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Redox Regulation of PTEN by S-Nitrosothiols

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Running Title: Redox regulation of PTEN by S-nitrosothiols

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Abbreviation used: CSNO, S-nitrosocysteine; GSNO, S-nitrosoglutathione; SNAP, S-nitroso-N-acetylpenicillamine; GSSG, oxidized glutathione; NEM, N-ethylmaleimide; IA, iodoacetamide; MPB, N^{α}-(3-maleimidylpropionyl)biocytin; PtdIns (3, 4, 5)P_3, phosphatidylinositol 3, 4, 5-trisphosphate; DTT, dithiothreitol. GSH, reduced glutathione; DMEM, Dulbecco's modified Eagle's medium; HBSH, Hanks' balanced salt solution buffered with 10 mM N-2-hydroxyethylpiperazine-N´–2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylaminde gel electrophoresis.

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Abstract

PTEN is a PtdIns(3,4,5,)P_3 phosphatase which regulates many cellular processes. Activity of the enzyme is dependent on the redox state of the active site cysteine such that oxidation by H_2O_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 co-incubated 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. While oxidation of PTEN by H_2O_2 led to formation of an intramolecular disulfide, oxidation of PTEN by CSNO appeared 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 similar manner as 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.

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Introduction

PTEN (phosphatase with sequence homology to tensin) is a member of the protein tyrosine phosphatase superfamily which dephosphorylates phosphatidylinositol-3,4,5-trisphosphate (Maehama et al., 2001; Maehama and Dixon, 1999). 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 is associated with a vast number of human cancers particularly in advanced stages (Sun et al., 1999; Parsons, 2004; Stambolic et al., 1998; Leslie and Downes, 2004).

Less is known about post translational modification and regulation of PTEN activity. PTEN function and cellular localization appears 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 (Kwon et al., 2004; Lee et al., 2002; Leslie et al., 2003). In common with other members of the protein tyrosine phosphatase superfamily, PTEN has a C(X_5)R signature motif at its active site where the environment surrounding the cysteinyl thiol leads to a low pKa (~5) and increased redox sensitivity (Maehama et al., 2001; Maehama and Dixon, 1999; Leslie and Downes, 2004; Denu and Dixon, 1998). Indeed it has been recently shown that H_2O_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 appears to act to preserve the reversibility of redox modification by preventing ‘over’ oxidation to irreversible oxidation states (Sohn and

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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). Althgough the role of redox regulation has not been established, it has been proposed that H_2O_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).

While it is clear that H_2O_2 can regulate PTEN activity, the effect of other signaling molecules which act as oxidants has not been studied. In this regard, nitric oxide is known to regulate activity of proteins by reversibly nitrosating cysteine residues (Gow et al., 2004; Stamler et al., 1997) 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 (Gow et al., 2004; Stamler et al., 1997; Hogg, 2002). Which protein cysteines are targeted by nitrosothiols depends on many factors including thiol pKa, steric factors and other factors which 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-nitrosocysteine 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 have 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.

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Experimental Methods

Cloning, Expression, and Purification of Recombinant PTEN. Human PTEN cDNA was obtained by RT-PCR from total RNA isolated from human lymphocytes using the primer pair (forward) 5'>TAGCATCCATGACAGCCATCATCAAAGAG>3'; (reverse) 5'>TAGAATTCTCAGACTTTTGTAACTTGTG>3' and cloned into a prokaryotic expression vector pQE30 (Qiagen, Valencia, CA) for expression of the protein with a histidine tag at 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+}-NTA-agarose column (Qiagen, Valencia, CA). The purified protein was desalted using a Centriprep YM-10 filter (Amicon, Inc., Beverly, MA), concentrated against 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 10% glycerol, and 5 mM DTT and stored at -80ºC.

Cell Culture and Treatment: A431 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) with high glucose supplemented with 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 250 ng/ml amphotericin B and incubated at 37ºC in 7.5% CO_2 in air. To begin an experiment, confluent monolayers of A431 cells were rinsed three times with Hanks' balanced salt solution (Gibco, Grand Island, NY) buffered with 10 mM N-2-hydroxyethylpiperazine-N'–2-ethanesulfonic acid (HBSH, pH 7.4) and incubated in HBSH at 37ºC.

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 25ºC. Following incubation, samples were alkylated with 40 mM NEM in electrophoresis gel-

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loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) and subjected to sodium dodecyl sulfate (SDS)-polyacrylaminde gel (10%) electrophoresis (PAGE). Proteins were transferred electrophoretically to a PVDF membrane (Invitrogen, Carlsbad, CA) and detected by immunoblot after blocking with 5% powered milk in TBS-T (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) using mouse monoclonal antiPTEN as primary antibody (PTEN A2B1, Santa Cruz Biotechnology, Santa Cruz, CA) and horse radish peroxidase-conjugated antimouse IgG BD Biosciences/Transduction Labs (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 (non-reducing conditions) and analyzed by immunoblot using an antibody which specifically recognizes glutathione-protein adducts (Virogen, Watertown, MA).

Oxidation of cellular PTEN: A431 cells were treated with various agents in HBSH at 37ºC. Cells were washed three times with ice-cold HBSH and incubated on ice for 30 min with lysis buffer A (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, 5 µg/ml pepstain A) containing 40 mM N-ethyl maleimide (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 12,000 g for 5 min at 4ºC. The supernatant was removed for protein analysis using the Micro-BCA assay (Pierce, Rockford, IL). PTEN was visualized by immunoblot as above.

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Determination of PTEN thiol oxidation in intact cells: To determine the effect of oxidants on PTEN thiol oxidation, we used MPB as previously described (Li and Whorton, 2003). Briefly, A431 cells were exposed to CSNO at 37ºC for 20 min, rinsed three times with ice-cold HBSH 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, 5 µg/ml pepstain A) containing 10 mM iodoacetamide. Lysates were collected by scraping, and subjected to centrifugation at 12,000 g at 4ºC for 5 min. The supernatant was applied to a PD-10 desalting column equilibrated with lysis buffer B to remove excess iodoacetamide. The eluate was treated with 60 mM DTT at 4ºC for 30 min to reduce all oxidized thiols. Samples were re-applied to a PD-10 column equilibrated with 8 M urea in lysis buffer B to remove excess DTT. The eluate containing 8 M urea was incubated for 30 min to denature proteins and subsequently applied to a PD-10 column to remove excess urea. Eluates were then treated with 100 µM N^{α}-(3-maleimidylpropionyl)biocytin (MPB) for 1 h at 4ºC to biotinylate that fraction of thiol groups which had been oxidized in the original sample. The samples were again applied to a PD-10 column to remove excess MPB and eluates treated with 60 µM glutathione, precleared by incubation with 2 µg of mouse IgG and 20 µl of protein G PLUS agarose (Santa Cruz Biotechnology) at 4ºC for 30 min and centrifuged at 1,000 g at 4ºC for 5 min. Aliquots containing equivalent amounts of total cellular protein were immunoprecipitated using 1 µg of mouse monoclonal antiPTEN by incubation at 4ºC for 2 h. Immune complexes were precipitated by incubation with 20 µl of protein G PLUS agarose at 4ºC overnight, and collected by centrifugation. Immunoprecipitates were washed four times with lysis buffer B,

