, by ligating

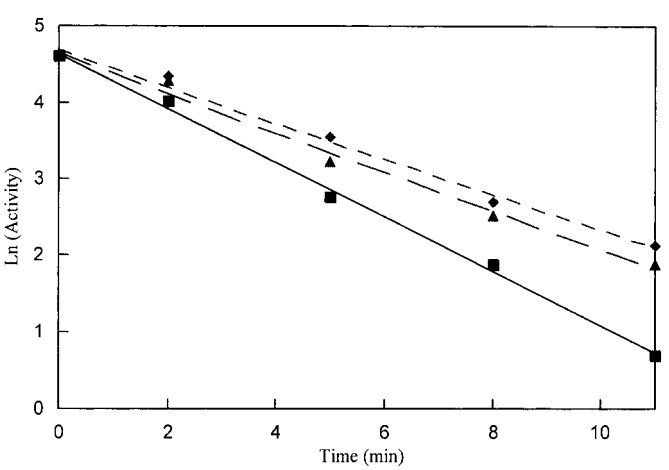

FIG. 4. Concentration effect of competitive inhibitor phosphate on the inactivation of *Yersinia* PTPase by SNAP. Phosphate concentration: (■) 0 mM; (▲) 30 mM; (◆) 40 mM.

TABLE 3

Kinetic Parameters for the Inactivation of *Yersinia* PTPase by Single- and Poly-S-nitrosylated Human Serum Albumin

Inhibitor	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I (M^{-1} min^{-1})
Single-S-nitrosylated HSA	0.594	1.258	472
Poly-S-nitrosylated HSA	0.0128	0.0107	1188

free metals and scavenging reactive species and serving as a transport molecule for nitric oxide. In fact, the main predominant redox form of NO *in vivo* is the *S*-nitroso adduct of serum albumin. S-nitrosylation of this protein endows it with potent and long-lasting endothelium-derived relaxing factor (EDRF)-like effects of vasodilation and platelet inhibition that are mediated by guanylate cyclase activation (28). This well characterized 66-kDa protein contains 35 cysteines that form 17 disulfide bonds with one free thiol group in the native conformation. Stamler's method (28) was used to nitrosylate this free sulfhydryl group and obtain the single-S-nitrosylated HSA. If reduced the serum albumin with excess of DTT, about half of the (native) cysteine residues become exposed and sustain nitrosylation to form the poly-S-nitrosylated HSA (29).

Incubation of *Yersinia* PTPase with both *S*-nitrosoproteins resulted in a time- and concentration-dependent loss of the enzyme activity. The calculated second-order rate constants for single-S-nitrosylated HSA and poly-S-nitrosylated HSA inactivation of *Yersinia* PTPase are 472 and 1188 M^{-1} min^{-1}, respectively (Table 3). These second rate constants are much higher than those from low molecular weight *S*-nitrosothiols. Poly-S-nitrosylated HSA exhibited stronger inhibition to PTPase than single-S-nitrosylated HSA did. These data suggest that the multiple copies of —*S*-NO group in the protein greatly enhance the transfer NO to the target enzyme. Furthermore, the inactivation of enzyme could be recovered by the addition of DTT and partially prevented in the presence of inorganic phosphate (data not shown). These results indicate that S-nitrosylated proteins are good inactivators of PTPases and have the same inactive mechanism for PTPases as low molecular weight *S*-nitrosothiols.

In the past few years, it has been shown that NO produced in biological systems has been implicated in a number of physiological processes which include smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission, and immune regulation (8–12). However, the half-life of NO is very short, because it easily reacts with molecular oxygen and other reactive oxygen species as well as free thiols in, for example, glutathione and proteins to form *S*-nitrosothiols. *S*-nitrosothiols functionally mimic NO and are also thought to play important roles in storing, transporting and delivery of NO *in vivo* (28, 35). There are a number of cases in which enzymes or receptor proteins modulate their biological activities through the S-nitrosylation of exposed and reactive cysteine residues. These enzymes include ornithine decarboxylase (36), HIV-1 protease (37, 38), papain (39), glyceraldehyde-3-phosphate dehydrogenase (40), and others (41). Transfer of the nitrosyl moiety from NO donor to the thiol group of protein has been suggested to be a signaling mechanism whereby nitric oxide controls cellular processes. In this paper, we demonstrated that not only low molecular weight *S*-nitrosothiols, but also S-nitrosylated proteins could serve as good inactivators to PTPases through a S-nitrosylation process. This finding points to a possibility that the endogenous S-nitrosylated albumin may function as an inhibitor for a variety of cysteine dependent enzymes. Our results also show the inhibition of PTPases is a transient phenomenon, because the inactivation of PTPase can be reverted by dithiothreitol (DTT), which means the enzyme can regenerate its original activity through the action of free thiols in biological systems, like cysteine and reduced glutathione. Therefore, the reversible covalent modification of reactive protein thiols by S-nitrosothiols represents a interesting type of regulation of biological functions.

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