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Original article

Redox-based probes as tools to monitor oxidized protein tyrosine phosphatases in living cells

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ABSTRACT

Reversible oxidation of protein tyrosine phosphatases (PTPs) has emerged as an important regulatory mechanism whereby reactive oxygen species (ROS) inactivates the PTP and promotes phosphorylation and induction of the signaling cascade. The lack of sensitive and robust methods to directly detect oxidized PTPs has made it difficult to understand the effects that PTP oxidative inactivation play in redox signaling. We report the use of redox-based probes to directly detect oxidized PTPs in a cellular context, which highlights the importance of direct approaches to assist in the study of physiological and pathophysiological PTP activity in redox regulation. We also demonstrate, as a proof-of-concept, that these redox-based probes serve as prototypes for the design and development of a new class of inhibitors for phosphatases. We envision a nucleophile reacting with the oxidized inactive catalytic cysteine to generate an irreversible thioether adduct which prevents the phosphatase from being reactivated and ultimately fortifies the signaling cascade. Our results reveal the potential of translation of our redox-based probes, which are used to understand redox cell circuitry and disease biology, to small-molecule nucleophile-based inhibitors, which may treat diseases associated with redox stress. This may have implications in the treatment of type 2 diabetes and cancer.

1. Introduction

Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) are formed by the partial reduction of oxygen. Cellular ROS can be generated endogenously by univalent reduction of molecular oxygen to generate $O_2^{\bullet -}$, which can dismutase spontaneously or thorough the assistance of superoxide dismutase to give H_2O_2. Superoxide formation typically results from the premature leakage of electrons from the electron transport chain, byproducts of xanthine and aldehyde oxidases, or induced through growth factor stimulation [1–3]. Substantial evidence indicates ROS functions as a signaling molecule during signal transduction in a diverse range of biological processes to mediate distinct physiological responses such as proliferation, differentiation, and apoptosis [4,5].

Protein tyrosine phosphatases (PTPs) are crucial regulators of signal transduction. This class of enzymes function as antagonists towards protein tyrosine kinases (PTKs) to control reversible tyrosine phosphorylation, which governs fundamental physiological functions such as cellular growth, proliferation, differentiation, survival, metabolism, and motility [6]. PTPs are tightly regulated by several mechanisms ranging from differential expression, subcellular localization, limited proteolysis, post-translational modifications, ligand binding, and dimerization [7]. The PTP catalytic domain contains a unique microenvironment in which the catalytic cysteine exhibits a depressed pK_a and therefore exists as a thiolate anion at physiological pH. This facilitates the phosphatases to carry out their enzymatic function but also renders them sensitive to oxidation [8]. Upon exposure of the phosphatase to H_2O_2, the catalytic cysteine residue is converted to a sulfenic acid (RSOH), which results in PTP inactivation. Reactions with cellular thiols can restore the oxidized PTPs to the catalytically active form [9]. Therefore, reversible PTP oxidation has emerged as an important cellular regulatory mechanism whereby ROS, such as H_2O_2 generated from physiological responses, promotes phosphorylation and induction of the signal cascade by oxidizing and inactivating the PTP at its catalytic site.

The lack of sensitive and robust methods for detecting oxidized phosphatases, as opposed to abundant redox-sensitive enzymes, has made understanding redox regulation of phosphatases in the cellular context challenging. The majority of approaches available to detect PTP oxidation are indirect and not apt for cell based

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studies [10]. In-gel activity assays use radioactively labeled substrate to monitor reversible PTP oxidation [11]. The in-gel phosphatase assay is biased towards non-membrane bound phosphatases and is not quantifiable. Since cysteine thiols are known to react with electrophiles, a modified cysteinyl-labeling assay was developed that relies on biotinylated thiol-alkylating agents to expose reversibly oxidized phosphatases [12]. The modified cysteinyl-labeling assay is not a targeted approach and suffers from high background of non-PTP proteins (Fig. 1a). An alternative strategy to survey oxidized PTPs is to use an antibody generated against the conserved signature motif of phosphatases harboring the terminally oxidized (RSO_3H) active-site cysteine [13,14]. This immunochemical approach does not differentiate between phosphatases regulated by reversible oxidation (RSOH) from those that are inherently hyperoxidized (RSO_2H, RSO_3H).