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resuspended in 40 µl of 2X electrophoresis gel-loading buffer without DTT, separated on a 10% SDS-polyacrylamide gel, and transferred to PVDF membranes. After blocking with 5% powered milk in TBS-T (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20), biotinylated PTEN was visualized by incubation with Neutr-Avidin 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 55ºC 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 previously described (Li and Whorton, 2002; Li and Whorton, 2003; Leslie et al., 2003) was used to measure PTEN phophatase activity. After treatment with nitrosothiols, A431 cells were rinsed with ice-cold HBSH, 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, 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 2 times with lysis buffer A containing 2 mM DTT, 2 times with buffer C (100 mM Tris-HCl, pH 7.5, 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) P_3 was measured in a 96-well plate using published procedures (Campbell et al., 2003). Briefly,

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the immunoprecipitated PTEN was incubated with 40 µM PtdIns (3,4,5) P_3 in buffer C at 37ºC 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 to 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)) x 100%.

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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 25ºC 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 (figure 1A). Treatment with other nitrosothiols produced a similar pattern (figure 1B and 1C). 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 antigenecity determined by immunoblot, it appears 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 since 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 likely a thiol since it is reversible by DTT. In addition, we found that the antibody (mouse monoclonal antiPTEN) 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

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using A431 cells. It has been reported that cellular responses to nitrosothiols require transmembrane movement of CSNO mediated by system L amino acid transporters (Li and Whorton, 2003; Zhang and Hogg, 2004; Li and Whorton, 2005). Activity of other nitrosothiols has been shown to require co-incubation with L-cysteine and subsequent formation of L-CSNO via transnitrosation reactions (Padgett and Whorton, 1995; Li and Whorton, 2003; Mallis et al., 2001). 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 HBSH containing CSNO, GSNO, SNAP or equimolar mixtures of GSNO and L-cysteine or SNAP and L-cysteine. After incubation for 20 min at 37ºC, cells extracts were prepared in lysis buffer A and analyzed by immunoblot. Incubation of A431 cells with CSNO lead to oxidation and decreased intensity of the PTEN protein band (figure 2). Furthermore, while GSNO or SNAP alone did not alter the appearance of the PTEN band, combinations of these nitrosothiols with L-cysteine also lead to loss of signal. Since CSNO was prepared by mixing L-cysteine with $NaNO_2$ immediately prior to addition to cells, experiments were done to test the effect of $NaNO_2$. Treatment of cells with H_2O_2 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 (figure 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 figure 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. Importantly, modification by CSNO was different from that seen with H_2O_2. When A431 cells were incubated with increasing concentrations of H_2O_2 we found that the PTEN band was shifted to one with a lower apparent molecular size. High concentrations (1 mM) lead to nearly complete conversion to this species (figure 3D). In studies by others, PTEN oxidation by H_2O_2 has also been shown to result in formation of an intracellular disulfide between Cys 124 and Cys 71 and a shift in mobility (Lee et al., 2002). As seen in figure 3, the PTEN disulfide is recognized by the mouse monoclonal antibody used in this study with no evidence for loss of band intensity on the immunoblot except after prolonged incubation. It would appear 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 (figure 3E). At 1 mM CSNO, oxidation of PTEN was maximal by approximately 20 min. Likewise, PTEN oxidation by H_2O_2 was maximal by 10-20 min. As mentioned above, prolonged incubation with H_2O_2, appeared to lead to loss of PTEN protein perhaps due to over oxidation of thiol groups (figure 3F).

Loss of PTEN signal on immnoblot was a function of the antibody used. As stated above when we used monoclonal antibodies directed towards C-terminal fragements 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 (figure 3G).

In order to show oxidation of cellular PTEN thiols groups by CSNO, we used an approach we had previously employed 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 prevent artifactual oxidation which may occur during sample processing. PTEN thiol groups which 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) (figure 4B). Very little PTEN was oxidized in control cells. Concentrations in the range of 0.5 mM CSNO led to significant labeling. This response roughly corresponds to the modification of PTEN shown by immunoblot (figure 3). Increased labeling with higher concentrations of CSNO (2.5 mM) most likely 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 figure 2-4 suggest that S-nitrosothiols oxidize

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PTEN in intact cells by oxidatively modifying cysteine thiol groups and suggest that the mechanism involved requires the uptake of CSNO (figure 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 H_2O_2 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. In order to investigate this possibility, we used an indirect assay (Li and Whorton, 2003; Leslie et al., 2003) in which free thiols in reduced PTEN are alkylated by preparation of cell lysated in the presence of iodoacetamide. Those thiols which existed in the oxidized state prior to acetylation, are subsequently reduced and activity measured. 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 prior to reduction with DTT. This portion represented total PTEN activity and was used to calculate the fraction inhibition (figure 5A). For untreated cells, activity detected by this assay should be near zero if the enzyme is in the fully reduced active state prior to alkylation by iodoacetamide.

For these experiments, A431 cells were exposed to CSNO, lysates prepared in the presence of iodoacetamide and activity of PTEN was measured using PtdIns(3,4,5)P_3 as substrate. As shown in figure 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 minute reaching a maximum at about 5 minutes. The degree of inhibition gradually decreased

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after about 20 minutes (figure 5C). Both the time course and extent of inhibition are likely governed by activity of cellular mechanisms which 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 minutes (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.

