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Original Research Communication

Sensitivity of Protein Tyrosine Phosphatase Activity to the Redox Environment, Cytochrome *c,* and Microperoxidase

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ABSTRACT

Protein tyrosine phosphatase activity depends on a catalytic thiolate group on an acidic cysteine residue that is sensitive to reactive oxygen species. Representative of this family of enzymes is protein tyrosine phosphatase 1B (PTP1B), a major target for type 2 diabetes therapy. PTP1B is sensitive to hydrogen peroxide (H_2O_2) *in vitro* and in cells. It is also sensitive to glutathionylation by glutathione disulfide (GSSG). The sensitivity of PTP1B to the redox state of its environment was partially characterized *in vitro* by examination of phosphatase activity in the presence of various concentrations of glutathione (GSH) and GSSG. Enzyme sensitivity to glutathionylation was dependent on the amount of available thiol groups and increased as GSH concentration was increased. The half-inhibitory concentration for H_2O_2 was much less than that of GSSG in the presence of low concentrations of GSH, indicating that reaction with H_2O_2 is much more likely than is glutathionylation by GSSG. PTP1B and a related oxidant-sensitive phosphatase, PTEN, were also sensitive to the lipid peroxidation by-product 4-hydroxynonenal. Futhermore, PTP1B was inhibited by cytochrome *c* and microperoxidase. Taken together, these data suggest that not only H_2O_2, but also a variety of redox-active metabolites and hemes can oxidatively inactivate PTPs with potentially profound implications for signal transduction. *Antioxid. Redox Signal.* 7, 1078–1088.

INTRODUCTION

Protein tyrosine phosphatases (PTPs or PTPases) have become recognized in recent years as critical components of cell signaling, contributing specific regulatory mechanisms to the vast network of kinase transduction cascades that exists in all cells. PTP active-site chemistry centers on the thiolate group of a low pK_a cysteine residue located in a conserved active-site motif. This highly nucleophilic group makes the initial attack on the phosphate group of the enzyme's substrate (Fig. 1A). This feature of the PTP makes it highly susceptible to electrophilic modification, and the catalytic cysteine residue is therefore considered a likely target for cellular regulation of these regulatory enzymes by oxidative modification, especially by reactive oxygen or nitrogen species. For example, it is known that hydrogen peroxide (H_2O_2) is generated in 3T3-L1 adipocytes in response to insulin treatment (12). H_2O_2 reacts with the active site of protein tyrosine phosphatase 1B (PTP1B) (4), a particularly well studied PTP that is a major target for type 2 diabetes therapy, and insulin directs 3T3-L1 cells to oxidize the enzyme in a reversible manner via a process that is not yet understood (12). PTP oxidation may occur following stimulation of NADPH oxidases downstream of phosphatidylinositol 3-kinase (14).

There are multiple oxidation states for the PTP active site, all of which are inactive enzyme states. Reaction of PTP1B with H_2O_2 results in a short-lived sulfenic acid species, R-SOH, which is reversible by reaction with low-molecular-weight thiol compounds, such as glutathione (GSH) and thioredoxin (Fig. 1B). Sulfenic acid formation in some PTPs, such as Cdc25 (16, 18) and PTEN (phosphatase/tensin homologue mutated on chromosome 10) (11), is reversible by disulfide bond formation with another cysteine residue in the enzyme. Additional oxidation of the sulfenic acid species re-

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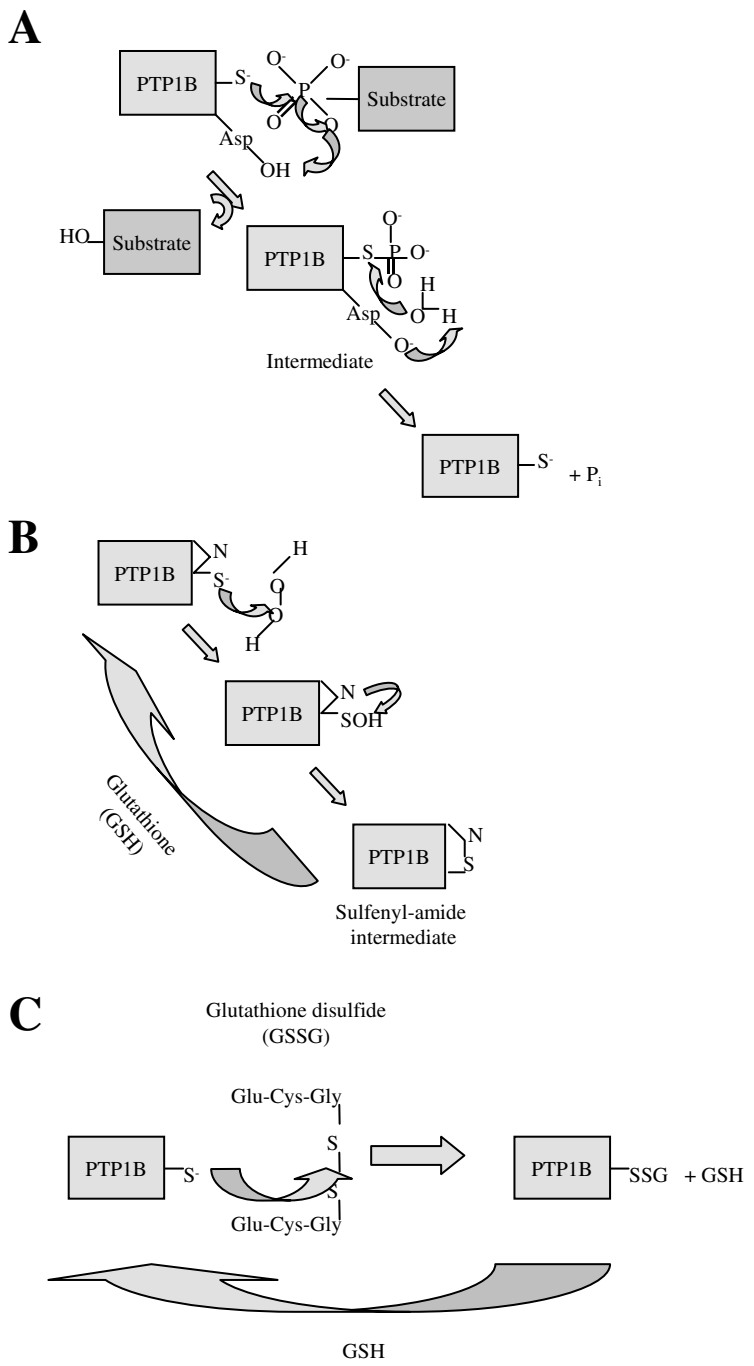

FIG. 1. Mechanisms of PTP1B activity and regulation. (**A**) Catalytic mechanism of phosphate hydrolysis. (**B**) Oxidative inhibition and regeneration. (**C**) Glutathionylation by GSSG. See text for details.

sults in irreversible sulfinic acid (R-SO_2H) and sulfonic acid (R-SO_3H) oxidation states (17, 19). Irreversible oxidation of PTP1B is prevented by the rapid reaction of R-SOH with the backbone amide group of an adjacent serine residue, resulting in the formation of a five-membered sulfenyl-amide ring (15, 19). In the case of Cdc25 and PTEN, the intramolecular disulfide bond likely serves a similar purpose (15, 16, 18).