Direct approaches to monitor protein oxidation currently rely on nucleophiles that exploit the selective reaction between RSOH (Fig. 1b). The most common example is 5,5-dimethyl-1,3-cyclohexanedione (dimedone) which generates a stable thioether adduct upon reacting with the RSOH [15]. The chemoselective reaction of several dimedone-based probes has been applied to detect oxidized proteins *in vitro* and *in situ* [16]. Based on the success of this critical reaction, several probes to exclusively monitor PTP oxidation have been developed. These PTP redox-based probes (RBPs) are composed of: 1) a dimedone-based warhead that forms a covalent adduct with the oxidized active-site cysteine; 2) a module that directs binding to the PTP catalytic site; and 3) a reporter tag used for the identification, purification, or direct visualization of the labeled protein [17]. Additionally, single-chain variable fragment (ScFv) antibodies directly detect unique conformational changes associated with oxidized PTP1B [18]. Though the conformation sensing antibodies provides a direct approach to monitor PTP oxidation, they are specific for a single protein and may not be used to monitor oxidation of the entire classical PTP family.

The low cellular abundance of signaling proteins has made the detection of oxidized PTPs difficult. Herein, we report the use of the RBPs to detect oxidized phosphatases in cells and to investigate PTP regulation in redox signaling (Fig. 1c). Literature has reported that the bioorthogonal reaction is enhanced when the chemical reporter harbors an alkyne handle and is used in combination with an azide bearing detection tag [19]. In an effort to circumvent detection limitations of the low abundant phosphatases, we synthesized alkyne analogues of our previously reported RBPs to give the parent compound (DYn-0), biphenyl (BiPhYn-1), and naphthyl (NaphYn-1) probes (Fig. 1d). We've also adopted a more robust ligand for the Huisgen [3 + 2] cycloaddition reaction (click chemistry). We report the use of a more reactive tris(triazolylmethyl)amine-based ligand – BTTP as our ligand of choice for the bioorthogonal chemical reaction, as opposed to TBTA, to append reporter tags to the low abundant probe-modified proteins [20].

2. Results

The catalytic cysteine thiolate of PTP1B reacts with H_2O_2 to yield the RSOH, which rapidly condenses with the main-chain nitrogen of an adjacent serine residue to give the cyclic sulfenamide [21,22]. To determine whether dimedone could trap the PTP1B-SOH intermediate, we performed *in vitro* experiments with dimedone and the resulting protein S-dimedone adduct was detected using an