Role of mixed disulfide formation in oxidation of PTEN: It is well known that both H_2O_2 and CSNO target proteins primarily through oxidation of cysteine thiols. Although oxidation of PTEN by H_2O_2 appears to produce a disulfide within the protein, exposure to CSNO does not (figure 3). This suggests that CSNO produces other oxidized species. Several modifications are possible. PTEN thiols may undergo transnitrosation reactions leading to S-nitrosation of the protein (PTEN-SNO). Once formed, nitroso derivatives of PTEN may subsequently react with intracellular small molecule weight thiols (such as GSH) to produce mixed disulfides via glutathionylation (PTEN-SSG). Alternatively, CSNO may react with cellular GSH forming GSNO which subsequently modifies PTEN. Finally, PTEN may react directly with CSNO to form a mixed disulfide between PTEN and cysteine (PTEN-SSC). Differential reduction using either DTT or ascorbate can help distinguish among these possibilities. Treatment of oxidized PTEN will reduce both nitrosothiols and mixed disulfides while ascorbate will only reduce nitrosothiols (Holmes and and Williams, 1998; Williams, 1999; Barnett et al., 1994; 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

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immunoblot (figure 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 prior to preparation of extracts (figure 6A) or extracts were prepared and subsequently treated with reducing agents (figure 6B). As can be seen, DTT completely reversed oxidation caused by either H_2O_2 or CSNO while ascorbate did not. These data are consistent with those in figure 1 which suggest that thiol oxidation by CSNO leads to loss of antibody recognition and suggest that the species responsible is not PTEN-SNO. Formation of PTEN-SSC or PTEN-SSG is certainly possible. Since GSH concentrations in cells are much higher than that of CSH and since incubation of cells with CSNO 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 while 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; Casagrande et al., 2002; Mallis et al., 2001). In these experiments cells were either incubated with diamide (figure 7A), or extracts were prepared and subsequently incubated with GSSG (figure 7B). In either case, oxidation of PTEN resulted in formation of a species which 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 antiPTEN antibody. As shown in figure 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 which recognizes glutathione-protein adducts we see glutathinylated protein. Reduction with DTT abolished the signal (figure 7D). Taken together with data using GSSG and diamide, it appears that glutathionylation of PTEN leads to loss of antibody recognition and strengthens our proposal that CSNO leads to glutathionylation in intact cells.

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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; Kwon et al., 2004; Leslie et al., 2003). In this regard, the role 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_2O_2 in several ways. Exposure to H_2O_2 leads to reversible formation of a 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 sulfinic acid and is likely critical to the signaling role of H_2O_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. Since the monoclonal antibody used in these studies was raised against a pepide (residues 388-400) from the C-terminus of PTEN, it is possible that some change in this region is

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responsible. However, there are no cysteine residues in or near this area. Thus oxidation in other parts of the enzyme apparently render this area inaccessible on immunoblot.

The effect of CSNO on PTEN is due to modification of protein thiols. This is clear since 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. While 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 which 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. Interestingly 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. While 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; Li and Whorton, 2003; Nemoto and Finkel, 2002; Zhang and Hogg, 2004). Recently it has been shown that amino acid transporters of the system L family (LAT1 and LAT2) are carriers of

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CSNO (Li and Whorton, 2003; Nemoto and Finkel, 2002). This is likely important since 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 following 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 since 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. Due to the low pKa and concomitant reactivity of the active site cysteine, it is likely that this residue is targeted by CSNO. PTEN activity appears to recover quickly. Although we have not identified which redox mechanisms are 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; Padgett and Whorton, 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_2O_2, PTEN inhibition was found to increase signaling through the PI3 kinase pathway and increase phosphorylation of Akt (Leslie et al., 2003; Kwon et al., 2004). In fact inhibition of both PTEN and PTP1B by H_2O_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 which may be synthesized intracellularly or which 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). However, in general, 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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Figure Legends

Figure 1. Oxidation of purified recombinant PTEN by S-nitrosothiols. Purified recombinant human PTEN was incubated with the indicated concentrations of CSNO (A), GSNO (B), or SNAP (C) in the presence of 20 mM Tris-HCl (pH 6.7) at 25ºC for 10 min. Following treatment, samples were alkylated with 40 mM N-ethylmaleimide in electrophoresis gel-loading buffer with (far right lane) or without 50 mM DTT (other lanes), and fractionated by SDS-PAGE on a 10% gel followed by immunoblot analysis with a mouse monoclonal antibody to PTEN. Decreased PTEN band intensity represents loss of antibody recognition. All blot data are representative of at least three separate experiments.

Figure 2. CSNO, but not GSNO or SNAP, oxidizes PTEN in A431 cells. A431 cells were treated with 1 mM of the various agents indicated with or without 1 mM cysteine at 37ºC for 20 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 with a mouse monoclonal antibody to PTEN. All blot data are representative of at least three separate experiments.

Figure 3. Oxidation of PTEN in A431 cells by S-nitrosothiols is concentration and time dependent. A431 cells were incubated with indicated concentrations of (A) CSNO, (B) SNAP + cysteine, (C) GSNO + cysteine, (D) H_2O_2 at 37ºC for 20 min or for increasing periods of time with (E) 1 mM CSNO, (F) 1 mM H_2O_2 or (G) 1 mM CSNO. 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 with a mouse monoclonal antibody to PTEN. Immunoblot in panel G was done using a polyclonal antibody raised against full length PTEN. All blot data are representative of at least three separate experiments.

Figure 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 which 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 37ºC 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 N α -(3-maleimidylpropionyl) biocytin (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 localized with Neutr-Avidin linked to HRP and visualized with ECL Plus (B, upper blot). Blots were stripped, reprobed with the rabbit polyclonal antibodies to PTEN, and visualized with goat anti-rabbit IgG conjugated to HRP followed by ECL Plus to show total PTEN protein (B, lower 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-

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SH, activity) in intact cells following exposure to oxidants and was used in the following experiments. (B) A431 cells were treated with increasing concentrations of CSNO at 37ºC for 20 min before lysis in lysis buffer with or without the alkylating agent, iodoacetamide (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 37ºC for indicated times. Phosphatase activity was determined as described. Data represent means $\pm$ SE, n=4.

Figure 6. Selective reduction of oxidized PTEN in A431 cells by DTT or ascorbate. (A) A431 cells were treated with H_2O_2 (1 mM) or CSNO (1 mM) as indicated for 20 min at 37ºC followed by incubation with DTT (50 mM) or ascorbate (50 mM) for additional 10 min. Cellular protein extracts were prepared in lysis buffer containing 40 mM N-ethylmaleimide, fractionated by nonreducing SDS-PAGE on a 10% gel followed by immunoblot. (B) A431 cells were treated with CSNO (1 mM) or H_2O_2 (1 mM) as indicated for 20 min at 37ºC. After stimulation, the cells were incubated with lysis buffer on ice for 30 min, collected by scraping and centrifuged. The supernatant was incubated with the indicated concentrations of ascorbate or DTT at 4ºC for 30 min, treated with 40 mM NEM and analyzed by immunoblot. All blot data are representative of at least three separate experiments.

