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Oxidative inactivation of the lymphoid tyrosine phosphatase mediated by both general and active site directed NO donors

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ABSTRACT

Oxidative modification of protein tyrosine phosphatases (PTPs) has recently been recognized as an important regulatory mechanism in biological systems. Reported herein is the oxidative inactivation of the lymphoid tyrosine phosphatase (LYP) with both the general nitrosating reagent sodium nitroprusside (SNP) and also a novel peptide-based nitrosating reagent, Ac-ARLIEDNE(HcyNO)TAREG-NH_2, where HcyNO = *S*-nitrosohomocysteine. The SNP oxidatively inactivated LYP with a k_{inact} of 0.383 per min and a K_I of 27.4 μM and mixed-type inactivation kinetics. The peptide was a competitive LYP inactivator with a k_{inact} of 0.0472 per min and a K_I of 7.00 μM. LYP nitrosation by SNP was characterized by the addition of several NO moieties to the enzyme, while oxidation of LYP by the peptide did not result in the formation of a LYP–NO adduct. We propose that general NO donors promiscuously nitrosate any free cysteine residue while the active-site directed peptide selectively oxidizes the catalytic cysteine residue, resulting in the formation of a disulfide bond between the catalytic cysteine residue and a second cysteine in the active site.

Protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) maintain a dynamic equilibrium of tyrosine phosphorylation which plays an essential role in a multitude of cellular signaling pathways ranging from controlling cell growth and differentiation to metabolism and immunology.[1,2] It is increasingly apparent that oxidative inhibition of PTP activity is key to the biological regulation of PTP/PTK dependent cellular signaling pathways.[3–5] As an example, engagement of the T cell receptor (TCR) results in a spatially and temporally regulated chain of PTK/PTP activation and inactivation events.[6,7] Both PTPs and PTKs serve as active mediators of TCR signaling, play positive and negative roles in signaling pathways, and have tightly regulated activities.[8] Upon exposure to a variety of stimuli, T cells produce reactive oxygen and nitrogen species such as hydrogen peroxide (H_2O_2) and nitric oxide (NO).[5,9,10] These reactive oxidizing species readily diffuse throughout the cell and therefore can react with a variety of different biomolecules. One of the outcomes of NO production is a net increase in phosphotyrosine levels in the cell, which has been proposed to be a result of PTP inactivation.[5]

PTPs contain a conserved active site based on the sequence I/V-H-C-X-X-G-X-X-R-S/T. The local environment in the catalytic pocket results in an activated Cys residue with increased nucleophilicity and susceptibility to oxidation.[10] Because of this, several PTPs are known to be inactivated by oxidation. In the presence of H_2O_2, the catalytic cysteine residue can be oxidized to a sulfenic acid. Similarly, upon exposure to NO, the catalytic cysteine residue can be converted to an *S*-nitrosocysteine. The sulfenic acid and *S*-nitrosocysteine species can be further converted into disulfide or cyclic sulfenyl amide intermediates, with each of these oxidation products retaining the ability to be reduced under physiologically relevant conditions (see Scheme 1). In addition to the reversibly oxidized species, irreversible oxidation of the catalytic cysteine to a sulfinic (SO_2H) or sulfonic acid (SO_3H) can also occur.[9]

The lymphoid tyrosine phosphatase (LYP or PTPN22) has been the subject of considerable interest of late due both to its key role as a negative regulator of early stage TCR signaling[7,11–14] and also its involvement in autoimmunity.[15–17] One of the major biological substrates of LYP is Y394 of Lck, a Src family tyrosine kinase that also plays a critical role in early T cell receptor signaling.[18] Interestingly, LckY394 exhibits increased phosphorylation under oxidizing conditions, indicating possible oxidative inactivation of LYP.[5] In addition, in the presence of H_2O_2 in vitro, the catalytic cysteine residue in LYP forms a disulfide bond with a second cysteine residue in close proximity to the active site, both reversibly inactivating the enzyme and also, presumably, protecting the enzyme from irreversible over-oxi-

Abbreviations: PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; LYP, lymphoid specific tyrosine phosphatase PTPN22; SNP, sodium nitroprusside; Hcy, homocysteine; TCR, T cell receptor; Lck, lymphoid specific protein tyrosine kinase; DiFMUP, difluoromethylumbelliferyl phosphate; ESI-MS, electrospray ionization mass spectrometry.