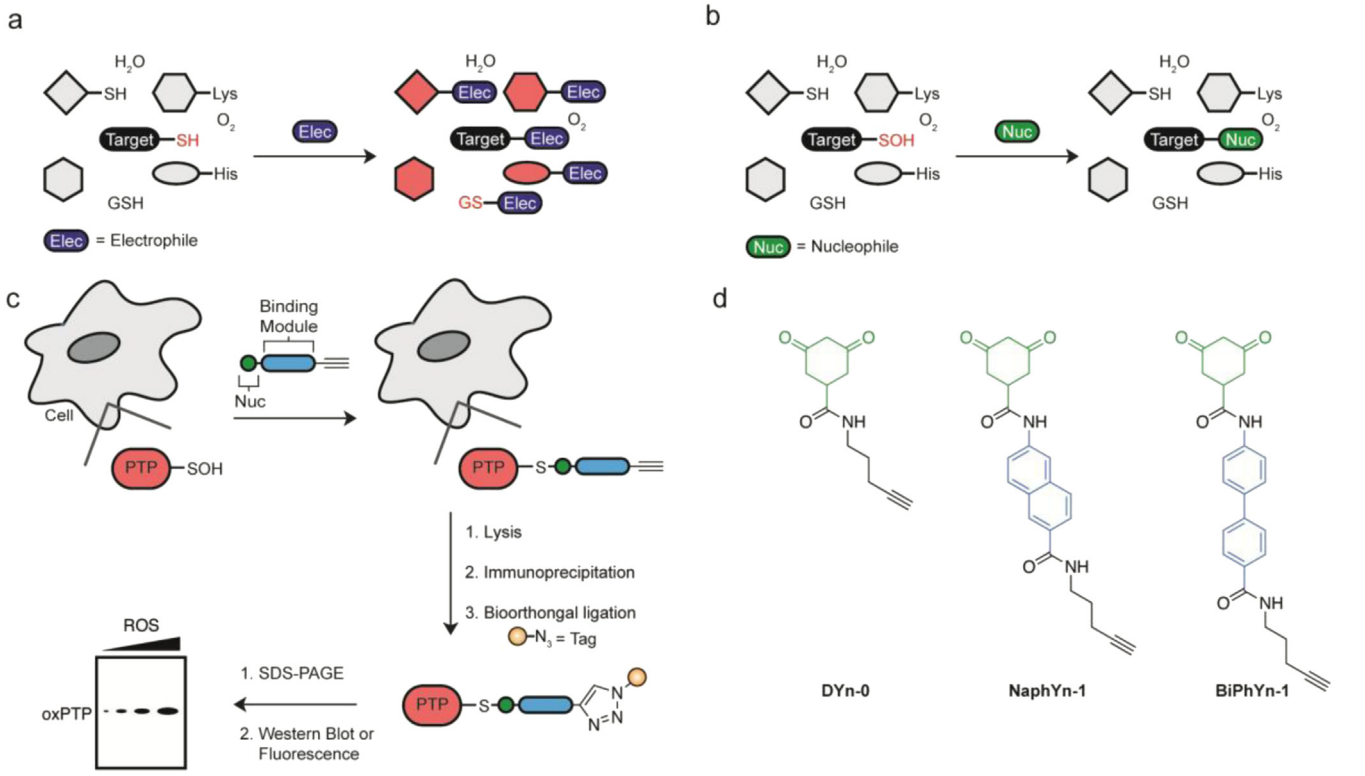

Fig. 1. (a) Electrophiles are promiscuous and may react with varying nucleophilic species found within a proteome. (b) Nucleophiles react selectively with the RSOH to generate a stable thioether bond. Figure adapted from Ref. [38]. (c) A direct approach to monitor the extent of PTP oxidation within a cell. Cells are treated with redox-based probes (RBPs) to selectively label the endogenously oxidized phosphatases. The cells are lysed and proteins of interest are immunoprecipitated. Bioorthogonal ligation is performed on the immunocomplex to append a reporter tag. Samples are resolved by SDS-PAGE and visualized by avidin blotting or fluorescence. (d) Structures of alkyne based RBPs used to directly monitor PTP oxidation.

immunochemical approach previously reported in our laboratory [23]. We treated recombinant PTP1B (aa 1-321) with increasing concentrations of dimedone in the presence of H_2O_2. A stable adduct between dimedone and oxidized PTP1B was generated and detected by the antibody (Supplementary Fig. 1). In order to evaluate the capacity of RBPs to react towards the oxidized phosphatase, we treated PTP1B with increasing concentrations of the RBPs in the presence of H_2O_2 followed by the conjugation of a biotin tag via bioorthogonal ligation and visualization by avidin blotting. The data demonstrates that RBPs have increased sensitivity towards the oxidized phosphatase as opposed to the parent compound (Fig. 2a). Carbon acids, such as dimedone, can be oxidized by H_2O_2 to generate a trione species, which could act as an electrophile and form an adduct with the thiol form of PTP1B. It is important to note that the concentrations of H_2O_2 required to effect such a chemical reaction are significantly higher (mM) than those used in these experiments (μM) (unpublished data). Nonetheless, to further rule out this possibility we generated the sulfenic acid form of PTP1B, quenched this reaction with catalase, and then exposed the oxidized enzyme to the RBPs. Using this alternate workflow, no differences in PTP1B labeling by RBPs were observed, as expected (Supplementary Fig. 2). A time dependent study with BiPhYn-1 and DYn-0 showed that BiPhYn-1 detects the oxidized PTP within 5 min as opposed to 30 min with the parent compound (Fig. 2b).

