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Original Contribution

The myeloperoxidase-derived oxidant hypothiocyanous acid inhibits protein tyrosine phosphatases via oxidation of key cysteine residues

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ABSTRACT

Phosphorylation of protein tyrosine residues is critical to cellular processes, and is regulated by kinases and phosphatases (PTPs). PTPs contain a redox-sensitive active site Cys residue, which is readily oxidized. Myeloperoxidase, released from activated leukocytes, catalyzes thiocyanate ion (SCN^-) oxidation by H_2O_2 to form hypothiocyanous acid (HOSCN), an oxidant that targets Cys residues. Dysregulated phosphorylation and elevated MPO levels have been associated with chronic inflammatory diseases where HOSCN can be generated. Previous studies have shown that HOSCN inhibits isolated PTP1B and induces cellular dysfunction in cultured macrophage-like cells. The present study extends this previous work and shows that physiologically-relevant concentrations of HOSCN alter the activity and structure of other members of the wider PTP family (including leukocyte antigen-related PTP, PTP–LAR; T-cell PTP, TC–PTP; CD45 and Src homology phosphatase-1, Shp-1) by targeting Cys residues. Isolated PTP activity, and activity in lysates of human monocyte-derived macrophages (HMDM) was inhibited by 0–100 μM HOSCN with this being accompanied by reversible oxidation of Cys residues, formation of sulfenic acids or sulfenyl-thiocyanates (detected by Western blotting, and LC–MS as dimedone adducts), and structural changes. LC–MS/MS peptide mass-mapping has provided data on the modified Cys residues in PTP–LAR. This study indicates that inflammation-induced oxidants, and particularly myeloperoxidase-derived species, can modulate the activity of multiple members of the PTP superfamily via oxidation of Cys residues to sulfenic acids. This alteration of the balance of PTP/kinase activity may perturb protein phosphorylation and disrupt cell signaling with subsequent induction of apoptosis at sites of inflammation.

1. Introduction

Phosphorylation of protein tyrosine residues represents an important regulatory component of cellular processes such as signal transduction and cell migration (reviewed [1]). The extent of cellular protein tyrosine phosphorylation is tightly controlled by the opposing activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs) [1]. PTP enzymes are part of a superfamily of proteins that contain a catalytic cysteine (Cys) residue at the active site; this site is highly susceptible to oxidation, allowing cells to rapidly regulate PTP function in response to stimuli. However, dysregulated PTP function, which may occur if the redox environment within cells is altered, has been linked to multiple human diseases, including cardiovascular disease and cancer (reviewed [2–4]).

Accumulating evidence implicates the heme enzyme myeloperoxidase (MPO) as an important risk factor for many inflammatory diseases including cardiovascular disease, arthritis,

Abbreviations: BSA, bovine serum albumin; CK, creatine kinase; DMSO, dimethylsulfoxide; DTNB, 5,5′-dithio-(2-nitrobenzoic acid); DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hank's buffered salt solution without calcium and magnesium; HMDM, human monocyte-derived macrophages; HRP, horseradish peroxidase; IAF, iodoacetamidofluorescein; LDH, lactate dehydrogenase; LPO, lactoperoxidase; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; PBS, phosphate-buffered saline; pNPP, p-nitrophenyl phosphate; PTP, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase-1B; PTP–LAR, leukocyte antigen-related protein tyrosine phosphatase; Shp-1, Src homology phosphatase-1; TBST, tris-buffered saline with Tween 20; TC–PTP, T-cell protein tyrosine phosphatase; TFA, trifluoroacetic acid; TNB, 5-thio-2-nitrobenzoic acid

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neurodegenerative diseases and some cancers (reviewed [5–7]). MPO is released by activated neutrophils, monocytes and some tissue macrophages at sites of inflammation, where it catalyzes the reaction of hydrogen peroxide (H_2O_2) with halide (Cl^-, Br^-) and pseudohalide (thiocyanate, SCN^-) ions to form hypohalous acids (HOCl, hypochlorous acid; HOBr, hypobromous acid; HOSCN, hypothiocyanous acid) [8]. These species are potent oxidants with antibacterial properties and play an important role in the immune system [9], however, their excessive or misplaced generation can result in damage to host cells and may contribute to disease.

Thiocyanate ions are a favored substrate for MPO and have a very high specificity constant for the enzyme [10]. Consequently, HOSCN is a major oxidant formed by MPO both with normal plasma levels of halides and thiocyanate (100–140 mM Cl^-, 20–100 μM Br^-, < 1 μM I^-, 20–120 μM SCN^- [10,11]), and people such as smokers, who can have markedly elevated levels of thiocyanate ions (up to 250 μM SCN^- [11–13]). SCN^- is also a favored substrate for multiple other peroxidases, including eosinophil peroxidase, lactoperoxidase and gastric peroxidase, as these members of the peroxidase superfamily do not readily oxidize Cl^- (reviewed [5,14]). HOSCN is also generated efficiently and rapidly via non-enzymatic reaction of HOCl and HOBr with SCN^- [15,16].

While HOCl and HOBr react rapidly with multiple biological targets (reviewed [17,18]), HOSCN reacts with considerable specificity with thiol groups (including glutathione (GSH) and protein Cys residues) [19] and selenols (e.g. selenocysteine [20]). This selectivity can result in significant damage to key cellular enzymes that contain Cys residues (e.g. [21–23]) or Cys-metal ion clusters [12,24].