Figure 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 37ºC 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 GSSG at 4ºC for 30 min, treated with 40 mM NEM followed by immunoblot analysis as described above. Some samples were treated with 100 mM DTT prior to analysis. (C) Recombinant PTEN was treated with 1 mM (C) or 5 mM (D) GSNO for 15 min or GSNO followed by 100 mM DTT for 10 min. Samples were separated by SDS-PAGE and analyzed by immunoblot. In (C) a monoclonal antibody was used while in (D) an antiglutathione antibody was used. Lane 1 is untreated sample, lane 2 is GSNO treated and lane 3 is GSNO + DTT treated. to PTEN. All blot data are representative of at least three separate experiments.

Figure 1

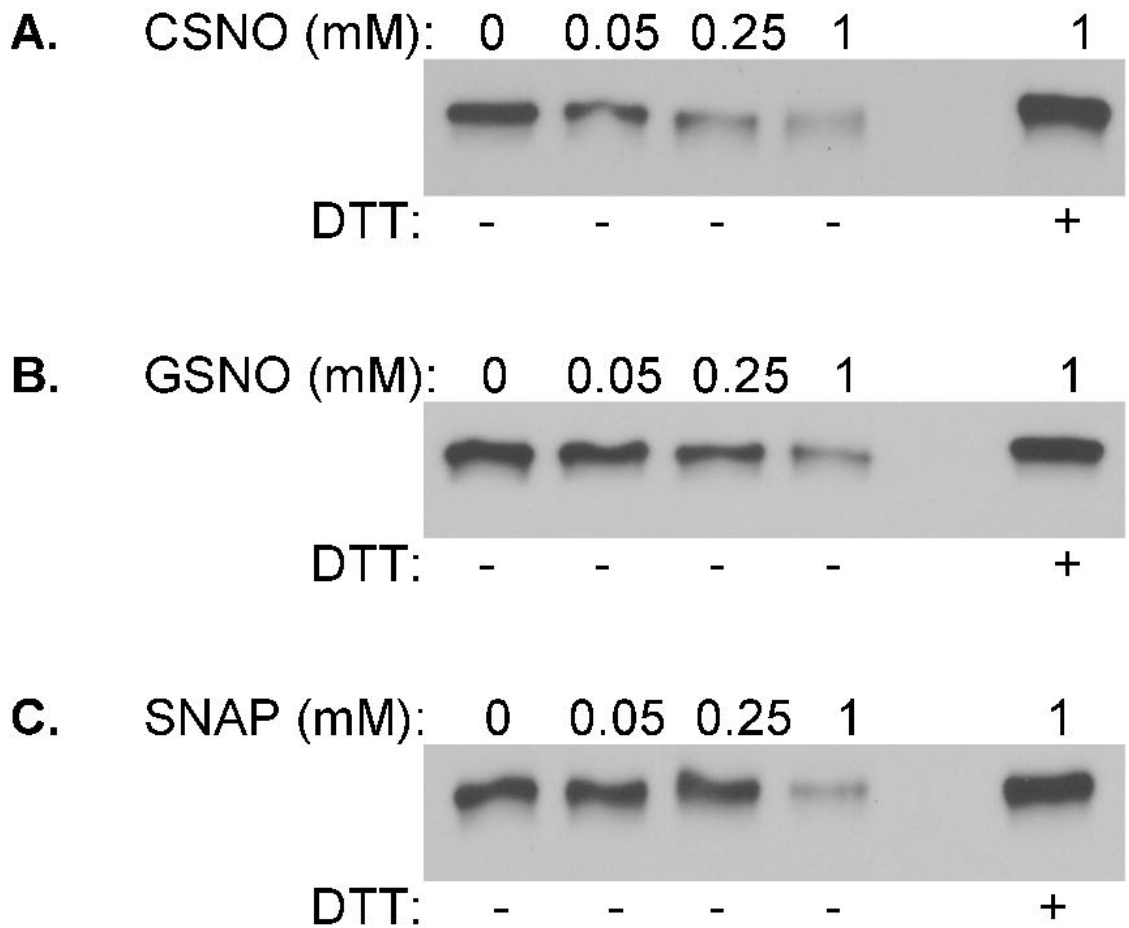

A.
CSNO (mM): 0 0.05 0.25 1 1
DTT: - - - - +
B.
GSNO (mM): 0 0.05 0.25 1 1
DTT: - - - - +
C.
SNAP (mM): 0 0.05 0.25 1 1
DTT: - - - - +

