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Endogenous *S*-sulfhydration of PTEN helps protect against modification by nitric oxide

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ABSTRACT

Hydrogen sulfide (H_2S) is a gaseous regulatory factor produced by several enzymes, and plays a pivotal role in processes such as proliferation or vasodilation. Recent reports demonstrated the physiological and pathophysiological functions of H_2S in neurons. PTEN is a target of nitric oxide (NO) or hydrogen peroxide, and the oxidative modification of cysteine (Cys) residue(s) attenuates its enzymatic activity. In the present study, we assessed the effect of H_2S on the direct modification of PTEN and the resulting downstream signaling. A modified biotin switch assay in SH-SY5Y human neuroblastoma cells revealed that PTEN is *S*-sulfhydrated endogenously. Subsequently, site-directed mutagenesis demonstrated that both Cys71 and Cys124 in PTEN are targets for *S*-sulfhydration. Further, the knockdown of cystathionine β-synthetase (CBS) using siRNA decreased this modification in a manner that was correlated to amount of H_2S. PTEN was more sensitive to NO under these conditions. These results suggest that the endogenous *S*-sulfhydration of PTEN via CBS/H_2S plays a role in preventing the *S*-nitrosylation that would inhibition its enzymatic activity under physiological conditions.

1. Introduction

Hydrogen sulfide (H_2S) is an endogenous gaseous mediator that produces gases such as nitric oxide (NO) and carbon monoxide (CO) [1]. H_2S is produced by several enzymes such as cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase of (3MST) [2]. In mammals, H_2S was detected in brain, liver, kidney, blood vessels, and pancreatic islets. It has a variety of physiological functions such as neurotransmission, vasodilation, insulin secretion, anti-inflammatory effects, and stimulating angiogenesis and cell proliferation [3–5]. H_2S also exerts protective effects in neurons after various neurotoxic chemical-induced brain injuries [6,7]. However, excess H_2S results in pathophysiological consequences such as Alzheimer's disease, hypertension, and type 1 diabetes.

H_2S can interact with sulfhydryl groups in the cysteine (Cys) residue(s) of selective proteins directly to generate a hydropersulfide moiety (-SSH) known as *S*-sulfhydration [8]. This post-translational modification leads to a conformational and subsequent altered protein function, which represents a novel and important redox signaling mechanism that regulates a variety of cellular functions. The development of a modified biotin-switch assay contributed to the identification of *S*-sulfhydryl proteins, such as PTP1B, NF-κB, Parkin, and MEK1 [9–12].

We demonstrated previously that PTEN is regulated by NO via *S*-nitrosylation [13]. This modification leads to the activation of Akt signaling by inhibiting PTEN. However, it remains unclear whether H_2S regulates PTEN via *S*-sulfhydration in neuronal cells. In the present study, we found that PTEN was *S*-sulfhydrated endogenously in the basal state. Interestingly, knocking down the H_2S-producing enzyme resulted in the *S*-nitrosylation of PTEN in a NOS inhibitor-sensitive manner. These results suggest that the *S*-sulfhydration of PTEN is a novel protective modification that prevents the inhibition of its enzymatic activity via *S*-nitrosylation in SH-SY5Y cells under conditions of oxidative stress.

2. Materials and methods

2.1. Materials

Biotin-HPDP was purchased from Pierce Chemical Co. (Rockford, IL, USA). N^G-Nitro-L-arginine methyl ester (L-NAME) and wortmannin were obtained from Dojindo (Kumamoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. All other reagents were from Sigma–Aldrich (St. Louis, MO, USA). Anti-PTEN,

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anti-Akt, anti-phospho-Akt (Ser473), and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-CBS antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Plasmids and cell culture

The entire coding sequence of human wild-type PTEN was amplified from a human cDNA library by polymerase chain reaction (PCR) [13]. To generate several mutants of PTEN, Cys was mutated to Ser using the Quikchange mutagenesis method (Stratagene) according to the manufacturer's directions.

SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.3. Knockdown of CBS using siRNA

The following siRNA sequences specific to human CBS were used: 5′-CCA UUG ACU UGC UGA ACU UTT-3′ and 5′-AAG UUC AGC AAG UCA AUG GTT-3′. MISSION siRNA Universal Negative Control for humans was used as the random siRNA. All siRNAs were designed and synthesized by Sigma-Aldrich Genosys Japan. SH-SY5Y cells were transfected with 100 pmol of siRNA using Lipofectamine 2000 (Invitrogen) in 60-mm dishes and then incubated for 48 h according to the manufacturer's protocol [14].