Glutathionylation is another proposed mechanism for regulation of PTPs (2). The active-site thiolate of PTP1B attacks the disulfide bond of glutathione disulfide (GSSG), resulting in a mixed disulfide, R-SSG (Fig. 1C). This modification is reversible by addition of a thiol, *e.g.,* R-SSG + GSH → R-S^- + H^+ + GSSG. The current work describes an attempt to characterize the sensitivity of PTP1B to the redox state of its environment, specifically to GSH, GSSG, and H_2O_2. We show that H_2O_2 was a much more potent inhibitor of PTP1B activity than was GSSG in this study.

In addition, a series of electrophilic compounds was investigated for reactivity with the PTP active site. The present study revealed that 4-hydroxynonenal (HNE), a lipid peroxidation product, is a mild inhibitor of PTP1B that reacts with the active-site cysteine residue of PTP1B, as well as that of PTEN. HNE may therefore participate in regulation of signal transduction, especially under conditions of oxidative stress.

Cytochrome *c* (cyt. *c*), which has recently been suggested to participate in redox signaling in cells (7), dramatically inhibited PTP1B, as did its proteolytic product, microperoxidase. Taken together, these data reveal that PTP1B is highly sensitive to various cellular redox influences and therefore probably acts as a redox sensor influencing intracellular signaling. As PTP1B activity is linked to diseases such as breast cancer and type 2 diabetes, as well as to neural signaling through insulin-like growth factor receptors, oxidative stress may contribute to cellular disease and neurodegeneration by disrupting signaling via this enzyme and related enzymes, such as the PTEN tumor suppressor phosphatase.

MATERIALS AND METHODS

PTP1B assay

PTP1B phosphatase activity was assayed using either *para*-nitrophenyl phosphate (pNPP; Sigma–Aldrich, St. Louis, MO, U.S.A.) or 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP; Molecular Probes, Eugene, OR, U.S.A.) as substrate. For assays with pNPP, 0.25 μg/ml PTP1B (Upstate, Lake Placid, NY, U.S.A.) was incubated with the indicated concentrations of GSH, GSSG, or inhibitory compound for 30 min in 0.2 ml of pNPP tyrosine assay buffer containing 25 m*M* HEPES, 0.1 mg/ml bovine serum albumin, 5 m*M* NaCl, and 2.5 m*M* EDTA, pH 7.2. To this was added 4 m*M* pNPP (0.25 ml final volume), followed by 30-min kinetic absorbance readings at 410 nm and 37°C. For assays with DiFMUP, 0.05 μg/ml PTP1B was incubated with GSH, GSSG, or inhibitory compound for 30 min in 0.2 ml of pNPP tyrosine assay buffer. To this was added 0.1 m*M* DiFMUP, followed by 33-min kinetic fluorescence readings at $355_{ex}/460_{em}$ and 37°C. V_{max} in milli (optical density units) per minute or relative fluorescence units per minute was calculated using SOFTmax. Enzyme activity was calculated as micromoles of substrate hydrolyzed per milligram of protein per minute. *p*-Nitrophenol (Sigma–Aldrich) and 6,8-difluoro-7-hydroxy-4-methylcoumarin (Molecular Probes) were used to generate standard curves.

PTEN expression and purification

6x-His-tagged human PTEN expression plasmid pHK101 was a kind gift of Dr. Chikashi Ishioka (Tohoku University, Sendai, Japan). PTEN was expressed in M15[pREP4] competent *E. coli* (QIAGEN, Valencia, CA, U.S.A.), and protein purification was carried out according to Han *et al.* (8), except that a His-Trap kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) was used for binding and elution of PTEN protein. Purification was confirmed by Coomassie staining of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels.

Detection of GSH oxidation

GSH oxidation was detected using 1 m*M* monochlorobimane (mBCl; Molecular Probes). Reaction of mBCl with GSH was followed by kinetic fluorescence assay at $355_{ex}/460_{em}$ and 37°C. V_{max} in relative fluorescence units per minute was calculated using SOFTmax.

Detection of reactive cysteine

PTP1B and PTEN were incubated with the indicated concentrations of HNE (Cayman Chemical, Ann Arbor, MI, U.S.A.) for 30 min in a buffer containing 25 m*M* HEPES, followed by addition of 0.5 m*M* *N*-(biotinoyl)-*N′*-(iodoacetyl)ethylenediamine (BIAM; Molecular Probes) for 30 min. Proteins were heat-denatured in SDS loading buffer and subjected to SDS-PAGE, followed by transfer to polyvinylidene difluoride (PVDF) membranes. Proteins were detected using antibody [anti-PTP1B (Transduction Laboratories, Mississauga, ON, Canada) or anti-PTEN (Upstate)] or horseradish peroxidase (HRP)-conjugated streptavidin (Pierce Biotechnology, Inc., Rockford, IL, U.S.A.).

RESULTS

Activity of human recombinant PTP1B toward the generic phosphatase substrate DiFMUP was measured fluorometrically in the presence of various concentrations of GSH after 30-min preincubation with various concentrations of H_2O_2 (Fig. 2). Phosphatase activity was highly dependent on thiol concentration. PTP1B activity was highly susceptible to inhibition by H_2O_2 ($IC_{50} \approx 5–15$ μ*M*) in the absence of GSH. Enzyme sensitivity to H_2O_2 decreased as GSH concentration was increased, indicating protection of the active site from oxidative inhibition by the reducing agent. Oxidation of PTP1B was therefore highly dependent on the amount of reducing groups in the environment. Similar results were seen when dithiothreitol (DTT) or L-cysteine was used in place of GSH and enzyme activity was measured using pNPP (data not shown).

