Redox Regulation of PTEN by S-Nitrosothiols

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ABSTRACT

PTEN (phosphatase with sequence homology to tensin) is a phosphatidylinositol 3,4,5-trisphosphate phosphatase that regulates many cellular processes. Activity of the enzyme is dependent on the redox state of the active site cysteine such that oxidation by H$_2$O$_2$ leads to inhibition. Because S-nitrosothiols are known to modify enzymes containing reactive cysteines, we hypothesized that S-nitrosothiols would oxidize PTEN and inhibit its phosphatase activity. In the present study, we show that S-nitrosocysteine (CSNO), S-nitrosoglutathione (GSNO), and S-nitroso-N-acetylpenicillamine (SNAP) reversibly oxidized recombinant PTEN. In addition, CSNO led to concentration- and time-dependent oxidation of endogenous cellular PTEN. However, in contrast, GSNO and SNAP were effective only when coincubated with cysteine, suggesting that these nitrosothiols must react with cysteine to form CSNO, which can be transferred across cell membranes. Oxidation of cellular PTEN resulted from thiol modification and led to reversible inhibition of phosphatase activity. Although oxidation of PTEN by H$_2$O$_2$ led to formation of an intramolecular disulfide, oxidation of PTEN by CSNO seemed to lead to formation of a mixed disulfide. Glutathionylation of cellular proteins by incubating cells with diamide or incubating cellular extracts with GSSG oxidized PTEN in a manner similar to that of CSNO. Overall, these data demonstrate for the first time that S-nitrosothiols oxidatively modify PTEN, leading to reversible inhibition of its phosphatase activity, and suggest that the oxidized species is a mixed disulfide.

PTEN (phosphatase with sequence homology to tensin) is a member of the protein tyrosine phosphatase superfamily, which dephosphorylates PtdIns(3,4,5)$P_3$ (Maehama and Dixon, 1999; Maehama et al., 2001). By reducing levels of PtdIns(3,4,5)$P_3$, PTEN regulates the overall activity of the phosphatidylinositol-3-kinase (PI3 kinase) signaling pathway, thereby inhibiting growth and survival (Stambolic et al., 1998; Sun et al., 1999; Parsons, 2004). As such, PTEN functions as a tumor suppressor, and mutations in the PTEN protein or deletion of the gene are associated with a vast number of human cancers, particularly in advanced stages (Stambolic et al., 1998; Sun et al., 1999; Leslie and Downes, 2004; Parsons, 2004).

Less is known about post-translational modification and regulation of PTEN activity. PTEN function and cellular localization seems to be regulated in part by phosphorylation of several serine and threonine residues (Miller et al., 2002). In addition, recent studies suggest that PTEN is redox-regulated (Lee et al., 2002; Leslie et al., 2003; Kwon et al., 2004). In common with other members of the protein tyrosine phosphatase superfamily, PTEN has a $\text{CX}_X\text{R}$ signature motif at its active site, where the environment surrounding the cysteinyl thiol leads to a low $pK_a$ (−5) and increased redox sensitivity (Denu and Dixon, 1998; Maehama and Dixon, 1999; Maehama et al., 2001; Leslie and Downes, 2004). Indeed, it has been shown recently that H$_2$O$_2$ effectively oxidizes and reversibly inhibits PTEN by forming an intramolecular disulfide between the active site cysteine 124 and cysteine 71 (Lee et al., 2002). Formation of a disulfide has been shown for other phosphatases and seems to act to preserve the reversibility of redox modification by preventing “overoxidation” to irreversible oxidation states (Sohn and Rudolph, 2003). Inhibition leads to increased PtdIns(3,4,5)$P_3$ levels and increased phosphorylation of Akt (Leslie et al., 2003; Kwon et al., 2004). Although the role of redox regulation has not been established, it has been proposed that H$_2$O$_2$ produced by cells in response to growth factor stimulation transiently inactivates PTEN allowing PtdIns(3,4,5)$P_3$ levels to increase sufficiently to produce downstream signals necessary for growth (Kwon et al., 2004). Although it is clear that H$_2$O$_2$ can regulate PTEN activity,
the effect of other signaling molecules that act as oxidants has not been studied. In this regard, nitric oxide is known to regulate activity of proteins by reversibly nitrosating cysteine residues (Stamler et al., 1997; Gow et al., 2004), thereby initiating cellular responses or regulating responses to other agonists. The ability of nitric oxide to oxidize cysteine residues requires reaction of NO with O$_2$ or metal ions to form nitrosating species, which may modify protein directly or react with small molecular weight thiols, such as cysteine, to form S-nitrosocysteine, an important mediator of NO biology (Stamler et al., 1997; Hogg, 2002; Gow et al., 2004). Which protein cysteines are targeted by nitrosothiols depends on many factors, including thiol $p_K_a$, steric factors, and other factors that may facilitate interactions between nitrosothiols and proteins. In the case of protein tyrosine phosphatase 1B, we have shown that the enzyme is reversibly inhibited by S-nitrosothiols, leading to increased phosphorylation of epidermal growth factor receptors (Li and Whorton, 2003). Whether PTEN is oxidized by nitrosothiols has not been investigated. In the present study, we studied the effects of nitrosothiols on PTEN activity. We show for the first time that S-nitrosothiols reversibly inhibit PTEN activity through formation of a protein mixed disulfide.