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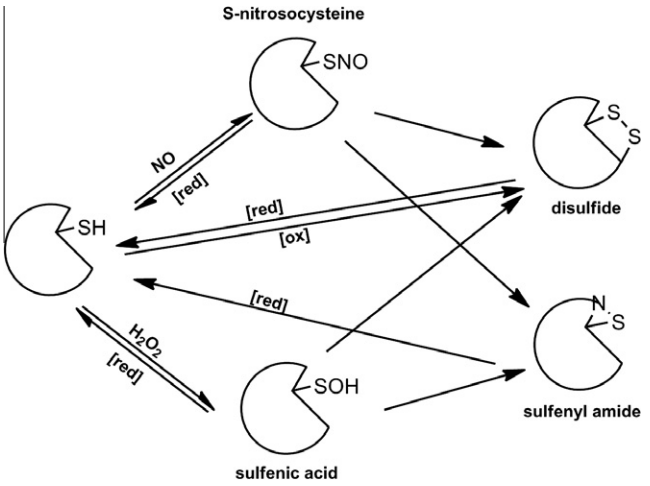

Scheme 1. PTP oxidation results in the formation of multiple reversibly oxidized species.

dation.[19] Although the biological regulation of LYP is not well understood, it is reasonable to expect that LYP is a candidate for biological oxidative regulation. Based on the importance of LYP in autoimmunity and the immune response, the evidence suggesting that LYP can be oxidatively inactivated both in vitro and in vivo, and the recognition that NO is a key signaling molecule in the immune system, we undertook a study of the oxidative inhibition of LYP activity by NO donors. The two main objectives of this work were to investigate the inhibitory potential of general NO donors with LYP and to develop a peptide-based, active site directed oxidizing agent.

The commonly used NO donor sodium nitroprusside (SNP) was chosen as a general NO donor to study oxidative inactivation of LYP. In addition, an active-site directed NO donor was designed based on the peptide sequence of the known LYP substrate LckY394. This peptide had the sequence Ac-ARLIEDNE(HcyNO)TAREG-NH_2, where the native tyrosine in the sequence was replaced by homocysteine (Hcy), a thiol-containing amino acid, that is readily nitrosated.[20] Both SNP and *S*-nitrosothiols release NO in the presence of thiolate anions and other reducing agents and can serve as oxidizing and/or nitrosating agents.[21] The activity of recombinant LYP was monitored in the absence and presence of the NO donors using the fluorogenic substrate 6,8-difluoromethylumbelliferyl phosphate (DiFMUP, Invitrogen) under reducing conditions (see Supplementary data for details). As shown in Figure 1, both compounds inactivated LYP in a time- and dose-dependent manner. The data shown in Figure 1 were fit to a single exponential function and the apparent inactivation constants thus obtained were used in a double-reciprocal plot to determine the K_I and k_{inact}.[22,23] For comparison, the SNP inactivated LYP with a k_{inact} of 0.383 per min and a K_I of 27.4 μM and the peptide had a k_{inact} of 0.0472 per min and a K_I of 7.00 μM. Inhibition by both compounds was readily reversible upon addition of L-cysteine (Figs. S2 and S3). The mechanism of inactivation was studied further using Lineweaver–Burk plots; SNP appeared to be a mixed-type LYP inactivator (Fig. S1) and the peptide showed competitive inactivation kinetics (Fig. S3). Importantly, LYP was not inactivated by the non-nitrosylated peptide (Fig. S5).

Based on the properties of the oxidizing agents and LYP, we hypothesized that LYP inactivation was due to oxidation and possibly nitrosation of the catalytic cysteine residue by both NO donors. However, LYP has eight free cysteine residues in addition to the catalytic cysteine, and we were interested to see whether exposure to the peptide and SNP resulted in the same form of oxidized LYP. To this end, we characterized the oxidized enzyme by ESI-MS. As indicated in Table 1, the ESI-MS spectrum of LYP in the presence of SNP shows several peaks, corresponding to LYP with 2, 3, 4, 5, 6, 7 and 8 NO moieties added (Fig. S6). This indicates that the general NO donor SNP nitrosates almost any available cysteine residue under the conditions of our experiment. In contrast, in the presence of the peptide, only one peak is observed in the ESI-MS (Table 1, Fig. S6). Interestingly, this peak does not represent an NO-adduct of LYP. The mass of the peak is 2 Da less than the parent enzyme, suggesting that a disulfide bond may have formed, presumably in the active site, upon oxidation by the active site directed peptide. This suggestion is consistent with data indicating that oxidation of LYP by hydrogen peroxide results in the formation of a disulfide bond between the catalytic cysteine residue and an adjacent cysteine in the active site.[19] Certainly the data indicate that general NO donors promiscuously oxidize and nitrosate the free cysteine residues on LYP, while the effect of the peptide is more subtle.