Figure 2

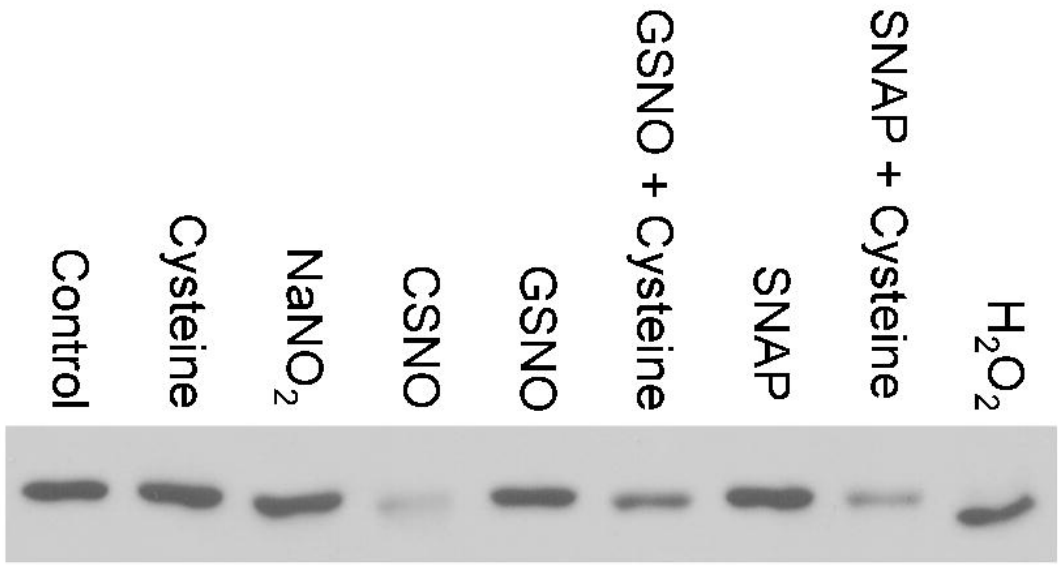

Control
Cysteine
$NaNO_2$
CSNO
GSNO
GSNO + Cysteine
SNAP
SNAP + Cysteine
H_2O_2

Figure 3

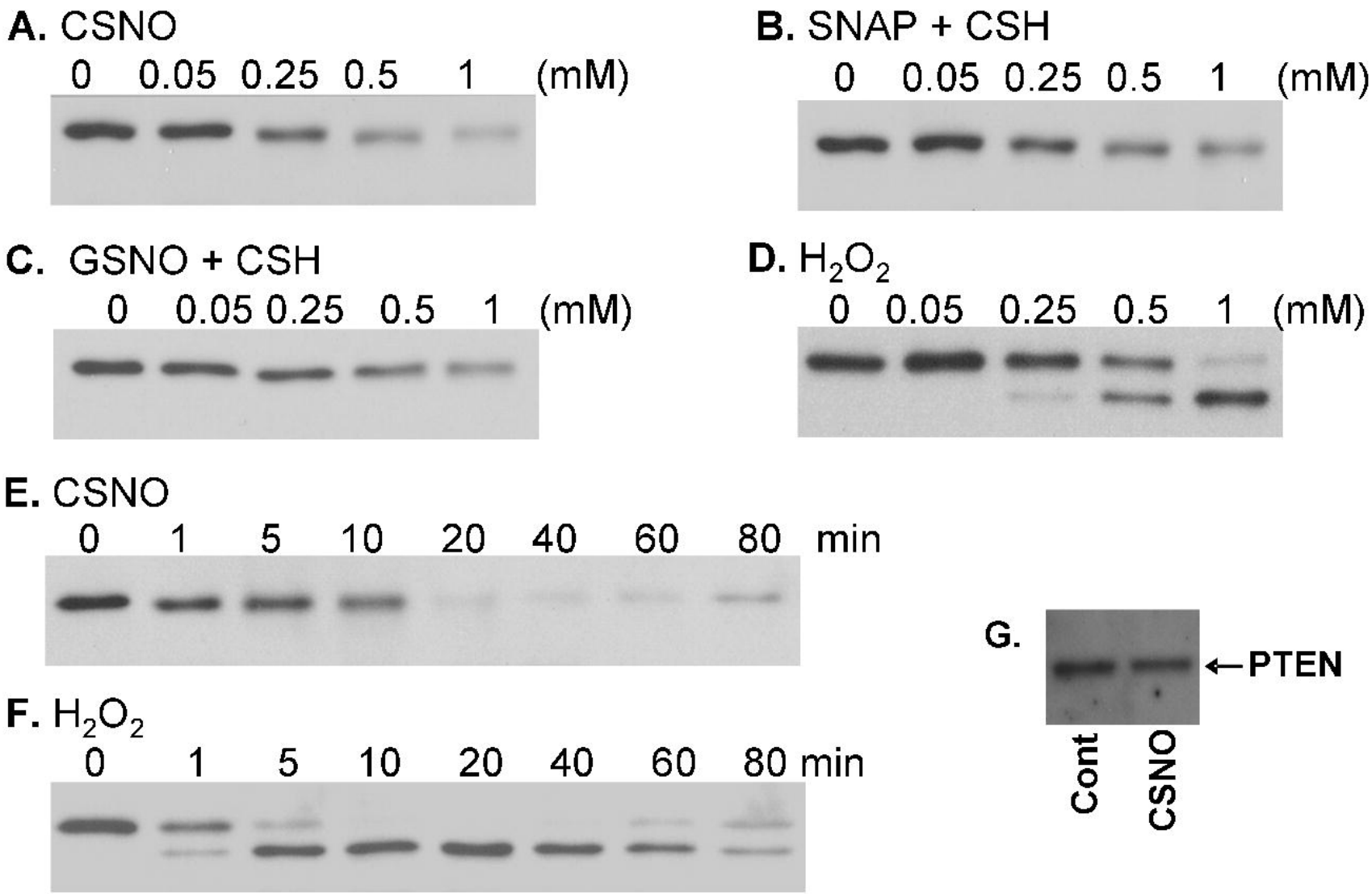
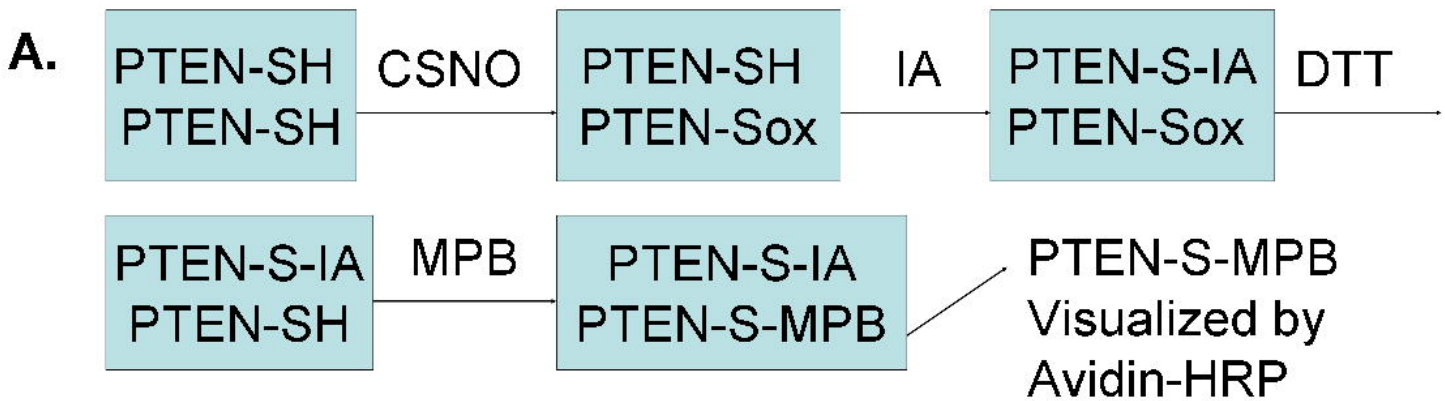