We have shown previously that HOSCN inhibits the activity of isolated PTP1B enzyme and alters mitogen-activated protein kinase (MAPK) signaling in J774.1 mouse macrophage-like cells [21]. Although a loss of Cys residues in isolated PTP1B was observed, these modifications were not examined in detail and whether this is a general phenomenon of relevance to other PTPs was not investigated. The present study therefore examined the hypothesis that this is a general phenomenon with PTPs, and also aimed to characterize the structural modifications that occur to the Cys residues of a number of isolated PTPs (leukocyte antigen-related PTP (PTP–LAR), T-cell PTP (TC–PTP), CD45 and Src homology-2 domain-containing phosphatase-1 (Shp-1)) on exposure to (patho)physiologically relevant concentrations of HOSCN. We show that HOSCN inactivates isolated PTPs via oxidation of particular Cys residues to the corresponding sulfenic acid; some of these changes are readily reversible. These studies were complemented by examination of PTP activity in primary human monocyte-derived macrophages (HMDM).

2. Materials and methods

2.1. Reagents

Aqueous solutions and buffers were prepared using nanopure water filtered through a four-stage Milli-Q system (Merck Millipore). Lactoperoxidase (LPO, from bovine milk; Calbiochem) was quantified by absorbance at 412 nm using a molar absorption coefficient, ε 112,000 M^{-1} cm^{-1} [25]. H_2O_2 (30% v/v, Merck) concentration was measured by absorbance at 240 nm, using ε 39.4 M^{-1} cm^{-1} [26]. PTP enzymes (human, recombinant) were purchased from Enzo Life Sciences (product numbers: PTP–LAR: BML-SE113; Shp-1: BML-SE334; TC–PTP: BML-SE114; CD45: BML-SE135). ThioGlo1 and *p*-nitrophenyl phosphate reagents were from Calbiochem. 5-iodoacetamidofluorescein (IAF), SDS-PAGE and Western blotting supplies were from Life Technologies. Precision Plus Kaleidoscope standards were from Bio-Rad. Pierce High Sensitivity Streptavidin HRP Conjugate was from Thermo Scientific. Western Lightning Plus ECL reagents were from Perkin Elmer. DCP-Bio1 was from Kerafast. Lymphoprep was from Axis-Shield PoC AS. All other chemicals were from Sigma-Aldrich.

2.2. Incubation of isolated PTPs with HOSCN

HOSCN was prepared enzymatically using bovine milk lactoperoxidase (LPO), as previously described [23,27]. The concentration of HOSCN was determined by reaction with TNB (5-thio-2-nitrobenzoic acid; prepared by alkaline hydrolysis of DTNB with NaOH), using ε 14,150 M^{-1} cm^{-1} at 412 nm [28]. Prior to oxidant treatment, dithiothreitol (DTT) was removed from PTP enzymes using Amicon Ultra 10 kDa centrifugal devices (Merck Millipore). For the enzyme activity and ThioGlo assays, PTPs were diluted to 1 μM in a buffer containing 25 mM Tris–HCl, pH 7.2, 50 mM NaCl, 2 mM EDTA and 0.01% Brij35 ('assay buffer'). For the gel-based analyses, 25 mM Tris–HCl, pH 7.2 was used in place of assay buffer. PTP enzymes (final concentration 500 nM) were exposed to 0–100 μM HOSCN in assay buffer for 5 min at 21 °C. In order to control for possible effects of SCN^-, or products generated on decomposition of the oxidant (e.g. ^-OCN), PTPs were also exposed to 50–100 μM SCN^- and up to 100 μM HOSCN that had been left to decompose overnight at 21 °C. Aliquots of control- and oxidant-treated PTPs were subsequently taken for analysis.

2.3. PTP activity assays

PTP activity was assessed as described previously [21] using the chromogenic substrate, *p*-nitrophenyl phosphate (pNPP), which is cleaved by PTPs to yield the *p*-nitrophenolate anion which absorbs at 405 nm. A 4 mM solution of pNPP substrate was prepared using 100 μL pNPP, 1.0 mL pNPP substrate buffer and water to 5.0 mL. Control and oxidant-treated PTP–LAR, TC–PTP and CD45 samples were added to wells of a 96-well plate containing assay buffer with 1 mg mL^{-1} BSA. The reaction was started by the addition of 2 mM pNPP substrate and the plate was incubated at 30 °C for 45 min. After this time, NaOH (10 mM) was added to each well and the plate was read at 405 nm. The concentration of the *p*-nitrophenolate anion formed, representing enzyme activity, was determined from a *p*-nitrophenol (pNP) standard curve. Control and oxidant-treated Shp-1 samples were added to wells of a 96-well tissue culture plate containing assay buffer with 1 mg mL^{-1} BSA. The reaction was started by the addition of 2 mM pNPP substrate and the plate was read at 405 nm and 30 °C once per min for 55 min, as per the manufacturer's instructions. Rates of pNPP hydrolysis were calculated from the linear portion of the curve using ε 18,000 M^{-1} cm^{-1} for the *p*-nitrophenolate anion [29].

2.4. Quantification of thiols

Following exposure of PTP enzymes to 0–100 μM HOSCN for 5 min at 21 °C and pH 7.2, free thiols were quantified by labeling Cys residues with the fluorescent dye, ThioGlo1 as described previously [30]. Control and oxidant-treated PTPs were diluted 1:3 in Tris–HCl, pH 7.2, and 25 μL of this solution were added to wells of a 96 well plate, followed by 25 μL diluted ThioGlo1 (prepared from a 1:100 dilution of 2.6 mM ThioGlo1 stock solution in acetonitrile). The plate was incubated for 5 min in the dark at 21 °C, with the fluorescence then measured at λ_{ex}=384 nm and λ_{em}=513 nm. Thiol concentrations were calculated relative to a standard curve constructed using glutathione.

2.5. Gel electrophoresis and Western Blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was