**Experimental Methods**

**Cloning, Expression, and Purification of Recombinant PTEN.** Human PTEN cDNA was obtained by RT-polymerase chain reaction from total RNA isolated from human lymphocytes using the primer pair 5'-TAGCATC-CATGACAGCCATCATCAAAG-3' (forward) and 5'-TA-GAATTCTCAGACTTTTGTACTTGTG-3' (reverse) and cloned into a prokaryotic expression vector pQE30 (QIAGEN, Valencia, CA) for expression of the protein with a histidine tag at the NH$_2$ terminus. The histidine-tagged wild-type PTEN protein was expressed in *Escherichia coli* according to standard procedures and purified by immobilized Ni$^{2+}$ affinity chromatography with the use of a Ni$^{2+}$-nitrilotriacetic acid agarose column (QIAGEN). The purified protein was desalted using a Centriprep YM-10 filter (Millipore Corporation, Billerica, MA), concentrated against 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2% deoxycholic acid sodium salt, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 5 µg/ml pepstatin A) containing 40 mM N-ethylmaleimide (NEM). NEM was included to preserve the protein thiol redox state present in intact cells by irreversibly blocking all unoxidized thiol groups. Lysates were sonicated and subjected to centrifugation at 12,000g for 5 min at 4°C. The supernatant was removed for protein analysis using the Micro-BCA assay (Pierce, Rockford, IL). PTEN was visualized by immunoblot as above.

**Determination of PTEN Thiol Oxidation in Intact Cells.** To determine the effect of oxidants on PTEN thiol oxidation, we used N^o-((3-maleimidylpropionyl)biocytin (MPB) as described previously (Li and Whorton, 2003). In brief, A431 cells were exposed to CSNO at 37°C for 20 min, rinsed three times with ice-cold HBSS, and lysed in lysis buffer B (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2% deoxycholic acid sodium salt, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 5 µg/ml pepstatin A) containing 10 mM iodoacetamide. Lysates were collected by centrifugation, and subjected to centrifugation at 12,000g at 4°C for 5 min. The supernatant was applied to a PD-10 column to remove excess MPB, and eluates were moved for protein analysis using the Micro-BCA assay (Pierce, Rockford, IL). PTEN was visualized by immunoblot as above.