A. CSNO
0 0.05 0.25 0.5 1 (mM)
B. SNAP + CSH
0 0.05 0.25 0.5 1 (mM)
C. GSNO + CSH
0 0.05 0.25 0.5 1 (mM)
D. H_2O_2
0 0.05 0.25 0.5 1 (mM)
E. CSNO
0 1 5 10 20 40 60 80 min
F. H_2O_2
0 1 5 10 20 40 60 80 min
G.
←PTEN
Cont
CSNO

Figure 4


A.
PTEN-SH
PTEN-SH
CSNO
PTEN-SH
PTEN-Sox
IA
PTEN-S-IA
PTEN-Sox
DTT
PTEN-S-IA
PTEN-SH
MPB
PTEN-S-IA
PTEN-S-MPB
PTEN-S-MPB
Visualized by
Avidin-HRP

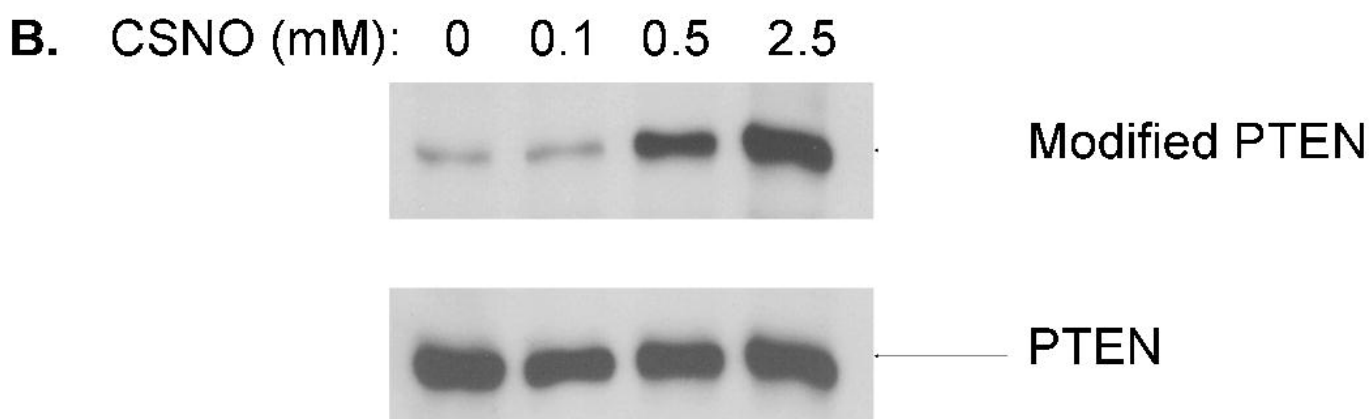

B.
CSNO (mM): 0 0.1 0.5 2.5
Modified PTEN
PTEN

Figure 5

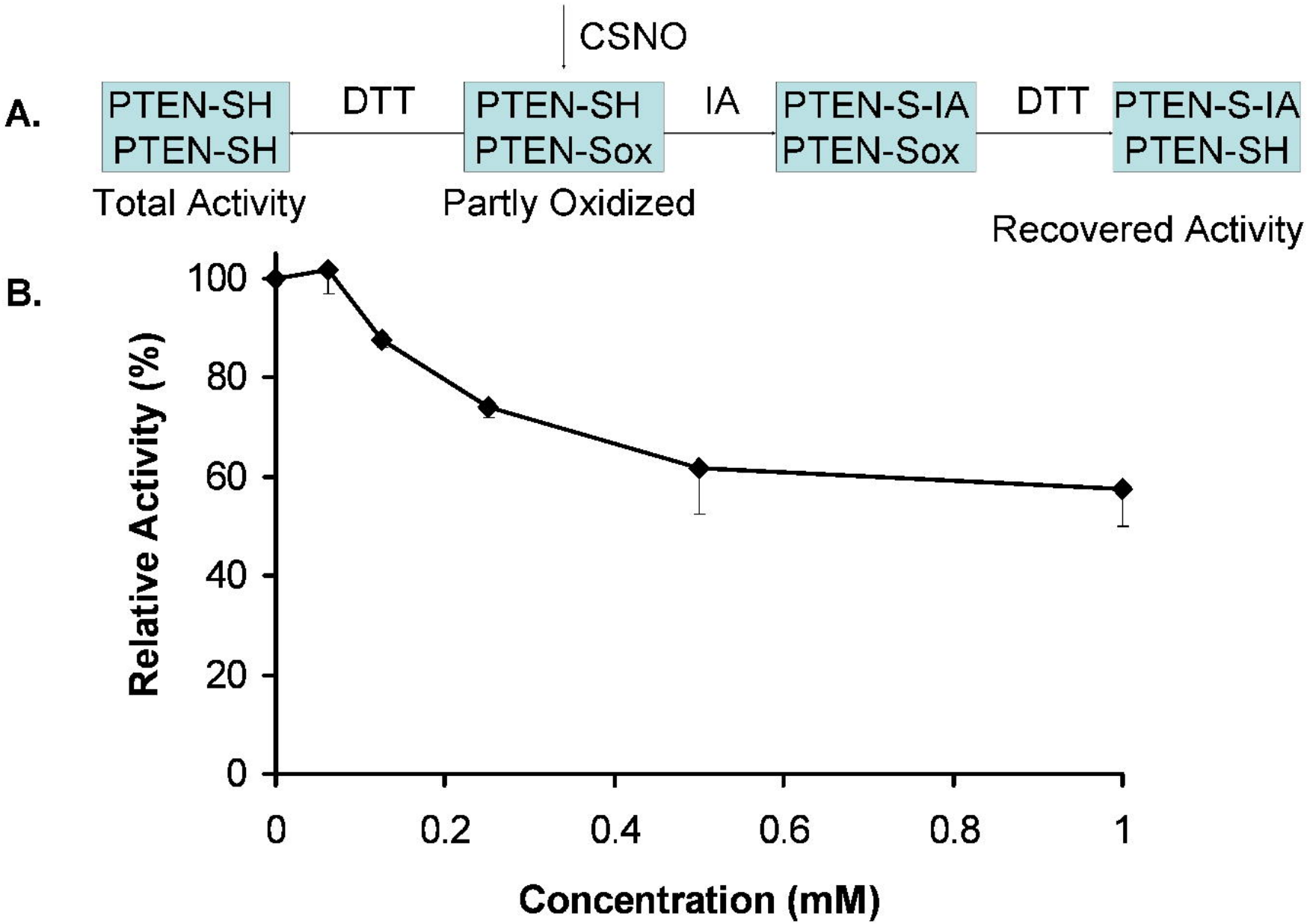

A.
CSNO
PTEN-SH
PTEN-SH
DTT
PTEN-SH
PTEN-Sox
IA
PTEN-S-IA
PTEN-Sox
DTT
PTEN-S-IA
PTEN-SH
Total Activity
Partly Oxidized
Recovered Activity
B.
Relative Activity (%)
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
Concentration (mM)

Figure 5 cont'd

C.