GSSG has been shown to be a potent reversible inhibitor of PTP1B via formation of a mixed disulfide (R-S-SG) with the active-site cysteine (2). In the current study, PTP1B activity was differentially sensitive to GSSG depending on GSH concentration (Fig. 3). Surprisingly, PTP1B susceptibility to inhibition by glutathionylation was proportional to [GSH], whereas inhibition by H_2O_2 was inversely proportional to [GSH]. These relationships are made especially clear when enzyme activity is plotted against the ratio of the molar concentration of H_2O_2 or GSSG to that of GSH (compare Fig. 2C and D with Fig. 3C and D). To address the issue of GSH/GSSG conversion, the experiment was repeated using DTT instead of GSH as reductant (data not shown). The correlation of enzyme inhibition to thiol concentration was seen again with DTT, as well as with L-cysteine (data not shown), when enzyme activity was plotted against the ratio of [GSSG] to [thiol]. The rate of GSSG regeneration in the presence of GSH may therefore be proportional to [GSH], resulting in an overall decrease in available enzyme.

Cyt. *c* is a heme-containing protein member of the electron transport chain in mitochondria. Its release from mitochondria is an established event during apoptosis, and cell death resulting from improper release of cyt. *c* is believed to contribute to the aging process, as well as to certain disease states (for a recent review, see 9). Recently, inhibition of cyt. *c* release was shown to induce activation of the nuclear transcription factor AP-2, and cyt. *c* was shown to oxidatively en-

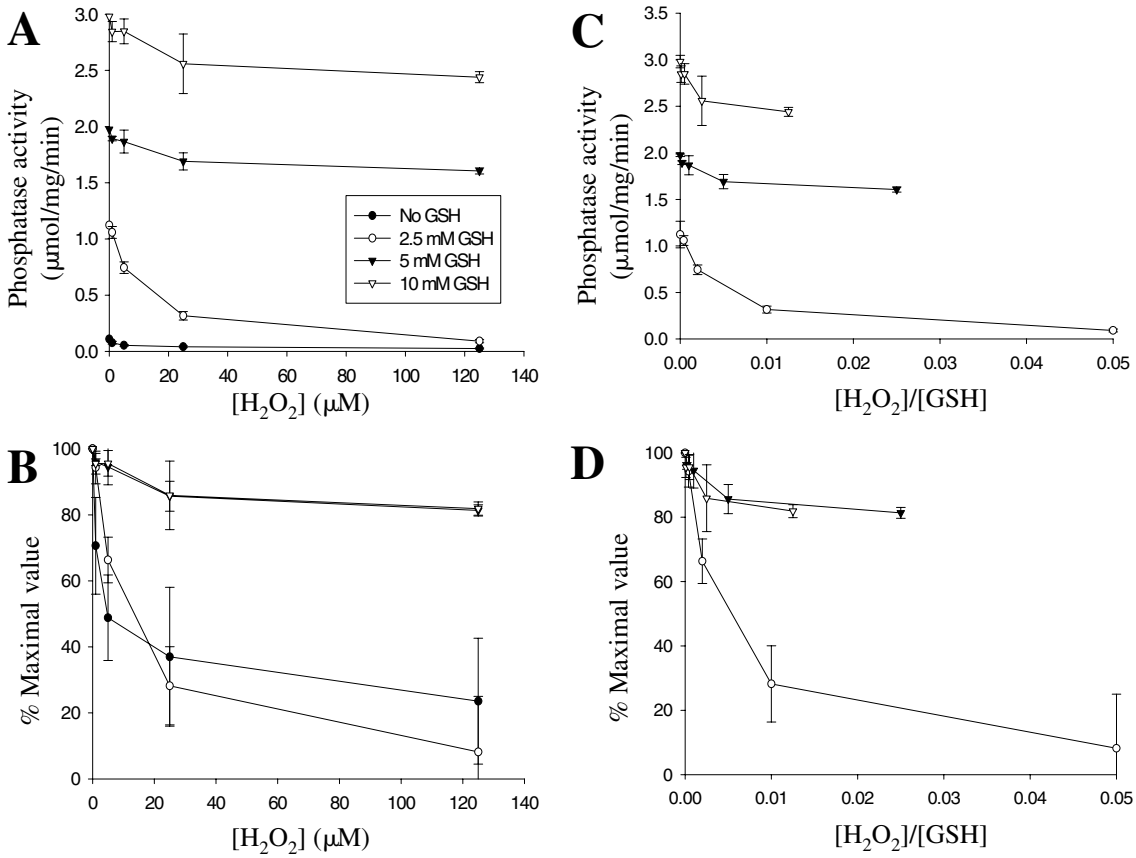

FIG. 2. Inhibition of PTP1B by H_2O_2 is dependent on [thiol]. All data are from one experiment ($n = 3$) and were corroborated by similar experiments. PTP1B was preincubated 30 min with the indicated concentration of H_2O_2 in the presence of the indicated concentration of GSH, followed by a 30-min incubation with DiFMUP substrate. Activity was measured in real time using a fluorescence microplate reader. (**A**) Activity reported as μmol of substrate hydrolyzed/mg of protein/min versus $[H_2O_2]$. (**B**) Activity reported as % maximal value versus $[H_2O_2]$. (**C**) Activity reported as μmol/mg/min versus H_2O_2/GSH (molar concentrations). (**D**) Activity reported as % maximal value versus H_2O_2/GSH. Error bars represent the standard deviation from the mean.

hance DNA binding by activator protein-2 (AP-2) *in vitro* (7). These data indicate novel roles for cyt. *c* involvement in cell signaling via its redox properties.

As PTPs are regulated by redox mechanisms, we asked whether cyt. *c* could inhibit PTP1B *in vitro.* Because of cyt. *c*'s peroxidative properties, such inhibition would likely take place by reduction of H_2O_2 and concurrent oxidation of the nucleophilic active-site cysteine residue of PTP1B. Thus, PTP1B would act as an electron donor, resulting in its own inhibition. Experimentally, this reaction is complicated by the fact that H_2O_2 itself inhibits PTP1B efficiently. As an initial experiment, PTP1B was preincubated with various concentrations of cyt. *c* for 30 min, and enzyme activity toward the DiFMUP substrate was followed for 33 min. Cyt. *c* alone did not hydrolyze the substrate (data not shown). Interestingly, cyt. *c* inhibited PTP1B in the absence of H_2O_2 and in the presence of as much as 10 m*M* GSH (Fig. 4). Efficiency of inhibition decreased as [GSH] was increased. The mechanism of cyt. *c* inhibition of PTP1B in the absence of H_2O_2 is not understood, but it is possible that the redox properties of cyt. *c* are unrelated to this observation. Equally interesting was the observation that inhibition of PTP1B by H_2O_2 was increased by addition of 8.1 μ*M* (0.1 mg/ml) cyt. *c* in the absence of GSH (see Fig. 5B), whereas cyt. *c* had very little effect on H_2O_2 inhibition in the presence of 2.5 (Fig. 5C), 5, or 10 m*M* GSH (data not shown). This is probably due to oxidation of GSH rather than PTP1B, because GSH was oxidized by H_2O_2 more efficiently in the presence of cyt. *c* than in its absence (see Fig. 5D).