**Oxidation of Purified PTEN.** Purified recombinant human PTEN dissolved in 20 mM Tris-HCl, pH 6.7, was incubated with S-nitrosothiols for 10 min at 25°C. After incubation, samples were alkylated with 40 mM NEM in electrophoresis gel-loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.1% bromphenol blue) and subjected to SDS-polyacrylamide gel (10%) electrophoresis (PAGE). Proteins were transferred electrophoretically to a PVDF membrane (Invitrogen) and detected by immunoblot after blocking with 5% powered milk in Tris-buffered saline-Tween 20 (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) using mouse monoclonal anti-PTEN as primary antibody (PTEN A2B1; Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated antitrust IgG (BD Transduction Laboratories, Lexington, KY) as secondary antibody. Immune complexes were visualized by chemiluminescence using ECL. In some experiments, purified PTEN was incubated with 5 mM GSNO, separated by SDS-PAGE (nonreducing conditions), and analyzed by immunoblot using an antibody that specifically recognizes glutathione-protein adducts (Virogen, Watertown, MA).
of 2× electrophoresis gel-loading buffer without DTT, separated on a 10% SDS-polyacrylamide gel, and transferred to PVDF membranes. After blocking with 5% powdered milk in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20, biotinylated PTEN was visualized by incubation with NeutrAvidin linked to HRP (Molecular Probes, Eugene, OR) followed by detection with ECL Plus. Blots were stripped using buffer containing 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS, at 55°C for 30 min, and reprobed with the rabbit polyclonal antibodies to PTEN (Upstate, Lake Placid, NY) and visualized with HRP-conjugated secondary antibody followed by ECL Plus.

Determination of Phosphatase Activity of PTEN. An indirect assay as described previously (Li and Whorton, 2002; Leslie et al., 2003; Li and Whorton, 2003) was used to measure PTEN phosphatase activity. After treatment with nitrosothiols, A431 cells were rinsed with ice-cold HBSS, lysed in lysis buffer B with or without iodoacetamide, collected by scraping, and centrifuged as described above. The supernatant was applied to a PD-10 column to remove excess iodoacetamide. The eluates were treated with 60 mM DTT and precleared with mouse IgG and protein G PLUS agarose as described above. PTEN immunoprecipitation was carried out with mouse monoclonal antibodies to PTEN and protein G PLUS agarose on aliquots containing equivalent amounts of total cellular proteins as described above. Immunoprecipitates were washed two times with lysis buffer A containing 2 mM DTT, two times with buffer C (100 mM Tris-HCl, pH 7.5, and 250 mM NaCl) containing 2 mM DTT, and resuspended in 40 μl of buffer C. Phosphatase activity of the immunoprecipitated PTEN against PtdIns(3,4,5)P3 was measured in a 96-well plate using procedures described previously (Campbell et al., 2003). In brief, the immunoprecipitated PTEN was incubated with 40 μM PtdIns(3,4,5)P3 in buffer C at 37°C for 40 min. The reaction was terminated with adding 200 μl per well of the phosphate detection reagent, BIOMOL GREEN (Biomol Research Labs, Inc., Plymouth Meeting, PA). After 30 min at room temperature, the absorbance at 595 nm was determined and compared with that of known phosphate standards. Total phosphatase activity of PTEN was determined in samples that were not treated with iodoacetamide and used to calculate relative phosphatase activity of PTEN defined as (1 – (recovered activity/total activity)) × 100%.

Results

In Vitro Oxidation of PTEN by S-Nitrosothiols. To investigate the effects of S-nitrosothiols on PTEN, we initially used purified recombinant PTEN. Purified PTEN was incubated with increasing concentrations of CSNO, GSNO, or SNAP 25°C for 10 min. After incubation, NEM was used to block all unoxidized thiol groups to terminate the reaction and to prevent further oxidation during subsequent processing. Exposure of the recombinant PTEN to CSNO resulted in a decrease in the appearance of PTEN in a concentration-dependent fashion as determined by immunoblotting (Fig. 1A). Treatment with other nitrosothiols produced a similar pattern (Fig. 1B and C). The loss of signal by immunoblot may be due to loss of protein during oxidation or to modification of important epitopes within the PTEN molecule, which leads to loss of antibody recognition. Because addition of DTT to oxidized PTEN (far right lanes) resulted in complete recovery of antigenicity determined by immunoblot, it seems that the latter is the case. In fact, the intensity of the PTEN band after DTT was consistently higher than seen in control untreated protein, suggesting that air oxidation had occurred in the control sample. This is expected because members of the protein tyrosine phosphatase superfamily are well known to be rapidly oxidized in air (Denu and Dixon, 1998). These data show that PTEN is oxidatively modified by nitrosothiols and that the functional group involved is probably a thiol because it is reversible by DTT. In addition, we found that the antibody (mouse monoclonal anti-PTEN) did not recognize oxidized PTEN. In subsequent experiments, we have used this property as an analytical tool.