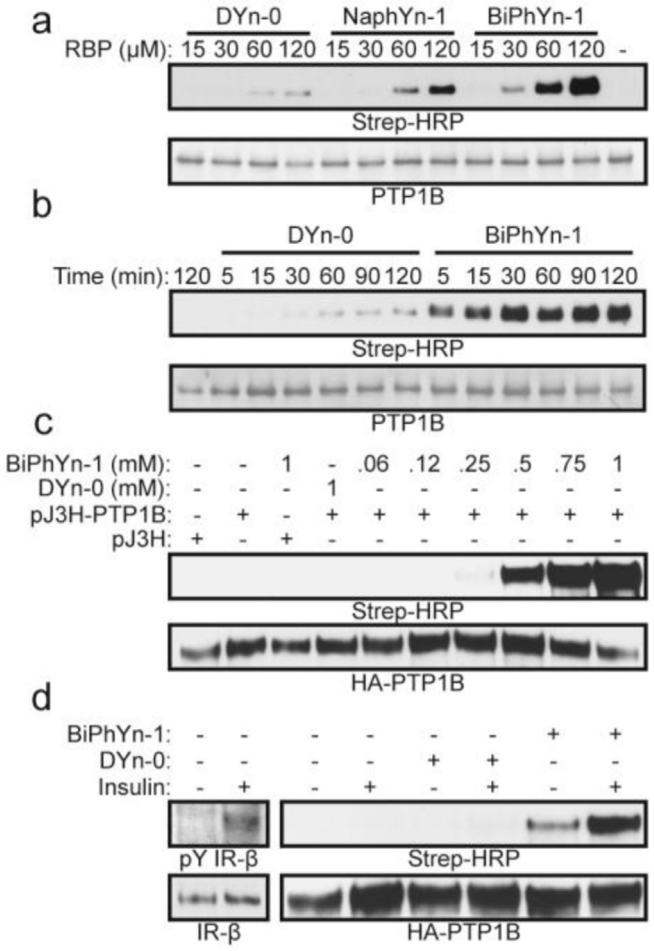

Fig. 2. RBPs exhibit enhanced selectivity towards oxidized PTP1B *in vitro* and *in situ*. Labeling of recombinant PTP1B (20 μM) with 10 equivalences H_2O_2 (200 μM) in the presence of 50 μM or increasing concentrations of RBP in (a) dose- and (b) time-dependent fashion displays RBPs selectivity towards oxidized PTP1B. (c) COS1 cells that had been transfected with pJ3H-PTP1B were treated with BiPhYn-1 and exhibit a dose-dependent detection of basal oxidized PTP1B. (d) 250 μM BiPhYn-1, but not DYn-0, detects increased levels of oxidized PTP1B initiated by insulin (100 nM) induced H_2O_2 production.

To determine whether the RBPs recognize and bind non-covalently to the phosphatase, we measured their potential to inhibit phosphatase activity using a fluorogenic substrate, 4-methylumberlliferyl phosphate (4-MUP). Aryl compounds can be prone to an aggregation-based mechanism of inhibition [24]. To test whether the RBPs inhibit PTP1B through such a mechanism, we performed activity assays in the presence of increasing concentrations of Triton-X 100 detergent. Notably, no differences were observed in the inhibition of PTP1B by BiPhYn-1 as the concentration of detergent was increased (Supplementary Fig. 3), indicating that this probe does not aggregate and subsequently inhibit PTP1B. BiPhYn-1 showed modest inhibition towards PTP1B ($IC_{50} = 0.49$ mM) as opposed to the parent compound DYn-0 ($IC_{50} = 4.7$ mM) and NaphYn-1 ($IC_{50} = 2.3$ mM) (Supplementary Fig. 4a). Since NaphYn-1 exhibited poor inhibition, we focused all subsequent efforts on further characterization of BiPhYn-1. First order reaction rates of the RBPs towards the RSOH were determined using a RSOH model system and revealed that the binding modules did not enhance the nucleophilicity of the dimedone warhead (Supplementary Fig. 4b) [25].

We used glutathione peroxidase 3 (Gpx3) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to evaluate the reactivity of the RBPs towards detection of RSOH in non-PTP proteins. Gpx3 and GAPDH were treated with BiPhYn-1 and DYn-0 in the presence of H_2O_2 followed by conjugation of the biotin tag and avidin blotting. Both compounds demonstrated equal labeling of non-PTPs suggesting that BiPhYn-1 maintains the ability to detect RSOH modifications in non-PTPs (Supplementary Fig. 5). The PTP super family is comprised of classical PTPs and dual specificity phosphatases (DUSPs), which dephosphorylate phospho-tyrosine and phospho-serine/threonine/tyrosine residues respectively. Since all PTPs contain a highly conserved catalytic site, we envisioned BiPhYn-1 would act as a global probe for all PTPs. We therefore tested the capacity of BiPhYn-1 in detecting a panel of oxidized PTPs in comparison to DYn-0. BiPhYn-1 was able to detect oxidized YopH, PTP1B, CDC25B, SHP-1, and VHR more robustly then DYn-0 (Supplementary Fig. 6). Taken together, these results designate the BiPhYn-1 RBP as a general tool for detecting oxidized PTPs.