2.4. Quantification of H_2S

The amounts of H_2S in culture medium were measured using the methyl blue method described previously [15]. Briefly, 75 μl of culture medium were mixed with 425 μl of distilled water, 125 μl of 1% zinc acetate, 100 μl of 20 μM *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl, and 100 μl of 30 μM $FeCl_3$ in 1.2 M HCl, and then incubated for 10 min at room temperature. Next, 250 μl of 10% trichloroacetic acid were added, and the mixture was centrifuged at 15,000 rpm at 4 °C for 5 min. The absorbance of the supernatant at 670 nm was then measured. All samples were assayed in triplicate, and H_2S concentrations were calculated against a calibration curve of NaHS.

2.5. Detection of sulfhydrated-PTEN using a modified biotin switch assay

The *S*-sulfhydration assays were performed as described previously [8,10,11]. Briefly, cells were collected in HEN buffer (250 mM HEPES-NaOH pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine) and centrifuged at 15,000 rpm for 10 min at 4 °C. Cell lysates were then added to blocking buffer (HEN buffer containing 2.5% SDS and 16 mM methyl methanethiolsulfonate [MMTS]) at 50 °C for 20 min while vortexing. To remove free MMTS, the blocked proteins were precipitated with acetone at −20 °C for 30 min. After acetone removal, the proteins were resuspended in HENS buffer (HEN buffer containing 1% SDS), and 4 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP) in dimethyl sulfoxide was added. After a 3-h incubation at 25 °C, biotinylated proteins were precipitated using streptavidin-agarose beads and then eluted on SDS–PAGE gels. Finally, they were analyzed using western blotting with anti-PTEN antibodies.

2.6. Detection of nitrosylated-PTEN using biotin switch assays

PTEN *S*-nitrosylation was detected using biotin switch assays as described previously [13,16].

2.7. Western blotting

SH-SY5Y cells were washed twice with ice-cold PBS, and then lysed in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1% NP40, and the complete protease inhibitors). Total cell lysates were centrifuged at 15,000 rpm for 30 min at 4 °C. Supernatants was boiled in Laemmli buffer for 5 min, and proteins (20 μg) were separated by electrophoresis on 10% polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were incubated with anti-CBS (1:2000), anti-PTEN (1:2000), anti-phospho-Akt (1:10,000), anti-Akt (1:5000), and anti-FLAG (1:50,000) antibodies, followed by anti-horseradish peroxidase-linked secondary antibodies. The antibody-reactive bands were revealed using chemiluminescence. Blots from Western analyses were quantified by ImageJ software (Ver. 1.48), and the relative ratio was calculated.

2.8. Statistical analysis

All experiments were repeated independently at least 3 times. Results are presented as the means ± S.E. Statistical comparisons were performed using Student's *t*-tests or one-way analysis of variance post hoc Bonferroni's test with Graphpad Prism 5 (Graphpad Software, La Jolla, CA, USA). $p < 0.05$ was considered to be significant.

3. Results

3.1. Effects of reduced CBS expression on PTEN sulfhydration

We first investigated the effect of reducing the amount of H_2S produced by enzymes to elucidate its endogenous role. The expression of CBS and CSE was abundant and very low, respectively, in SH-SY5Y cells [17]. Knocking down of CBS using siRNA reduced H_2S accumulation significantly (Fig. 1A). We next assessed the formation of *S*-sulfhydrated PTEN under these conditions. A modified biotin switch assay revealed significantly reduced PTEN *S*-sulfhydration in the quiescent state, and that this modification was sensitive to CBS expression (Fig. 1B). To identify the target sites of *S*-sulfhydration on PTEN, we mutated each cysteine to serine and then measured PTEN *S*-sulfhydration in SH-SY5Y cells transfected with expression vectors encoding either wild-type FLAG-PTEN or mutant forms of the protein after 24 h. The C71S and C124S mutants produced almost no signal, suggesting that both Cys-71 and Cys-124 are *S*-sulfhydration sites (Fig. 1C).