Microperoxidase is a proteolytic hemopeptide product of cyt. *c* with very high peroxidase activity (1). It typically consists of an eight- to 12-amino acid peptide conjugated to heme. Although no role for microperoxidase has been found in biology, we reasoned that it has the potential to be a signaling molecule based on the recent discoveries in cyt. *c* signaling mentioned above. Various concentrations of horse heart microperoxidase containing 11 amino acid residues (MP-11) were preincubated with PTP1B, and the enzyme activity was measured. MP-11 strongly inhibited PTP1B in the presence of 2.5 m*M* GSH and the absence of H_2O_2 (Fig. 6A). Unlike the case with cyt. *c*, the presence of 6.7 μ*M* (12.5 μg/ml) MP-11 prevented inhibition of PTP1B by H_2O_2 (Fig. 6B). This is probably due to reduction of H_2O_2 to H_2O by MP-11 using GSH as a hydrogen donor. Taken together, it is reasonable to conclude from these data that cyt. *c* and microperoxidase inhibit PTP1B in a nonoxidative manner. These results warrant

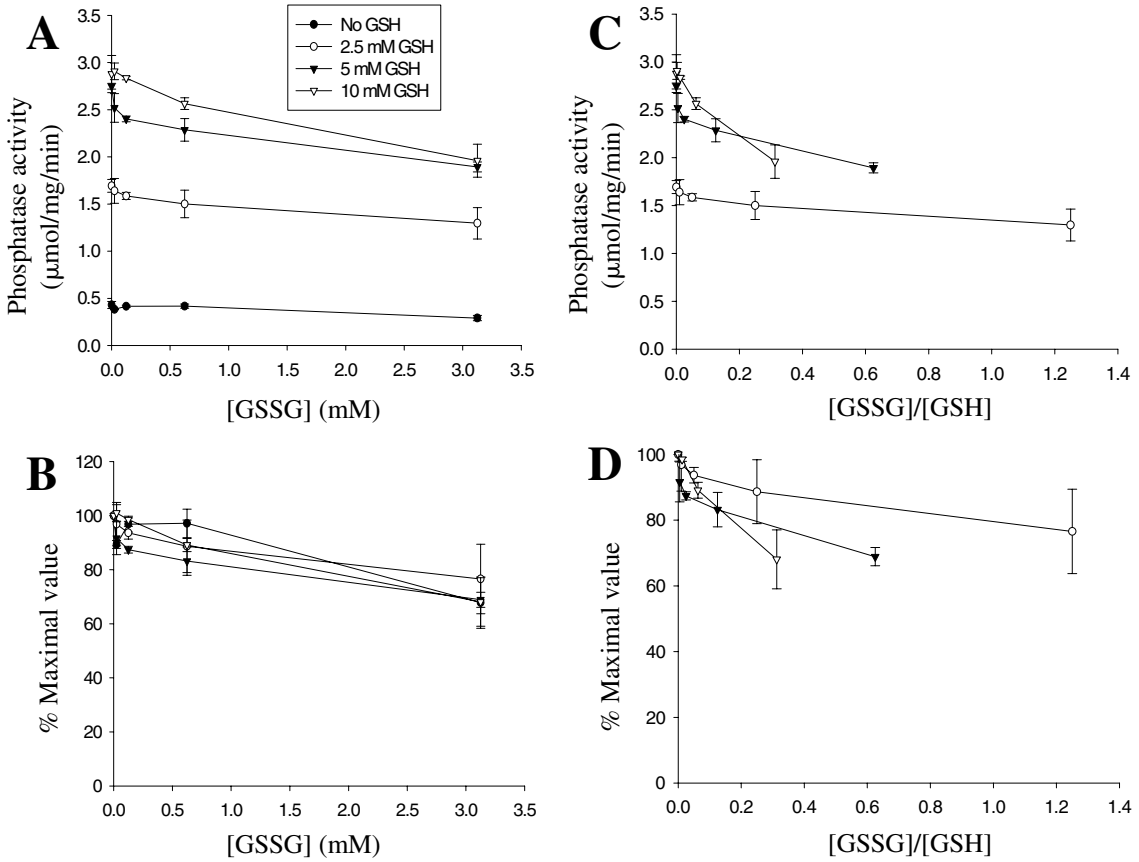

FIG. 3. Inhibition of PTP1B by GSSG is dependent on [thiol]. All data are from one experiment ($n = 3$) and were corroborated by similar experiments. PTP1B was preincubated 30 min with the indicated concentration of GSSG in the presence of the indicated concentration of GSH, followed by a 30-min incubation with DiFMUP substrate. Activity was measured in real time using a fluorescence microplate reader. (**A**) Activity reported as μmol of substrate hydrolyzed/mg of protein/min versus [GSSG]. (**B**) Activity reported as % maximal value versus [GSSG]. (**C**) Activity reported as μmol/mg/min versus GSSG/GSH (molar concentrations). (**D**) Activity reported as % maximal value versus GSSG/GSH. Error bars represent the standard deviation from the mean.