Catalytically inactive PTP1B (C215S) was generated to ensure RBPs were targeting the catalytic cysteine. A loss of signal was observed when labeling C215S with BiPhYn-1 in the presence of H_2O_2, as opposed to wild type (Supplementary Fig. 7a). Competition studies with phenyl vinyl sulfone (PVSF) [26], a mechanism based probe for PTPs, exhibited loss of detection of oxidized PTP1B with BiPhYn-1 in the presence of increasing concentrations of PVSF (Supplementary Fig. 7b) within cells. Finally, LC/MS/MS was used to map the site of modification by BiPhYn-1 (Supplementary Fig. 7c).

To ascertain whether BiPhYn-1 would detect oxidized phosphatases in a cellular context, COS1 cells were transfected with pJ3H-PTP1B to overexpress HA-tagged PTP1B (Supplementary Fig. 8). BiPhYn-1 was titrated into transfected cells for 1 h at 37 °C, 5% CO_2. The cells were washed then lysed followed by immunoprecipitation of HA-tagged PTP1B using anti-HA agarose resin overnight. The probe-labeled phosphatase was visualized by appending a biotin tag via bioorthogonal ligation of the immunocomplex followed by avidin blot. BiPhYn-1 detects basal levels of oxidized PTP1B in a dose dependent manner (Fig. 2c).

Several works have reported that PTP1B is transiently inactivated by H_2O_2 produced by various cell stimuli and that phosphatase inactivation is important for induction of optimal tyrosine phosphorylation response [27–29]. One signaling pathway for which H_2O_2 production occurs is in response to the hormone insulin [30]. To verify that BiPhYn-1 can detect increased oxidized

PTP1B upon ligand induced ROS formation, pJ3H-PTP1B transfected COS1 cells were treated with 100 nM insulin followed by the addition of BiPhYn-1. BiPhYn-1 was able to detect an increase in the levels of oxidized PTP1B upon insulin stimulation (Fig. 2d). This data suggests that the RBPs are robust tools to directly monitor levels of oxidized PTPs within a cell.

PTP1B has been shown to function as a negative regulator in the insulin signaling pathway. PTP1B influences the duration and amplitude of the insulin signaling response by catalyzing the removal of phosphoryl groups on tyrosine residues of the insulin receptor (IRβ, pYpY 1162/1163) and insulin receptor substrates protein (IRS-1) [31,32]. Since BiPhYn-1 was able to detect increased levels of oxidized PTP1B in cells, the next step was to determine if the RBPs could modulate the extent of tyrosine phosphorylation. We hypothesized that the BiPhYn-1 RBP would react with the oxidized inactivated PTP in cells to enable prolonged signaling and downstream physiological effects for further investigations (Fig. 3a). To this end, CHO cells that express the human insulin receptor (CHO/hIRc) were incubated with a range of concentrations of BiPhYn-1 followed by treatment with or without insulin. BiPhYn-1 was able to increase the phosphorylation levels of IRβ. The maximal effect of BiPhYn-1 on the levels of phosphorylation in CHO/hIRc cells peaked at 250 μM (Fig. 3b). At higher BiPhYn-1 concentrations the phosphorylation levels of the insulin receptor decreased potentially due to nonspecific effects. Due to the generic scaffold associated with the RBPs, it is speculated that the probes may behave promiscuously and give rise to off-target effects via the inhibition of opposing but structurally related phosphatases in the PTP super family, ultimately leading to the reduction in the overall levels of phosphorylation at high RBP concentrations [33].