3.2. Changes in the sensitivity to NO on PTEN by treatment with CBS siRNA

We demonstrated previously that PTEN is *S*-nitrosylated at Cys-83 in the presence of low concentrations of NO [13]. We hypothesized that preventing the *S*-sulfhydration of PTEN affects other oxidative modifications, such as *S*-nitrosylation and disulfide formation. Therefore, we next assessed the *S*-nitrosylation of PTEN in cells with or without CBS siRNA transfection. Interestingly, *S*-nitrosylated PTEN formation was detected in cells in which CBS had been knocked down, but not in control cells, in the absence of an NO donor (Fig. 2A). In addition, *S*-nitrosylation was increased significantly in CBS knockdown cells after challenge with very low concentrations of *S*-nitrosocysteine, a physiological NO donor, compared with random siRNA-transfected cells (Fig. 2A). To validate these observations, we next investigated the effect of L-NAME (an NOS inhibitor) on the formation of *S*-nitrosylated PTEN in CBS knockdown cells. *S*-nitrosylated PTEN formation almost disap-

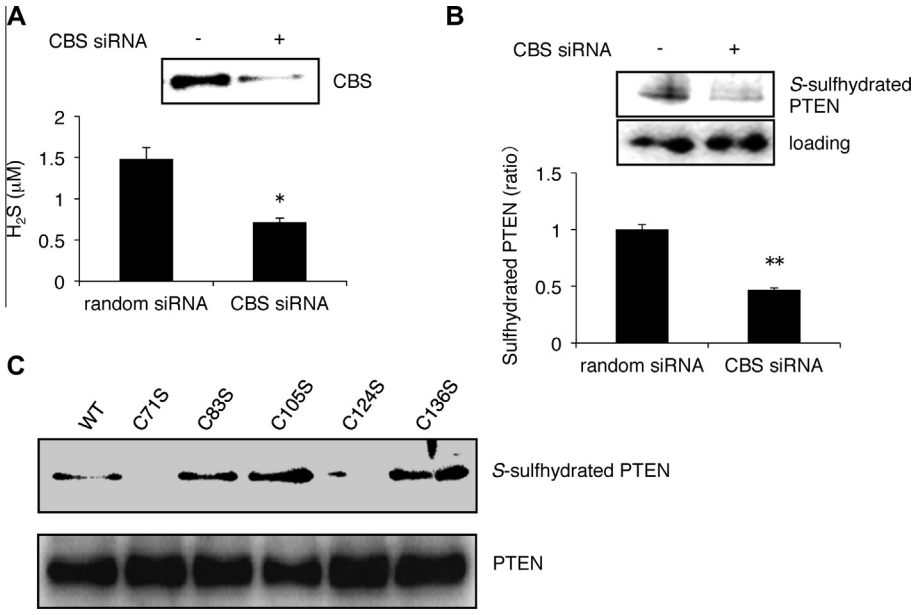

Fig. 1. *S*-sulfhydration of PTEN in SH-SY5Y cells. (A) Decrease in H_2S levels by treatment with or without *CBS* siRNA. H_2S production was measured using the methylene blue method. $^{*}p < 0.05$. (B) PTEN was partly *S*-sulfhydrated by intracellular H_2S produced by CBS. *S*-sulfhydrated PTEN was detected using the modified biotin switch method. $^{**}p < 0.01$. (C) The individual mutation of cysteine 71 (C71S) or cysteine 124 (C124S) to serine, but not other cysteine residues, diminished endogenous PTEN *S*-sulfhydration.