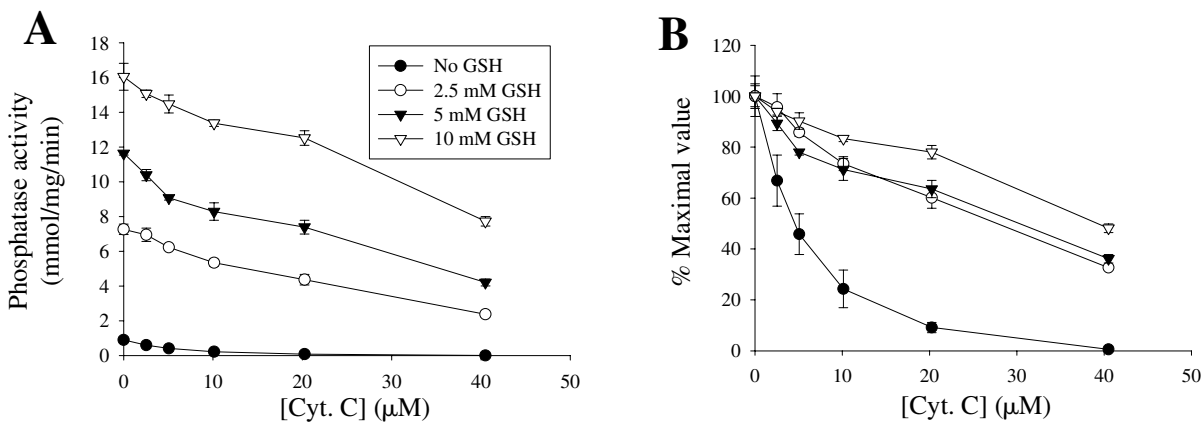

FIG. 4. PTP1B is inhibited by cyt. *c in vitro.* PTP1B was preincubated 30 min with the indicated concentration of cyt. *c* in the presence of the indicated concentration of GSH, followed by a 30-min incubation with DiFMUP substrate. Activity was measured in real time using a fluorescence microplate reader. (**A**) Activity reported as mmol of substrate hydrolyzed/mg of protein/min versus [cyt. *c*]. (**B**) Activity reported as % maximal value versus [cyt. *c*]. $n = 3$.

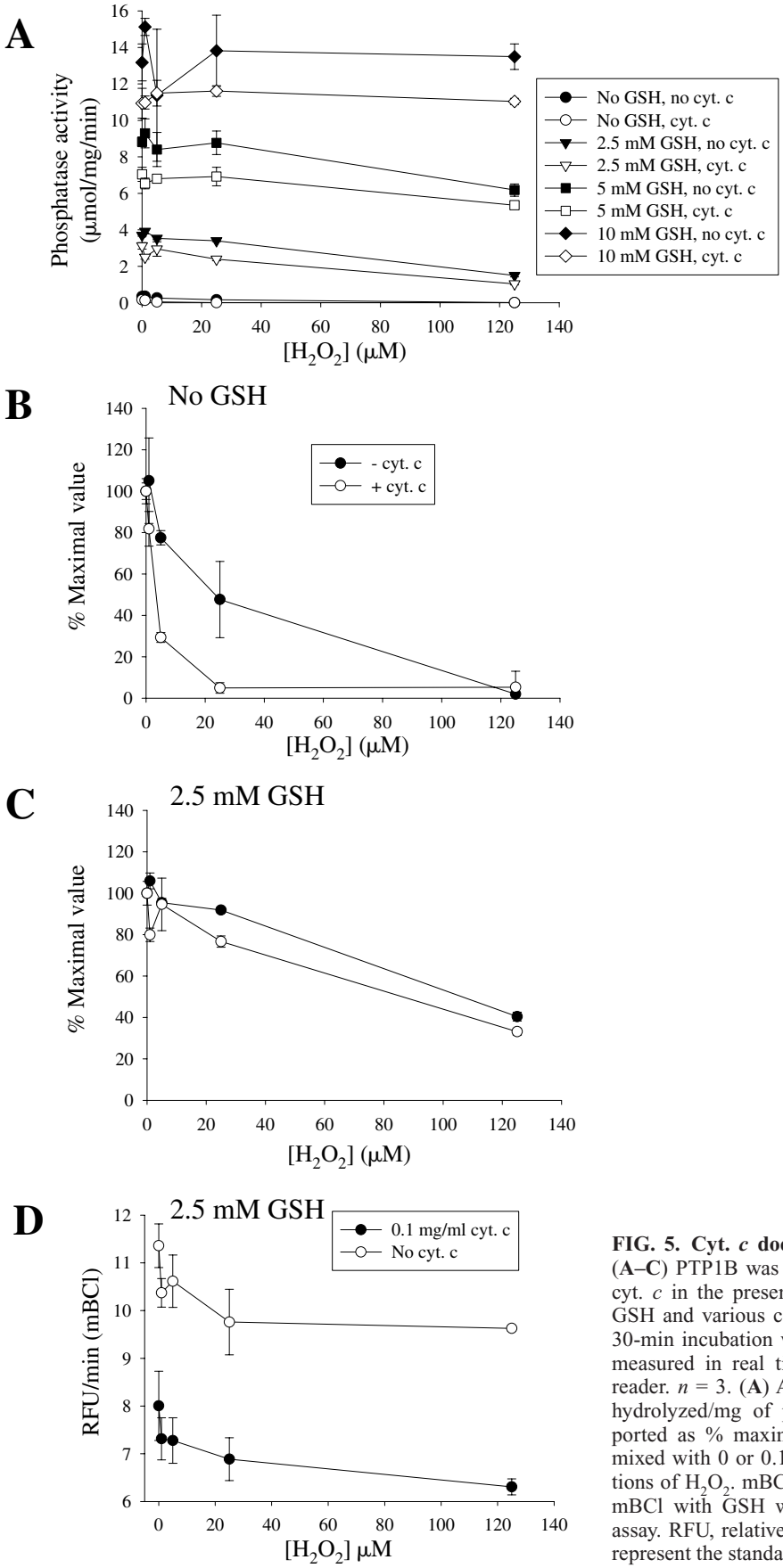

FIG. 5. Cyt. *c* does not use H_2O_2 to oxidize PTP1B. (**A–C**) PTP1B was preincubated 30 min with 0.1 mg/ml cyt. *c* in the presence of the indicated concentration of GSH and various concentrations of H_2O_2, followed by a 30-min incubation with DiFMUP substrate. Activity was measured in real time using a fluorescence microplate reader. $n = 3$. (**A**) Activity reported as μmol of substrate hydrolyzed/mg of protein/min. (**B** and **C**) Activity reported as % maximal value. (**D**) GSH at 2.5 m*M* was mixed with 0 or 0.1 mg/ml cyt. *c* and various concentrations of H_2O_2. mBCl at 1 m*M* was added, and reaction of mBCl with GSH was followed by kinetic fluorescence assay. RFU, relative fluorescence units. $n = 3$. Error bars represent the standard deviation from the mean.