Insulin increases glucose uptake in cells by stimulating the translocation of the glucose transporter (GLUT4) from intracellular sites to the cell surface [34]. We next sought to determine whether the increased levels of phosphorylation of IRβ translated into an increase in glucose uptake. A fluorescent derivative of glucose, 2-NBD Glucose (2-NBDG), was used to ascertain whether the RBPs could trap the oxidized PTP1B to enhance glucose uptake in cells. COS1 cells were treated with increasing concentrations of the RBPs followed by simultaneous treatment with insulin and 2-NBDG. A dose-dependent increase in 2-NBDG uptake was observed in cells treated with BiPhYn-1 as opposed to cells treated with DYn-0 (Fig. 3b,c). Taken together, this data conveys that the RBPs can be used as a direct approach to facilitate cellular investigation of PTP redox regulation in cell signaling.

3. Conclusion

In summary, we report the use of redox-based probes to directly detect and monitor oxidized phosphatases in a cellular setting. These RBPs can facilitate the detection of ROS-inactivated PTPs associated

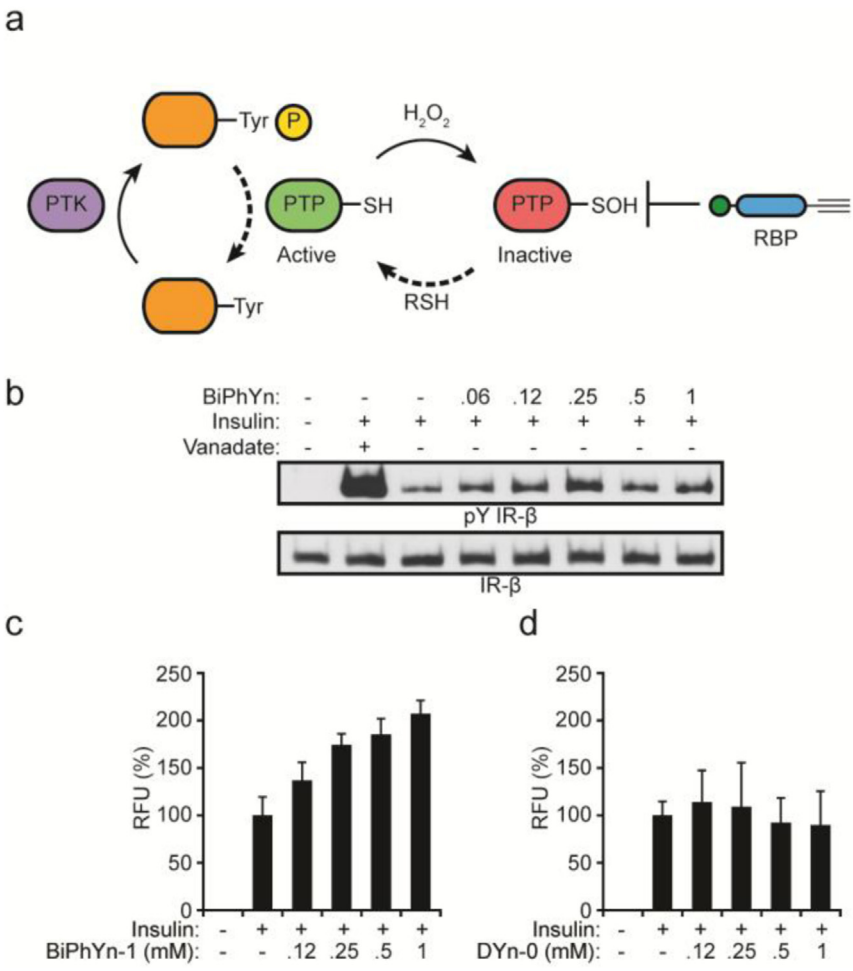

Fig. 3. (a) Reversible oxidative inactivation of protein tyrosine phosphatases (PTPs) is triggered by hydrogen peroxide (H_2O_2) reacting with the catalytic cysteine to generate a sulfenic acid (RSOH). Phosphatase activity is restored upon reaction with thiols. We hypothesize that trapping oxidized PTP1B with BiPhYn-1 can enhance tyrosyl phosphorylation of the insulin receptor β. (b) CHO/hIRc cells stimulated with 10 nM insulin in the presence of increasing concentrations of the RBP. (c, d) Enhanced glucose uptake observed in the presence of increasing concentrations of BiPhYn-1, but not DYn-0, when stimulating cells with 250 nM insulin in the presence of 50 μM 2-NBD glucose (2-NBDG).