Oxidation of PTEN in A431 Cells by S-Nitrosothiols. We next investigated whether endogenous cellular PTEN was a target for oxidation by extracellular oxidants using A431 cells. It has been reported that cellular responses to nitrosothiols require transmembrane movement of CSNO mediated by system 1-amino acid transporters (Li and Whorton, 2003, 2005; Zhang and Hogg, 2004). Activity of other nitrosothiols has been shown to require coinubcation with L-cysteine and subsequent formation of L-CSNO via transnitrosation reactions (Padgett and Whorton, 1995; Mallis et al., 2001; Li and Whorton, 2003). In the following experiments, we investigated the ability of various nitrosothiols to oxidize endogenous PTEN in A431 cells and examined the role of extracellular cysteine in this effect. Confluent monolayers of cells were rinsed free of growth medium and incubated in HBSS containing CSNO, GSNO, SNAP, or equimolar mixtures of GSNO and L-cysteine or SNAP and L-cysteine. After incubation for 20 min at 37°C, cells extracts were prepared in lysis buffer A and analyzed by immunoblot. Incubation of A431 cells with CSNO led to oxidation and decreased intensity of the PTEN protein band (Fig. 2). Furthermore, although GSNO or SNAP alone did not alter the appearance of the PTEN band, combinations of these nitrosothiols with L-cysteine also led to loss of signal. Because CSNO was prepared by mixing L-cysteine with NaNO2 immediately before addition to cells, experiments were performed to test the effect of NaNO2. Treatment of cells with H2O2 also oxidized
endogenous PTEN and produced a product with higher mobility on SDS-PAGE (see below). This is in contrast to the approximate equimolar potency of these nitrosothiols on purified PTEN (Fig. 1), further suggesting that CSNO specifically mediates cellular responses.

The effect of nitrosothiols on PTEN was concentration-dependent and increased with increasing incubation time. As shown in Fig. 3A, exposure of A431 cells to CSNO at concentration as low as 50 μM resulted in a decrease in the appearance of PTEN. At high concentrations (1 mM), essentially all of the cellular PTEN was oxidized. It is noteworthy that modification by CSNO was different from that seen with H₂O₂. When A431 cells were incubated with increasing concentrations of H₂O₂, we found that the PTEN band was shifted to one with a lower apparent molecular size. High concentrations (1 mM) led to nearly complete conversion to a product with a lower apparent molecular weight. Derivatizing reagents were removed after each step of processing. PTEN thiol groups that were oxidized by CSNO, GSNO, and H₂O₂ seemed to lead to loss of the disulfide between Cys 124 and Cys 71 and a shift in mobility (Lee et al., 2002). As seen in Fig. 3, the PTEN disulfide is recognized by the mouse monoclonal antibody used in this study with no evidence for loss of hand intensity on the immunoblot except after prolonged incubation. It would seem, then, that oxidation by CSNO does not lead to the same disulfide form of the enzyme and that these two oxidants, both of which target protein thiols, produce different oxidation products.

Oxidation of PTEN increased with increasing incubation time (Fig. 3D). At 1 mM CSNO, oxidation of PTEN was maximal by approximately 20 min. Likewise, PTEN oxidation by H₂O₂ was maximal by 10 to 20 min. As mentioned above, prolonged incubation with H₂O₂ seemed to lead to loss of PTEN protein, perhaps because of overoxidation of thiol groups (Fig. 3F).

Loss of PTEN signal on immunoblot was a function of the antibody used. As stated above when we used monoclonal antibodies directed toward C-terminal fragments of PTEN, we saw a decrease in signal when the protein was oxidized by CSNO. In contrast, when samples from cells were probed using a polyclonal antibody raised against the full-length protein, we saw very little loss of signal (Fig. 3G).