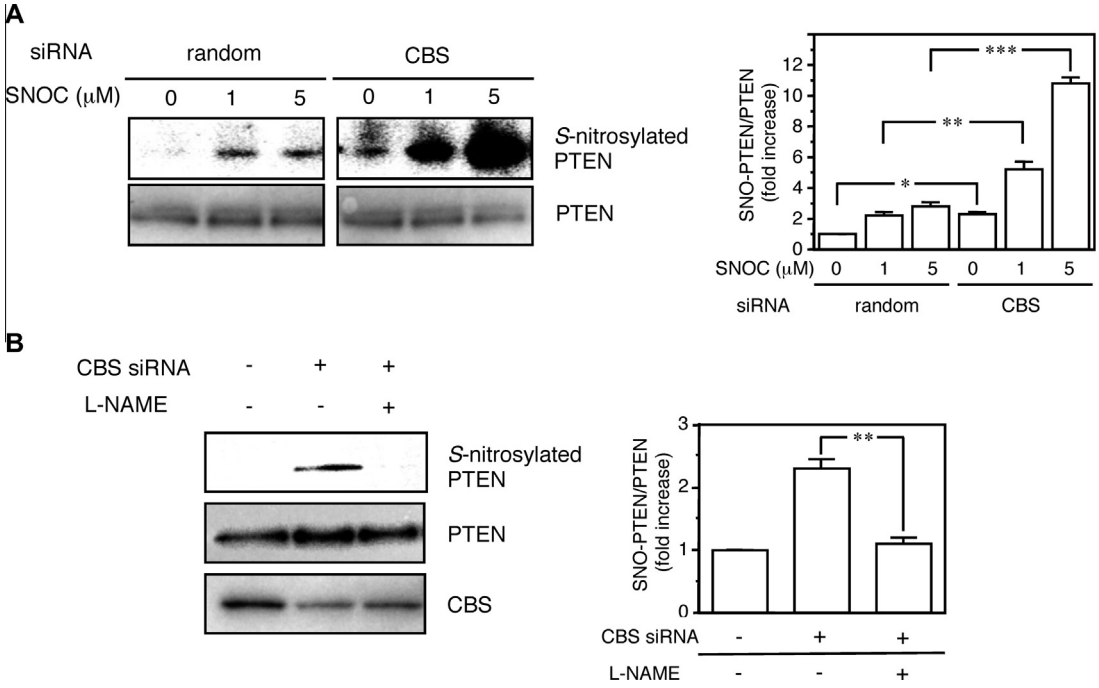

Fig. 2. Knockdown of CBS enhances the sensitivity of PTEN to NO. (A) Left panel: cells treated with control or *CBS* siRNA were treated with various concentrations of SNOC for 30 min, and PTEN *S*-nitrosylation was then assayed. Upper, *S*-nitrosylated PTEN; lower, total PTEN. Right panel: biotin-switch assay and Western analysis were quantified by ImageJ software; the relative ratio of SNO-PTEN to total PTEN was calculated for each sample. Values are means ± S.E., $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ between same concentrations of SNOC in random and CBS siRNA-treated cells. (B) Left panel: the endogenous *S*-nitrosylation induced by treatment with CBS siRNA was sensitive to a NOS inhibitor. Random (−) or CBS (+) siRNA-treated cells were incubated with 1 mM L-NAME for 16 h, and then assayed using the biotin switch method. Right panel: biotin-switch assay and Western analysis were quantified; the relative ratio of SNO-PTEN to total PTEN was calculated for each sample. Values are means ± S.E., $n = 3$. $^{**}p < 0.01$ by ANOVA.

peared after treatment with L-NAME, suggesting that endogenous NOS-derived NO plays a role in this modification (Fig. 2B).

3.3. Akt activation in CBS knockdown cells

Because treatment with *CBS* siRNA resulted in the formation of *S*-nitrosylated PTEN, we hypothesized that inhibiting PTEN activity by *S*-nitrosylation activated downstream signaling pathways such as Akt. Therefore, we next measured the levels of phosphorylated Akt (pAkt) in CBS knockdown cells. Fig. 3A demonstrates that pAkt levels were increased significantly in CBS, but not control, siRNA-transfected cells. The levels of Akt phosphorylation were sensitive to L-NAME and wortmannin, a PI 3-kinase inhibitor (Fig. 3B and C).