with specific cell signaling pathways and help to outline the physiological roles of redox regulation. Our experiments supports and communicates the importance and ease of incorporating chemoselective small molecule approaches to assist in the study of aberrant phosphatase activity brought about by increased ROS that is associated with pathophysiological states such as cancer. Additionally, PTP1B has been implicated as an important target for the treatment of type 2 diabetes and obesity. The development of cell permeable and bioavailable small molecule PTP inhibitors has been constrained by the highly conserved and highly polar phospho-tyrosine binding pocket [35]. As a result, many reported PTP inhibitors conform to highly charged anionic phosphatase mimetics that cannot cross the cell membrane [36]. Previously, aryl diketoacids were identified as pTyr surrogates that targeted and stabilized the "open" inactive conformation of PTP1B, thereby inhibiting the enzyme [37]. Recently it was demonstrated that stabilization of oxidized PTP1B, using single-chain variable fragment antibodies that target the unique conformation of oxidized PTP1B, enhanced phosphorylation and sustained insulin signaling [18]. We illustrate that our redox-based probes serve as a proof-of-concept for the development of a new class of small molecule inhibitors that target the oxidized inactive phosphatase via nucleophilic trapping of the oxidized catalytic cysteine. Our results revealed an increase in levels of phosphorylation of the insulin receptor as well as an increase in glucose uptake in the presence of the RPBs. This demonstrates that trapping of the oxidized phosphatase by way of small molecules bearing a nucleophilic site may be a practical means to inhibit further catalytic activity. The design of more selective and reactive "nucleophile-based inhibitors" may be used as a new approach for the treatment of diabetes and other disease states, which have been associated with aberrant phosphatase activity and amplified ROS production.

4. Materials and methods

More comprehensive experimental procedures, supplemental figures, and compound characterization can be found in the Supporting information.

4.1. Recombinant protein labeling with RBPs

PTP1B and C215S PTP1B were buffer exchanged using a Nap-5 column (GE Healthcare Illustra) pre-equilibrated with 50 mM HEPES, 100 mM NaCl, 1 mM EDTA, pH 7.0. Simultaneous labeling of PTP1B or C215S PTP1B was performed by taking 20 μM phosphatase and treating it with 10 equivalences (200 μM) of hydrogen peroxide and indicated concentrations of sulfenic acid probes (DYn-0, NaPhYn-1, BiPhYn-1, or dimedone) for 1 h at room temperature while rocking. Excess RBP and EDTA were removed by passing the samples through a P30 column (Bio-Rad) pre-equilibrated with 50 mM HEPES, 100 mM NaCl, 0.1% SDS, pH 7.4. Time-dependent analyses were performed by taking an aliquot of the reaction mixture at various time points and passing through a pre-equilibrated P30 column to quench the reaction. RBP modified proteins were then treated with 100 μM AzBiotin-PEG_4 (Invitrogen), a pre-mixed BTTP:$CuSO_4$ solution (200 μM BTTP:100 μM $CuSO_4$), and 2.5 mM sodium ascorbate for 1 h at room temperature while rocking. The click chemistry reaction was quenched with the addition of 1 mM EDTA. Protein samples were resolved by SDS-PAGE using Mini-Protean TGX 4–15% Tris-Glycine gels (BioRad) and transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad). The PVDF membrane was blocked with 3–5% BSA in TBST for 1 h at room temperature, washed with TBST (3×), and immunoblotting was performed with HRP-streptavidin (GE Healthcare, 1:80,000). The PVDF membrane was washed with TBST (3×) and developed with ECL Plus chemiluminescence (Pierce) and imaged by film. Equal loading of recombinant PTP1B was assessed by treating the membrane with a solution of 1:1 MeOH:R-250 Coomassie blue for 10 min, then allowing membrane to dry.

4.2. Cell culture

COS1 cells (ATCC) were maintained in a humidified atmosphere of 5% CO_2 at 37 °C and cultured in DMEM media (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin–streptomycin (Invitrogen), 1% GlutaMax (Invitrogen), and 1% non-essential amino acids (Invitrogen). CHO cells overexpressing the human insulin receptor (CHO/hIRc) were a kind gift from Dr. Michael L. Tremblay. CHO/hIRc cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C and cultured in Ham's F-12 media (Corning) supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% non-essential amino acids. For insulin treatment, cells were serum starved for 16 h prior to experimentation.