As shown here, LYP activity is affected by biologically relevant NO donors including peptide-based *S*-nitrosothiols. Oxidative

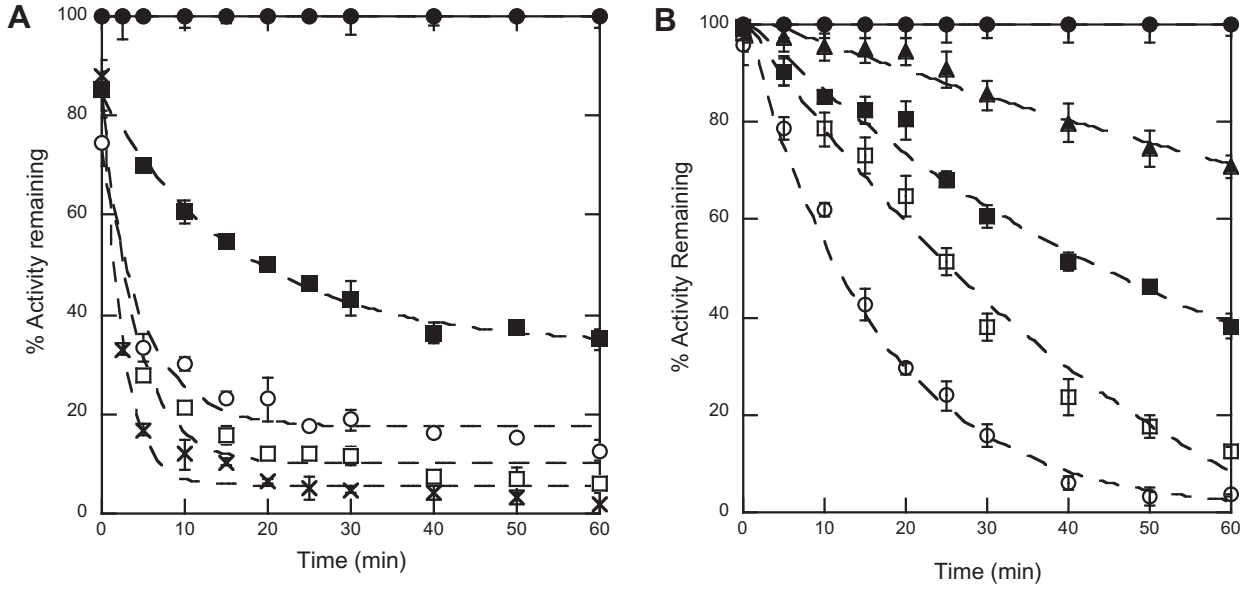

Figure 1. Time and concentration dependent inhibition of LYP activity by (A) SNP (where ● = 0 μM SNP, ■ = 5 μM SNP, ○ = 25 μM SNP, □ = 100 μM SNP, × = 250 μM SNP) and (B) Ac-ARLIEDNE(HcyNO)TAREG-NH_2 (where ● = 0 μM peptide, ▲ = 1 μM peptide, ■ = 5 μM peptide, □ = 20 μM SNP, ○ = 50 μM peptide).

Table 1
ESI-MS data for LYP + NO donors

LYP-$(NO)_X$ peak (in Da)	Difference from parent LYP peak (37805 Da)	*X*=	Relative % intensity
A. LYP + SNP			
37873	+68	2	55
37896	+91	3	100
37926	+121	4	80
37958	+153	5	55
37986	+181	6	25
38015	+210	7	17
38047	+242	8	20
B. LYP + Ac-ARLIEDNE(HcyNO)TAREG-NH_2			
LYP-$(NO)_X$ peak (in Da)	Difference from parent LYP peak (37800 Da)	*X*=	Relative % intensity
37798	−2	0	100

inactivation of LYP by NO donors is reversible, suggesting that this may be a relevant mechanism for biological regulation of LYP activity. Further characterization of the oxidized enzyme both in vitro and in vivo is currently underway in our laboratory.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.025.

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