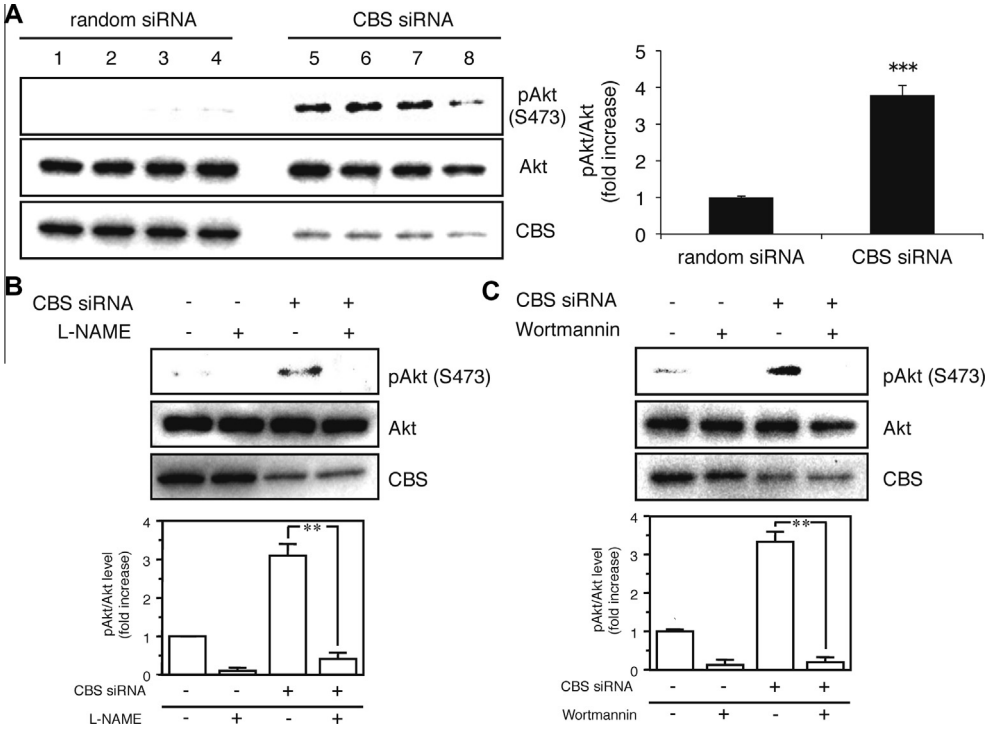

Fig. 3. CBS knockdown enhanced Akt activation in SH-SY5Y cells. (A) Cells were transfected with siRNA for 48 h, and were then further incubated for 24 h in DMEM supplemented with 1% FBS. Then, total lysates were analyzed by western blotting with anti-phospho Akt (pAkt), -Akt, and -CBS antibodies. $^{***}p < 0.001$. (B and C) The levels of pAkt induced by transfection with CBS siRNA were sensitive to NOS or PI3K inhibitors. Random or CBS siRNA transfected cells were incubated with 1 mM L-NAME (B) or 10 nM wortmannin for 24 h (C), and then total lysates were analyzed by western blotting. Intensity levels were quantified from blots by using ImageJ (1.48v) software. Values are means ± S.E., $n = 3$. $^{**}p < 0.01$ by ANOVA.

4. Discussion

The aim of this study was to elucidate the role of endogenous basal H_2S on PTEN activity and downstream signal transduction. PTEN is a phosphatase that regulates phosphatidylinositol triphosphate negatively, and some cysteine residues play key roles as catalytic or allosteric sites. We demonstrated previously that PTEN is *S*-nitrosylated by low concentrations of NO, which attenuates its enzymatic activity [13]. In contrast, treatment with high concentrations of hydrogen peroxide induced disulfide bond formation between Cys71 and 124 of PTEN [18]. This oxidized modification also inhibits its enzymatic activity. Because it is widely accepted that CBS and CSE are not activated by intracellular signaling or extracellular stimuli, unlike NOS, we hypothesized that endogenous H_2S might regulate PTEN activity via oxidative modification (*S*-sulfhydration). Therefore, we analyzed the effect of H_2S on PTEN activity.

A modified biotin switch assay revealed that PTEN is *S*-sulfhydrated in the quiescent state. Interestingly, NaHS did not affect this modification (data not shown). This suggests that endogenous levels of H_2S are sufficient for PTEN *S*-sulfhydration. In addition, knocking down CBS reduced the levels of sulfhydrated PTEN significantly. Surprisingly, this knockdown resulted in the *S*-nitrosylation of PTEN in an NOS-sensitive manner. These observations suggest that the very low levels of NO produced by NOS had a high affinity for PTEN in CBS-knockdown cells. Alternatively, *S*-sulfhydration might protect PTEN from *S*-nitrosylation. In support of this, Akt activation was detected in CBS-knockdown cells in an L-NAME-sensitive manner. Therefore, H_2S might protect against nitrosative stress in neuronal cells. Although *S*-sulfhydration causes conformational changes that alter protein function [12], it remains unclear why the de-sulfhydration of PTEN induces *S*-nitrosylation. Further structural experiments are required to answer this question.

In this study, we demonstrated that endogenous PTEN is modified by H_2S in SH-SY5Y cells, suggesting that nascent PTEN is a target for CBS-produced H_2S. Knocking down CBS reduced PTEN *S*-sulfhydration significantly and promoted Akt phosphorylation in a NOS inhibitor-dependent manner. Therefore, these results suggest that activation of the Akt pathway by a reduction in H_2S levels is dependent on endogenous NO. Alternatively, the reduced nascent state of PTEN in CBS knockdown cells might be more prone to nitrosative modification by basal NO and highly sensitive to exogenous NO. In addition, the concentration of H_2S in quiescent SH-SY5Y cells was ~1 μM, whereas NO was not detected by a Griess assay (data not shown). Because the concentration of H_2S is much higher than that of NO in basal SH-SY5Y cells, *S*-sulfhydration (but not *S*-nitrosylation) of PTEN was evident.

In conclusion, the current findings report for the first time that PTEN is modified by H_2S endogenously, and that preventing this modification by knocking down CBS renders PTEN sensitive to NO. Therefore, in the basal state H_2S functions as an endogenous regulator of PTEN in neuronal cells. Akt, a downstream kinase regulated by PTEN, has roles in several processes such as promoting cell proliferation and survival (anti-apoptosis), and inhibiting autophagy [19]. Because PTEN is a negative regulator of Akt signaling, the endogenous modification of PTEN by H_2S might maintain Akt activity.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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