4.3. Labeling of oxidized PTP1B in COS1 cells

COS1 cells were plated on 100 mm dishes and transfected at 90% confluency according to manufactures instructions with pJ3H-PTP1B (Addgene plasmid 8601) for 48 h. COS1 cells were then washed with PBS, lifted with 0.25% trypsin–EDTA, harvested by centrifugation at 1500 *g* for 2 min, and then resuspended in serum-free DMEM at a density of 3–4 × 10^6 cells/mL. The resuspended cells were treated with DMSO or the indicated concentration of the redox-based probe for 1 h at 37 °C in a 5% CO_2 humidified atmosphere with periodic gentle agitation. Following treatment, cells were collected and washed with PBS (3×). For insulin treatment, cells were cultured in a similar fashion as above with the exception of being serum starved for 16 h prior to the experiment. After serum deprivation, cells were treated with 100 nM insulin (Calbiochem) for 2 min followed by the addition of the 250 μM RBPs for 1 h at 37 °C in a 5% CO_2 humidified atmosphere with periodic gentle agitation.

4.4. Generation of cell lysate

COS1 and CHO/hIRc cells were harvested in a NP-40 lysis buffer [50 mM Tris–HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 50 mM NaF, 10 mM β-glycerolphosphate, 1 mM sodium vanadate, 1 × EDTA-free protease cocktail inhibitors (Roche), and 200 U/mL catalase (Sigma)]. After 20 min incubation on ice with frequent mixing, cell debris was removed by centrifugation at 14,000 rpm at 4 °C for 15 min. Protein concentrations were determined by BCA assay (Pierce).

4.5. Immunoprecipitation

HA-PTP1B was immunoprecipitated from 500 μg of cell lysate with 20 μL anti-HA agarose (Pierce) as specified by manufacturer. The following day, the resin was pelleted with a 10 s burst at 12,000 × *g* and the supernatant was saved. The resin was washed three times with TBST and twice with 50 mM HEPES, 100 mM NaCl, pH 7.4. The resin was then treated with 20 μL of a pre-mixed click chemistry mix (100 μM AzBiotin-PEG_4, 500 μM BTTP, 250 μM $CuSO_4$, and 2.5 mM sodium ascorbate in 50 mM HEPES, 100 mM NaCl, pH 7.4). The click reaction was allowed to mix for 1 h then terminated by the addition of 10 μL Laemmli sample buffer without β-Me and boiling for 10 min.

4.6. Detection of phosphorylated IRβ in CHO/hIRc cells

CHO/hIRc cells were plated onto 6-well pates and allowed to become adherent in complete media overnight at 37 °C in a humidified atmosphere of 5% CO_2. The cells were serum starved for

16 h prior to the experiment. CHO/hIRc cells were pre-treated with DMSO, 1 mM vanadate, and DYn-0 or BiPhYn-1 at various concentrations for 1 h at 37 °C, 5% CO_2. Media containing DMSO, vanadate, or RBPs was removed and cells were washed with PBS (3×) then stimulated with 10 nM insulin for 5 min. After stimulation, the media was removed and the cells were washed with ice cold PBS (3×). The cells were then lysed with the aid of a rubber policeman. 25 μg of clarified cell lysate was resolved by SDS-PAGE, transferred to PVDF membrane, and probed with appropriate antibodies.

4.7. 2-NBD glucose uptake assay

COS1 cells were plated in triplicate at a density of 3×10^4 cells/well in black clear bottom 96-well microplates (Corning) and allowed to become adherent in complete media overnight at 37 °C in a humidified atmosphere of 5% CO_2. The following day, the cells were gently washed with PBS (3×) and serum starved in glucose free DMEM (Corning) for 4 h at 37 °C, 5% CO_2. The cells were then treated with the RBPs for 1 h at 37 °C. Media containing RBPs was aspirated and glucose uptake was assessed with a Glucose Uptake Cell-Based Assay Kit (Cayman Chemicals) in which we treated cells with 50 μM 2-NBDG in the presence of 250 nM insulin (Calbiochem) for 15 min at 37 °C in a humidified atmosphere of 5% CO_2. Following stimulation, the cells were gently washed with PBS (2×) before the addition of 100 μL of the cell-based assay buffer. 2-NBDG uptake was measured using an EnVision plate reader (Perkin Elmer) with an excitation wavelength of 485 nm and emission wavelength of 535 nm.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.06.040.

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