To show oxidation of cellular PTEN thiol groups by CSNO, we used an approach that we had employed previously to demonstrate oxidation of other protein tyrosine phosphatases (Li and Whorton, 2003). In this assay, cells were lysed in lysis buffer containing iodoacetamide to irreversibly alkylate all reduced cysteine thiols in cellular PTEN and to prevent artifactual oxidation, which may occur during sample processing. PTEN thiol groups that were oxidized by treatment of cells with oxidants were subsequently reduced with DTT, biotinylated by reaction with MPB, and immunoprecipitated using a PTEN specific antibody. In these experiments, we found that reaction of PTEN with MPB was not efficient unless the protein was denatured by treatment with urea. Derivatizing reagents were removed after each step using a PD-10 desalting column and 60 μM GSH added to the final eluate to react with any excess MPB. Using this assay, we found that treatment of A431 cells with CSNO led to a concentration-dependent increase in thiol oxidation (MPB-labeling) (Fig. 4B). Very little PTEN was oxidized in control cells. Concentrations in the range of 0.5 mM CSNO led to significant labeling. This response corresponds roughly to the modification of PTEN shown by immunoblot (Fig. 3). Increased labeling with higher concentrations of CSNO (2.5 mM) probably represents labeling of multiple oxidized thiols within the PTEN molecule. When the MPB blot was stripped and reprobed with a rabbit antibody to PTEN, it can be seen that similar amounts of PTEN were loaded and recovered in each lane, suggesting that oxidative modification and subsequent derivatization do not alter the efficiency of immunoprecipitation or lead to other artifactual changes in protein recovery. Taken together, the results presented in Figs. 2 to 4 suggest that S-nitrosothiols oxidize PTEN in intact cells by oxidatively modifying cysteine thiol groups and suggest that the mechanism involved requires the uptake of CSNO (Fig. 2).

**Oxidative Inactivation of PTEN in A431 Cells by S-Nitrosothiol.** Because members of the protein tyrosine phosphatase family are rapidly inactivated by oxidation of the active site cysteine and because other oxidants such as H₂O₂ are known to reversibly inhibit PTEN activity (Lee et al., 2002; Leslie et al., 2003), it seems reasonable to propose that modification of PTEN by CSNO also inhibits PTEN. To investigate this possibility, we used an indirect assay (Leslie

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**Fig. 2.** CSNO, but not GSNO or SNAP, oxidizes PTEN in A431 cells. A431 cells were treated with 1 mM concentrations of the various agents indicated with or without 1 mM cysteine at 37°C for 20 min. Cellular protein extracts were prepared in lysis buffer containing 40 mM N-ethylmaleimide and fractionated by nondenaturing gel. All blot data are representative of at least three separate experiments.

**Fig. 3.** Oxidation of PTEN in A431 cells by S-nitrosothiols is concentration- and time-dependent. A431 cells were incubated with indicated concentrations of CSNO (A), SNAP + cysteine (B), GSNO + cysteine (C), or H₂O₂ (D) at 37°C for 20 min or for increasing periods of time with 1 mM CSNO (E), 1 mM H₂O₂ (F), or 1 mM CSNO (G). Cellular protein extracts were prepared in lysis buffer containing 40 mM N-ethylmaleimide and fractionated by nondenaturing SDS-PAGE on a 10% gel as described. Immunoblot analysis with a monoclonal antibody to PTEN. Immunoblot in G was done using a polyclonal antibody raised against full-length PTEN. All blot data are representative of at least three separate experiments.
et al., 2003; Li and Whorton, 2003) in which free thiols in reduced PTEN are alkylated by preparation of cell lysates in the presence of iodoacetamide. Those thiols, which existed in the oxidized state before acetylation, are subsequently reduced, and PTEN activity is measured as described under Materials and Methods. This fraction of activity represents the degree of oxidative inhibition occurring when cells are treated with oxidants. In each experiment, a portion of the lysate was not treated with iodoacetamide before reduction with DTT. This portion represented total PTEN activity and used to calculate the fractional inhibition (Fig. 5A). For untreated cells, activity detected by this assay should be near zero if the enzyme is in the fully reduced active state before alklyation by iodoacetamide.