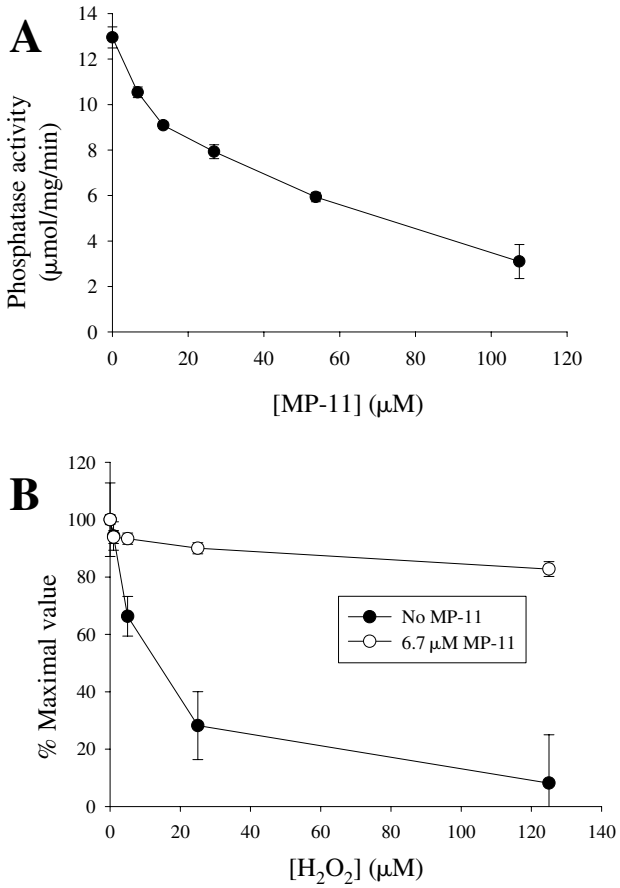

FIG. 6. PTP1B is inhibited *in vitro* by microperoxidase (MP-11), but MP-11 blocks inhibition by H_2O_2. (**A**) PTP1B was preincubated 30 min with the indicated concentration of MP-11 and 2.5 m*M* GSH, followed by a 30-min incubation with DiFMUP substrate. Activity was measured in real time using a fluorescence microplate reader. $n = 3$. (**B**) PTP1B was preincubated 30 min with the indicated concentration of H_2O_2 in the presence of 12.5 μg/ml MP-11 and 2.5 m*M* GSH, followed by incubation with DiFMUP. $n = 3$. Error bars represent the standard deviation from the mean.

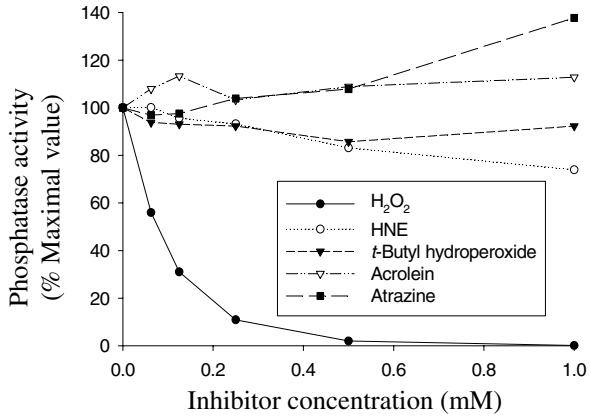

FIG. 7. HNE has a modest effect on PTP1B activity. PTP1B was preincubated 30 min with the indicated concentration of the indicated compound in the presence of 2.5 m*M* GSH, followed by a 30-min incubation with pNPP substrate. Activity was measured in real time using a microplate reader.

further investigation into the mechanism of PTP1B inhibition by these molecules.

The PTP active site is highly nucleophilic; therefore, an inhibitor screen was conducted using various electrophilic compounds (Fig. 7). PTP1B activity was assayed using pNPP as substrate after preincubation with each compound for 30 min. *tert*-Butyl hydroperoxide had little effect on PTP1B activity up to 1 m*M* as reported by Denu and Tanner (4). HNE, an aldehydic by-product of lipid peroxidation, had a significant inhibitory effect on PTP1B activity between 0.5 and 1 m*M*. HNE is a highly reactive compound that modifies amino acid residues of proteins, especially cysteine, histidine, and lysine (5). Acrolein (2-propenal), an unstable compound used in the manufacturing of plastics, is chemically similar to HNE, but surprisingly had no effect on PTP1B activity (Fig. 7). Atrazine (Sigma–Aldrich; Fig. 7), an electrophilic herbicide toxic to farm animals, and chlorpyrifos (Sigma–Aldrich; data not shown), an electrophilic insecticide, also had no effect.

As the major signaling targets for PTP1B and PTEN are located at the cell membrane (growth factor receptors for PTP1B and phosphatidylinositol for PTEN), we reasoned that reactive lipid oxidation products such as HNE could contribute to oxidative signaling as second messengers by inhibiting PTPs localized to the membrane. To determine whether PTP inhibition by HNE (see Fig. 7) was caused by modification of the active-site cysteine residue, an alkylation method adapted from that of Dr. Sue Goo Rhee and colleagues was used (10) (Fig. 8A). This method makes use of the selective reaction of iodoacetamide with cysteine residues that exhibit a low pK_a. The alkylation reaction competes with other oxidative reactions, such as the reaction with H_2O_2. BIAM was used to label purified PTPs (glutathione *S*-transferase-tagged PTP1B or His-tagged PTEN tyrosine/phosphoinositide phosphatase) possessing available active sites after prior incubation of the enzymes with various concentrations of HNE at room temperature. The modified proteins were subjected to SDS-PAGE and transferred to PVDF membrane, which was then probed with streptavidin-HRP. As shown in Fig. 8B and D, low concentrations of HNE effectively attenuated alkylation of both phosphatases. An upper band was present in those blots representing the experiments with PTEN, and multiple bands were present in blots representing PTP1B experiments. These may represent protein labeled multiple times with BIAM on residues outside the active site. In the case of PTEN, these multiply-labeled molecules were not noticeably affected by preincubation with HNE. Detection of PTEN using polyclonal PTEN antibody was blocked by HNE (data not shown), apparently due to epitope masking resulting from modification of residues outside the active site. Detection of PTP1B using polyclonal PTP1B antibody was not affected by preincubation with HNE (Fig. 8D, lower panel).