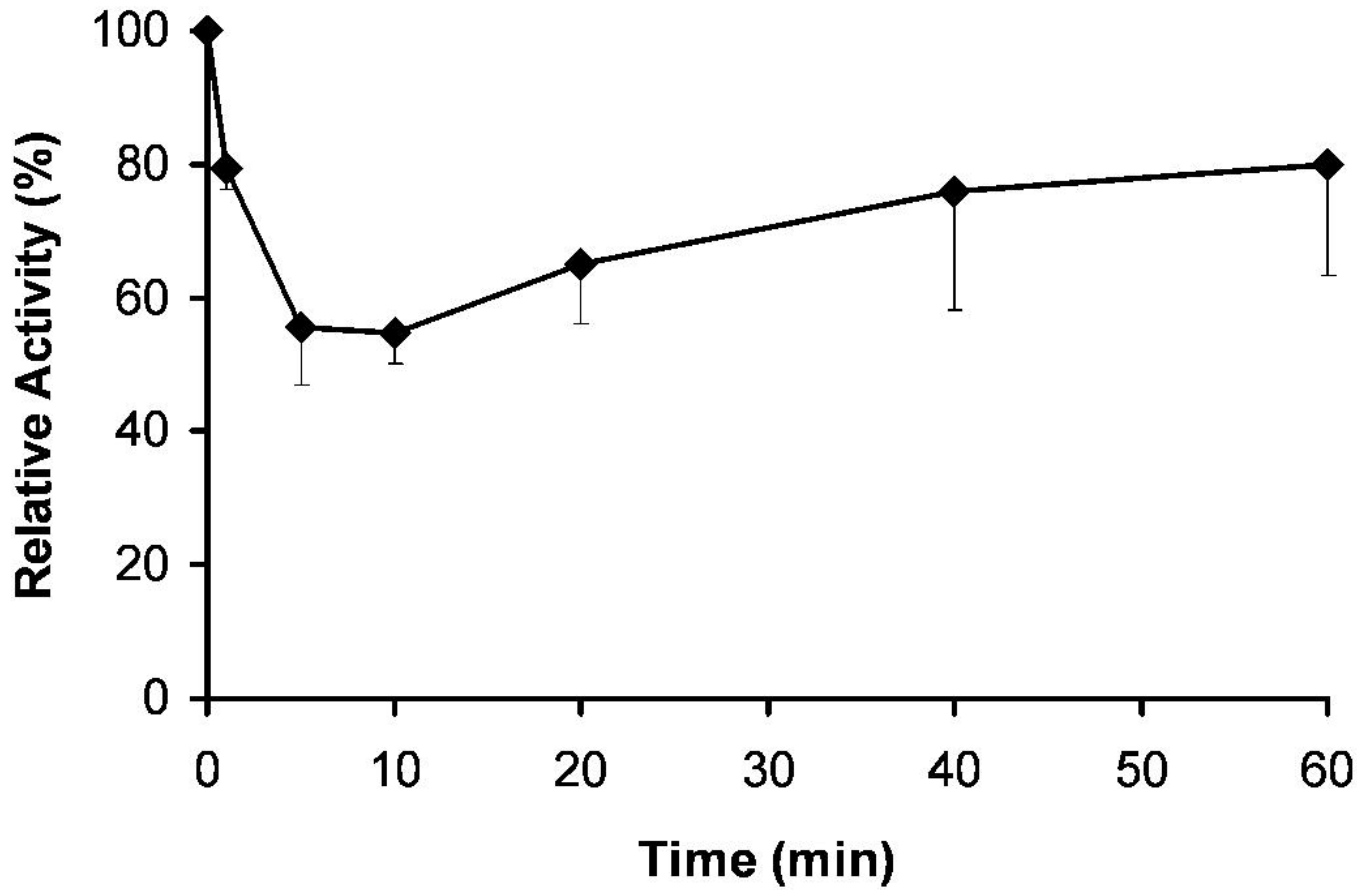


Figure 6

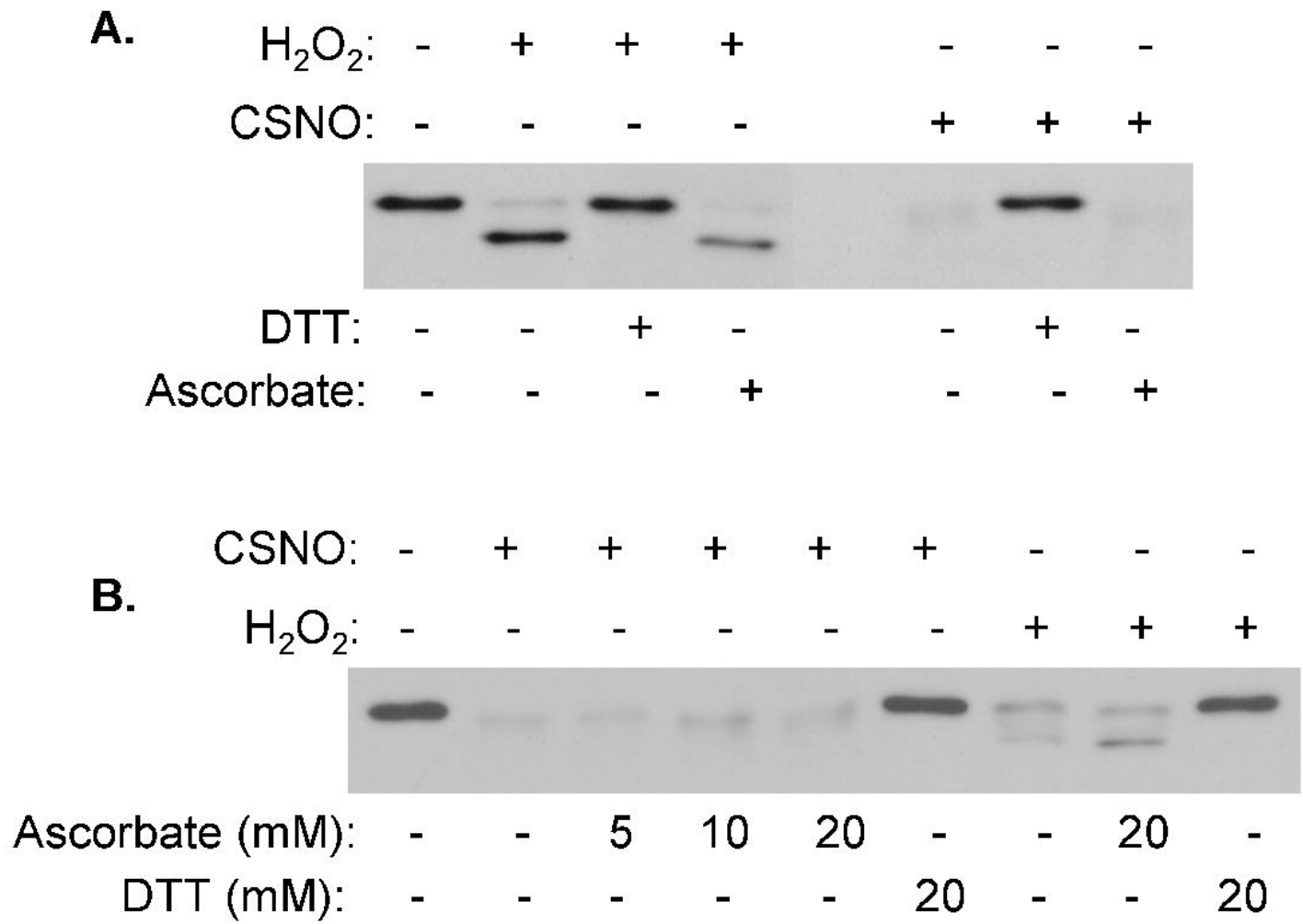
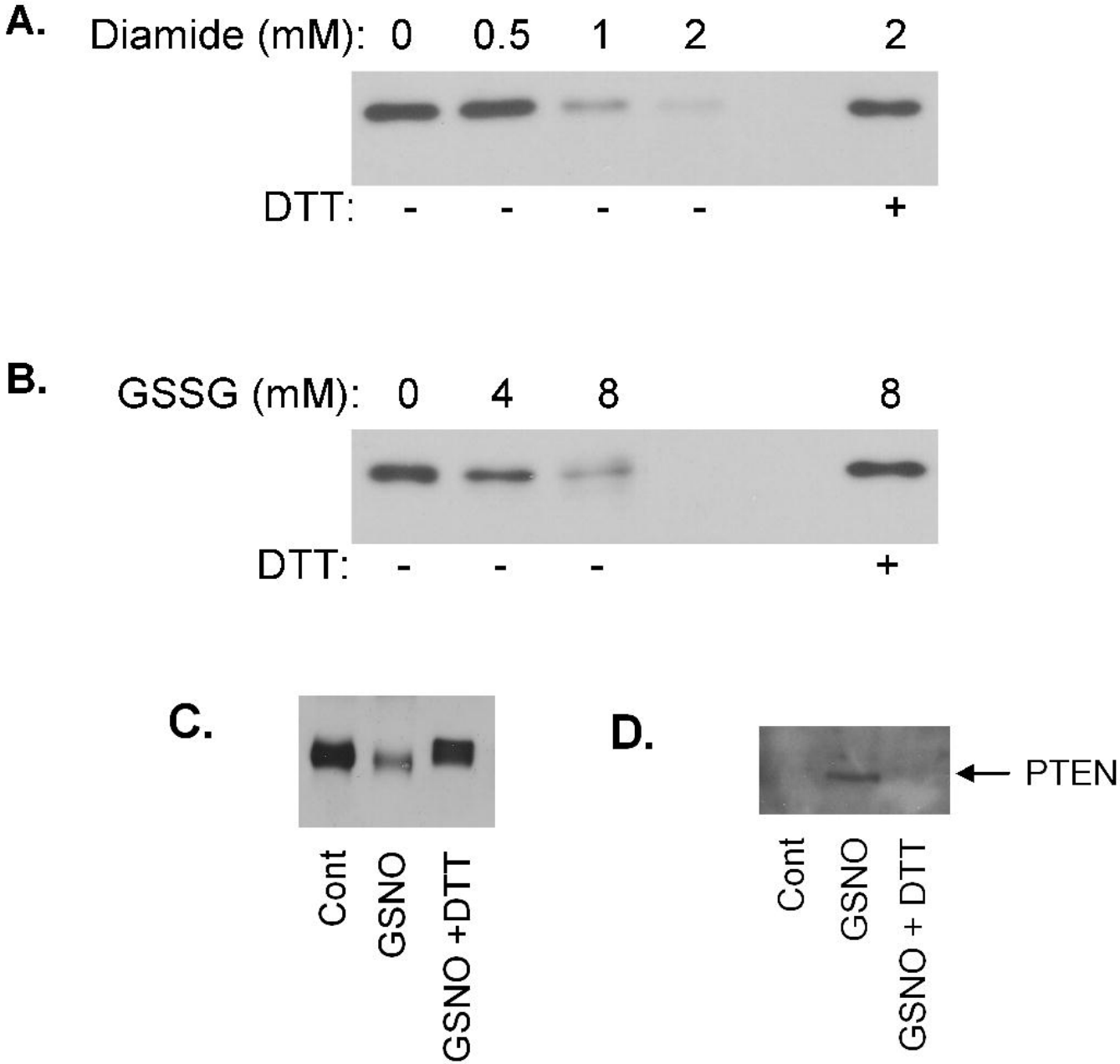

A.
H_2O_2: - + + + - - -
CSNO: - - - - + + +
DTT: - - + - - + -
Ascorbate: - - - + - - +
B.
CSNO: - + + + + + - - -
H_2O_2: - - - - - - + + +
Ascorbate (mM): - - 5 10 20 - - 20 -
DTT (mM): - - - - - 20 - - 20

Figure 7


A.
Diamide (mM): 0 0.5 1 2 2
DTT: - - - - +
B.
GSSG (mM): 0 4 8 8
DTT: - - - +
C.
Cont
GSNO
GSNO +DTT
D.
PTEN
Cont
GSNO
GSNO + DTT