For these experiments, A431 cells were exposed to CSNO, lysates were prepared in the presence of iodoacetamide, and activity of PTEN was measured using PtdIns(3,4,5)P3 as substrate. As shown in Fig. 5B, exposure of A431 cells to CSNO led to inhibition of PTEN phosphatase activity in a concentration-dependent fashion. Maximal inhibition occurred around 0.5 mM. Using 0.5 mM CSNO, inhibition was evident within 1 min, reaching a maximum at approximately 5 min. The degree of inhibition gradually decreased after approximately 20 min (Fig. 5C). Both the time course and extent of inhibition are probably governed by activity of cellular mechanisms that operate to reverse oxidation of PTEN thiols and restore activity. In fact, when recombinant PTEN was incubated with 0.5 mM CSNO, inhibition reached 100% within 15 min (data not shown). These findings demonstrate that exposure of cells to S-nitrosothiols leads to inhibition of cellular PTEN in intact cells. Inhibition is reversible by thiol reductants, indicating that oxidation of a critical thiol group is the likely mechanism.

Fig. 4. Oxidative modification of PTEN in A431 cells by CSNO. A, the approach used to determine the oxidation state of cellular PTEN by CSNO is shown. PTEN-S-MPB represents that fraction of PTEN that was oxidized in the intact cells by CSNO. This approach is used in B, in which A431 cells were incubated with indicated concentrations of CSNO at 37°C for 20 min. After incubation, cell protein extracts were prepared in lysis buffer containing iodoacetamide (IA), reduced by DTT, denatured by urea, and labeled with MPB (see text). Modified PTEN was immunoprecipitated with the mouse monoclonal antibodies to PTEN, separated by SDS-PAGE on a 10% gel, and transferred to a PVDF membrane. The MPB-modified PTEN was visualized by Neutr-Avidin linked to HRP and visualized with ECL Plus (B, top blot). Blots were stripped, reprobed with the rabbit polyclonal antibodies to PTEN, and visualized with goat antirabbit IgG conjugated to HRP followed by ECL Plus to show total PTEN protein (B, bottom blot). All blot data are representative of at least three separate experiments.

Fig. 5. Oxidation inactivation of PTEN in A431 cells by CSNO. A, the indirect method used to analyze the effect of CSNO on PTEN activity is shown. This method allows determination of the ratio between oxidized (PTEN-Sox, inactive) and reduced (PTEN-SH, activity) in intact cells after exposure to oxidants and was used in the following experiments. B, A431 cells were treated with increasing concentrations of CSNO at 37°C for 20 min before lysis in lysis buffer with or without the alkylating agent, IA (10 mM). Samples were applied to a PD-10 desalting column to remove excess IA followed by reduction with 60 mM DTT. PTEN was immunoprecipitated with mouse monoclonal antibodies to PTEN under reducing conditions before PTEN was assayed. Phosphatase activity of the immunoprecipitated PTEN against PtdIns(3,4,5)P3, was measured. C, A431 cells were incubated with 0.5 mM CSNO at 37°C for indicated times. Phosphatase activity was determined as described. Data represent means ± S.E., n = 4.
reduce both nitrosothiols and mixed disulfides, whereas ascorbate will only reduce nitrosothiols (Barnett et al., 1994; Holmes and Williams, 1998, 1999; Xian et al., 2000; Dairou et al., 2003). In any case, it should be pointed out that modification leads to a change in antibody recognition and apparent loss of PTEN protein on immunoblot (Fig. 1). To elucidate the mechanism responsible for oxidation of PTEN in cells by CSNO, A431 cells were incubated with CSNO or $H_2O_2$. The cellular protein extracts were treated with ascorbate or DTT followed by alkylation with NEM. Modification was examined by immunoblot using two approaches. First, after incubation with oxidants, whole cells were incubated with reducing agents before preparation of extracts (Fig. 6A) or extracts were prepared and subsequently treated with reducing agents (Fig. 6B). As can be seen, DTT completely reversed oxidation caused by either $H_2O_2$ or CSNO whereas ascorbate did not. These data are consistent with those in Fig. 1 suggesting that thiol oxidation by CSNO leads to loss of antibody recognition and that the species responsible is not S-nitrosoated PTEN. Formation of PTEN sulfides via glutathione or cysteine is certainly possible. Because GSH concentrations in cells are much higher than that of cysteine and because incubation of cells with certain GSH may elevate cytosolic concentrations of GSSG, it seems most likely that a mixed disulfide with this molecule is important. To examine the potential role of mixed disulfides, we used either diamide or GSSG to oxidize cellular PTEN. Both reagents are known to glutathionylate cellular proteins. GSSG modifies protein thiols by reacting directly with reactive protein thiols, whereas diamide produces mixed disulfides in proteins by initially reacting with protein thiols followed by reaction of the diamide product with cellular GSH (Kosower and Kosower, 1987; Mallis et al., 2001; Casagrande et al., 2002). In these experiments, cells were incubated with diamide (Fig. 7A) or extracts were prepared and subsequently incubated with GSSG (Fig. 7B). In either case, oxidation of PTEN resulted in formation of a species that is completely reversed by DTT and not recognized by the monoclonal antibody.