Although the alkylation reaction with iodoacetamide is selective for the active-site cysteine, it is not entirely specific and occurs with other cysteine residues as well. HNE, too, reacts with all cysteine residues, but may be selective for the active-site cysteine because this cysteine is ionized at neutral

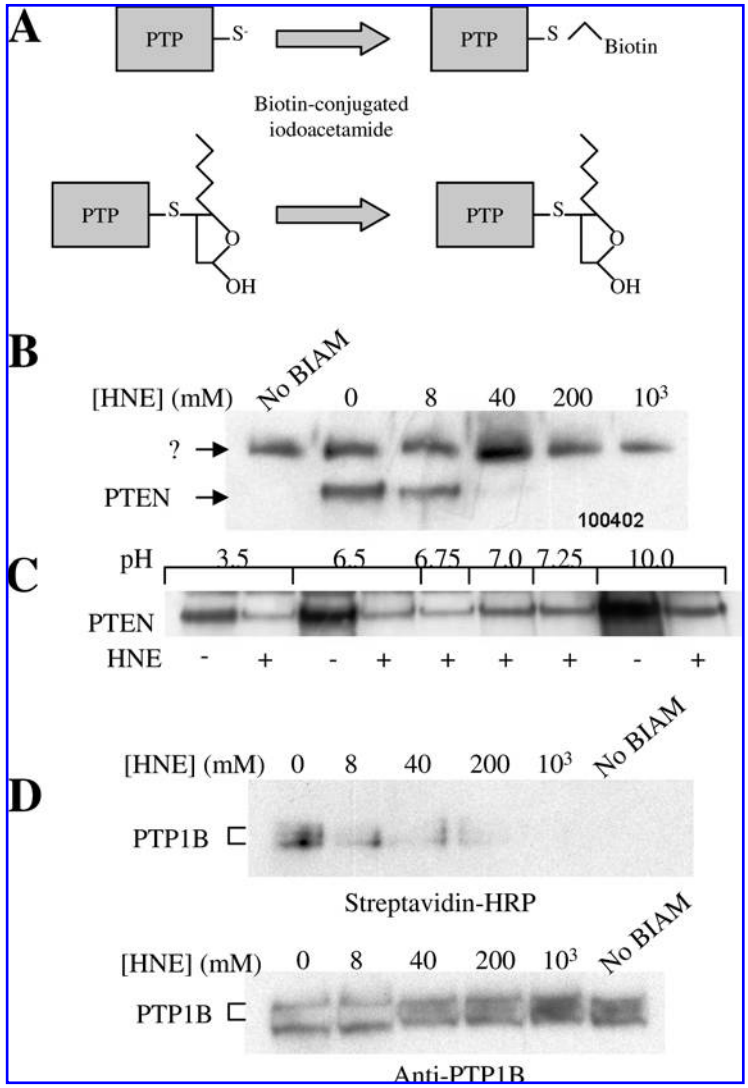

FIG. 8. HNE modification of PTEN and PTP1B prevents alkylation of the catalytic cysteine. (**A**) Schematic for the detection of reactive cysteine residues. The enzyme is alkylated using BIAM (see text) unless it has been preincubated with a certain concentration of HNE. (**B**) The alkylated enzyme, in this case PTEN preincubated with the indicated concentration of HNE, is detected by western blot using HRP-streptavidin as probe. PTEN (arrow) was identified by comparison with molecular weight standards. Protein preincubated with ≥40 μ*M* HNE was not detected. Upper band (?) is an unidentified protein possibly representing multiply-alkylated PTEN. (**C**) PTEN was (+) or was not (−) preincubated with 10 μ*M* HNE at the indicated pH, followed by alkylation with BIAM at the same pH and detection by western blot as in B. (**D**) (**Upper panel**) PTP1B was preincubated with the indicated concentration of HNE, alkylated using BIAM, and detected by western blot as in B. (**Lower panel**) Alkylated PTP1B (**upper panel**) was detected by western blot using PTP1B antibody. PTP1B was separated into two or three bands on the gel for unknown reasons.

pH. Therefore, recombinant PTEN, which has nine cysteine residues outside the active site, was incubated with 10 μ*M* HNE followed by BIAM at various pH in order to determine the efficiency of HNE modification and alkylation in relation to the ionization state of active-site and non–active-site cysteine residues. At pH 3.5, all cysteine residues, including the active-site cysteine, are protonated and would therefore be expected to react more slowly with HNE, as well as with BIAM, than at neutral pH, at which the active-site cysteine is ionized. At pH 10.0, all cysteine residues are ionized and are expected to react more quickly than at neutral pH. PTEN was found to react with BIAM more slowly at pH 3.5 and more quickly at pH 10.0 than at pH 6.5–7.25 in the absence of HNE (Fig. 8C). Alkylation efficiency did not change from pH 6.5–7.25 (data not shown). HNE dramatically attenuated alkylation at each pH tested, but the attenuation was most dramatic around neutral pH, especially at pH 6.5 and 6.75. These data indicate that both HNE and BIAM react more efficiently with a preionized cysteine than with a protonated cysteine that must be deprotonated before a reaction can take place. HNE modification may therefore be selective for active-site cysteine residues.

DISCUSSION

PTP1B can be considered a model for the family of PTPs because of the conserved active-site motif. Certain members of this family are more difficult to study. PTEN, for example, catalyzes dephosphorylation of lipid substrates that require careful preparation and is assayed less accurately than PTP1B, which is assayed using simple colorimetric or fluorometric compounds. The findings related to oxidative modifications of the active site reported in this work may be applied to other PTPs because of the similarity of the active sites.

GSH is oxidized in the presence of reactive oxygen species such as H_2O_2 and forms a disulfide bond with a second molecule of GSH, resulting in the formation of GSSG. Glutathionylation of cysteine by GSSG is reversed by another equivalent of GSH, resulting in the restoration of PTP activ-

ity, as well as the formation of a new GSSG disulfide. In the study by Barrett *et al.* (2), glutathionylation by GSSG was examined kinetically using 1 m*M* diamide to convert 2 m*M* GSH to GSSG or using molar concentrations (25 or 50 m*M*) of GSSG in large excess over enzyme concentrations for calculation of kinetic constants. Enzyme activity was restored by addition of 10 m*M* DTT.

We found that PTP1B is much more sensitive to inhibition by H_2O_2 than by GSSG. It may be concluded from this that oxidation of PTP1B in cells occurs more efficiently by direct reaction with H_2O_2 than by GSSG formed by oxidation of two GSH. We also observed that PTP1B activity is preserved in the presence of H_2O_2 in proportion to thiol concentration. This was not true when GSSG was used as inhibitor, and, in fact, increasing thiol concentration in the presence of GSSG appeared to increase the enzyme's sensitivity to glutathionylation. Oxidation of PTP1B by H_2O_2 in the presence of GSH results in the consumption of two GSH and the formation of one GSSG to regenerate the active enzyme, as shown in Scheme 1 [the formation and breakdown of the sulfenylamide ring after sulfenic acid formation (see above) are not shown]:

$$\text{PTP1B-S}^- + \text{H}_2\text{O}_2 + 2\ \text{GSH} \rightarrow \text{PTP1B-SOH} + \text{OH}^- + 2\ \text{GSH}$$

$$\rightarrow \rightarrow \text{PTP1B-S-SG} + 2\ \text{OH}^- + \text{GSH} + \text{H}^+$$

$$\rightarrow \text{PTP1B-S}^- + 2\ \text{OH}^- + \text{GSSG} + 2\ \text{H}^+$$

Scheme 1

The newly formed GSSG has the potential to react with the enzyme and inactivate it by glutathionylation. Addition of H_2O_2 to the PTP/GSH system therefore decreases the reductive potential of the buffer and creates an additional inhibitor. When all H_2O_2 is consumed, [GSSG] is equal to $[H_2O_2]_{original}$ and cycles with GSH (see below). Additionally, two GSH have been consumed per each molecule of H_2O_2 added. If DTT or L-cysteine is used instead of GSH, GSSG formation will not occur, and the final step in Scheme 1 will not occur. Therefore, the reductant is not depleted in those cases.