Incubation of recombinant PTEN with GSNO also leads to glutathione addition. To show this, we incubated purified protein with 1 mM GSNO and analyzed products by immunoblot using the monoclonal anti-PTEN antibody. As shown in Fig. 7C, GSNO led to oxidation and loss of signal. As before, treatment with DTT restored antigenicity, supporting our early conclusions. When purified protein was incubated with GSNO and then probed on immunoblot with an anti-GSH antibody that recognizes glutathione-protein adducts, we noted glutathionylated protein. Reduction with DTT abolished the signal (Fig. 7D). Taken together with data using GSSG and diamide, glutathionylation of PTEN seems to lead to loss of antibody recognition, which strengthens our proposal that CSNO leads to glutathionylation in intact cells.

**Discussion**

Redox regulation of protein function through activity of oxidants on cysteine thiols is increasingly appreciated as an important cell signaling mechanism. Recently, oxidants including $H_2O_2$ and superoxide produced by NADPH oxidases, have been shown to reversibly inhibit PTP1B and regulate signals generated by receptor tyrosine kinases (Bae et al., 1997; Bae et al., 2000). It has also been found that $H_2O_2$ reversibly inhibits PTEN in cells, leading to an increase in signaling through the PI3-kinase pathway (Lee et al., 2002; Leslie et al., 2003; Kwon et al., 2004). In this regard, the role of PTEN in these signaling pathways is most likely related to the redox state of cysteine residues, with oxidation leading to signal activation and reduction inactivating PTEN.

**Fig. 7.** Oxidative effects of diamide and oxidized glutathione (GSSG) on PTEN in A431 cells. A, A431 cells were treated with the indicated concentrations of diamide for 20 min at 37°C followed by incubation with or without 20 mM DTT for an additional 10 min. Cellular protein extracts were prepared in lysis buffer containing 40 mM N-ethylmaleimide and fractionated by nonreducing SDS-PAGE on a 10% gel followed by immunoblot analysis. B, A431 cells were incubated with lysis buffer on ice for 30 min, collected by scraping, and centrifuged. The supernatant was incubated with the increasing concentrations of diamide or DTT at 4°C for 30 min and treated with 40 mM NEM, and analyzed by immunoblot. All blot data are representative of at least three separate experiments.
of other biological oxidants, such as nitrosothiols, is less well studied. However, nitrosothiols are well recognized to target cellular thiols, and our current studies show for the first time that PTEN is reversibly inactivated by nitrosothiols.

Oxidative modification of PTEN by nitrosothiols differs from oxidation caused by H$_2$O$_2$ in several ways. Exposure to H$_2$O$_2$ leads to reversible formation of an intramolecular disulfide (Lee et al., 2002). Other phosphatases display a similar feature in which oxidation of the active site cysteine to a sulfenic acid results in rapid disulfide formation through reaction with a nearby cysteine (Sohn and Rudolph, 2003). This mechanism protects the enzyme from undergoing further irreversible oxidation to sulfonic acid and is probably critical to the signaling role of H$_2$O$_2$. CSNO-mediated oxidation proceeds through a different mechanism. Treatment of PTEN with CSNO leads to an apparent loss of protein by immunoblot. This effect is completely reversed by treatment with DTT, suggesting that thiol oxidation caused by CSNO leads to a loss in antibody recognition. Because the monoclonal antibody used in these studies was raised against a peptide (residues 388–400) from the C terminus of PTEN, it is possible that some change in this region is responsible. However, there are no cysteine residues in or near this area. Thus, oxidation in other parts of the enzyme apparently renders this area inaccessible on immunoblot.