When GSSG is added to the PTP/GSH system in the absence of added H_2O_2, GSH is not depleted, as shown in Scheme 2:

$$\text{PTP1B-S}^- + \text{GSSG} + \text{GSH} \rightarrow \text{PTP1B-S-SG} + \text{GS}^- + \text{GSH}$$

$$\rightarrow \text{PTP1B-S}^- + \text{GS}^- + \text{GSSG} + \text{H}^+$$

$$\rightarrow \text{PTP1B-S}^- + \text{GSSG} + \text{GSH}$$

Scheme 2

This results in a continuous cycle in which [GSSG] and [GSH] remain constant. In either of the above cases, it seems intuitive that an increase in [GSH] should result in a decrease of enzyme sensitivity to H_2O_2 or GSSG if the concentrations of these inhibitors are held constant. However, as [GSH] was increased in this study, the sensitivity of PTP1B to GSSG increased (see Fig. 3C and D), whereas the sensitivity to H_2O_2 decreased (see Fig. 2C and D). Substituting DTT or L-cysteine for GSH in Scheme 2 breaks the cycle and results in the potential for GSSG reformation with a constant reductant concentration. The sensitivity of the enzyme to GSSG is increased with increasing [DTT] and with increasing [L-cysteine]. The cause may be an increasing rate of GSSG formation when GSSG is added to PTP1B plus increasing [thiol], resulting in a decrease in available active sites that perhaps does not occur when H_2O_2 is added. Thus, the differing enzyme sensitivities may be due to a difference in the kinetics of GSSG formation between the two cases, or perhaps to a difference in enzyme conformation at different [thiol].

Cyt. *c* may play several roles in signaling outside the mitochondrion. Besides its involvement in apoptosis, it has been shown to play a possible role in regulation of transcription in the nucleus by oxidizing AP-2 (7). The iron-containing heme group coordinated by cyt. *c* is central to its oxidative properties, and cyt. *c* might oxidize many molecules in the cytosol and the nucleus. Interestingly, in the current study, when H_2O_2 was introduced *in vitro* in combination with a low concentration of cyt. *c* and 2.5 m*M* GSH, PTP1B activity was not decreased further than with H_2O_2 alone (see Fig. 5C), whereas in the presence of microperoxidase (MP-11), activity was protected from inhibition by H_2O_2 (see Fig. 6B). In theory, cyt. *c* and MP-11, a more powerful peroxidase, would use H_2O_2 as a peroxidative substrate, reducing H_2O_2 to H_2O and amplifying oxidation of another molecule. The data presented make it questionable whether inhibition of PTP1B by cyt. *c* (see Fig. 4) and by MP-11 (see Fig. 6A) occurs by oxidation or by another mechanism. Cyt. *c* oxidized GSH in the presence of H_2O_2 (see Fig. 5D), and it may oxidize GSH much more readily than PTP1B, especially in this case in which [GSH] >> [PTP1B].

HNE and related aldehydes are produced as a result of oxidation of lipids, such as arachidonic acid. They are therefore considered products of oxidative stress, and they furthermore react easily with many reactive groups on various macromolecule types, particularly with proteins, lipids, and nucleic acids. Cysteine is a particularly vulnerable amino acid residue and has been studied extensively as a target of HNE modification (5). This modification is not readily reversible, and although HNE had only a small effect on PTP1B activity *in vitro* as measured using a colorimetric substrate (see Fig. 7), it was interesting to note that it prevented PTP1B and PTEN from reacting with a thiol-reactive compound, iodoacetamide. We have observed that HNE masks PTEN epitopes recognized by a polyclonal PTEN antibody (detection by Coomassie stain is not affected; unpublished observations), which is not surprising considering the high reactivity of HNE with amino acid side chains. We cannot, therefore, rule out the possibility that HNE blocks active-site labeling with iodoacetamide by reacting with a residue other than the catalytic cysteine. The experiment shown in Fig. 8C addresses the question of whether this catalytic cysteine, which is ionized at neutral pH, is particularly vulnerable to modification by HNE or by BIAM. The results suggest that this is likely the case (as discussed in Results). HNE and similar molecules, therefore, may be important in cell signaling because of their high reactivity with such enzyme sites, especially in the case of an enzyme that performs its biological action in a lipid-

rich environment, such as the cell membrane, as is the case for both PTEN and PTP1B.

Perspectives

PTP1B is now a popular target for type 2 diabetes therapy and may play a role in breast cancer. Its major molecular substrates in these diseases are the insulin receptor, insulin-like growth factor receptor, and insulin receptor substrate-1, all of which cease participation in insulin signaling cascades when dephosphorylated by PTP1B (6, 13), and c-Src, which is activated by dephosphorylation and contributes to breast cancer. PTP1B has been shown to be elevated in breast tumors (20), as well as in breast cancer cells, and to activate c-Src in these cells (3). The inhibitory molecules mentioned in this study appear on the surface to be of little use as PTP1B inhibitors in these diseases, but it is helpful to understand the chemistry of this important family of signaling enzymes and to identify molecules in the cell that could inhibit these enzymes and thus, at low concentrations, act as signaling messengers *in vivo.* Because of the remarkable gains in insulin signaling in PTP1B-deficient mice, many researchers are studying compounds that may be of use as drugs for targeting PTP1B in insulin-sensitive cells (6, 13). These compounds show promise for future treatments of type 2 diabetes, obesity, and possibly cancer.

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ABBREVIATIONS

AP-2, activator protein-2; BIAM, biotin-conjugated iodoacetamide; cyt. *c*, cytochrome *c*; DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; DTT, dithiothreitol; GSH, glutathione; GSSG, glutathione disulfide; HNE, 4-hydroxynonenal; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; mBCl, monochlorobimane; MP-11, microperoxidase with 11-amino acid peptide; pNPP, *para*-nitrophenyl phosphate; PTEN, phosphatase/tensin homologue mutated on chromosome 10; PTP, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase 1B; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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