The effect of CSNO on PTEN is caused by modification of protein thiols. This is clear because oxidation is reversed by DTT, and the oxidized enzyme is labeled with MPB. Among several products, oxidation of PTEN thiols by CSNO may lead to formation of nitrosylated protein, a mixed disulfide with cysteine, or a mixed disulfide with GSH. We used differential reduction of oxidized protein by DTT and ascorbate (Xian et al., 2000; Dairou et al., 2003) to address these possibilities. Whereas DTT easily reversed the modification caused by CSNO, ascorbate did not, suggesting that nitrosylated protein was not the end product detected on immunoblot. It should be mentioned that nitrosylation of critical thiols might act to produce an intermediate S-nitrosylated PTEN that may subsequently react with cellular GSH to form the mixed disulfide, which then accumulates. On the other hand, critical PTEN thiols may react with CSNO to directly form a mixed disulfide with cysteine. It is interesting that when cells or extracts from cells are treated to produce protein mixed disulfides (glutathione adducts), we observed the same loss in PTEN antibody recognition that we saw with CSNO. Although this is not definitive proof of mixed disulfide formation, the data clearly support the proposal.

Oxidation of PTEN by nitrosothiols is mediated by CSNO. GSNO and SNAP are not active unless cysteine is included in the medium. This is similar to what we and other have reported, leading to the suggestion that a specific transport mechanism exists for movement of CSNO across biological membranes (Mallis et al., 2001; Nemoto and Finkel, 2002; Li and Whorton, 2003; Zhang and Hogg, 2004). It has recently been shown that amino acid transporters of the system L family (LAT1 and LAT2) are carriers of CSNO (Nemoto and Finkel, 2002; Li and Whorton, 2003). This is likely to be important because CSNO would have ready access to PTEN (and other reactive proteins) in the vicinity of the membrane, and entry of CSNO may readily alter the activity of proteins, including PTEN, within this compartment. It should be pointed out that the effects observed may be indirect after entry of CSNO into cells. Alternative mechanisms for PTEN oxidation, which occurs after transport of CSNO, include oxidation by intracellular GSNO, which may result from transnitrosation reactions between CSNO and GSH. In addition, because GSSG has been observed to increase in cells treated with CSNO, S-thiolation is also possible.

Oxidation of PTEN by CSNO leads to reversible inhibition of its activity against PIP$_3$. Because of the low pK$_a$ and concomitant reactivity of the active site cysteine, it is likely that this residue is targeted by CSNO. PTEN activity seems to recover quickly. Although we have not identified the redox mechanisms involved, previous work by our group has shown that the glutathione redox cycle is critical in both limiting and reversing the oxidative effects of nitrosothiols (Padgett and Whorton, 1997, 1998). Inhibition of PTEN activity would presumably lead to an increase in cellular PIP$_3$, and activation of downstream targets of this lipid. Data regarding Akt phosphorylation in response to CSNO have not been published. In the case of H$_2$O$_2$, PTEN inhibition was found to increase signaling through the PI3 kinase pathway and to increase phosphorylation of Akt (Leslie et al., 2003; Kwon et al., 2004). In fact, inhibition of both PTEN and PTP1B by H$_2$O$_2$ have been shown to be important in growth factor signaling (Kwon et al., 2004). It might be expected that nitrosothiols would increase signaling through the PI3 kinase pathway as well. Although our data clearly show that PTEN is reversibly inhibited in cells exposed to extracellular CSNO, it is not clear whether CSNO, at concentrations that could be synthesized intracellularly or that occur in extracellular fluids, will produce similar levels of inhibition or alter downstream signaling pathways regulated by PTEN. Our previous studies have shown that CSNO potently inhibits PTP1B and increases the level of phosphorylation of the epidermal growth factor receptor (Li and Whorton, 2003). In general, however, nitrosothiols inhibit rather than stimulate growth (Bauer et al., 2001). Further work on differential effects of biological oxidants on this signaling pathway is needed.


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