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Cigarette smoke extract amplifies NADPH oxidase-dependent ROS production to inactivate PTEN by oxidation in BEAS-2B cells

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is widely recognized as a global public health problem and the third leading cause of mortality worldwide by 2020. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual-specificity protein and lipid phosphatase that plays an important role in COPD. However, the redox regulation of PTEN in the development of COPD was poorly studied. Our results showed that cigarette smoke extract (CSE) could oxidize PTEN in a time-dependent manner in BEAS-2B cells, whereas PTEN oxidation exposed to CSE was delayed compared to that of H_2O_2. Additionally, we found that ROS derived from DUOX1 and 2 of NADPH oxidases were mainly responsible for oxidative inactivation PTEN, also simultaneously led to Trx-1 inactivation by dimerization. Oxidative mechanism of PTEN exposed to CSE was mediated by forming a disulfide bond between $Cys^{71}$and Cys^{124}, similar to H_2O_2. Inactivation of PTEN resulted in the increased phosphorylation of Akt. In conclusion, CSE exposure could elevate the intracellular ROS mainly from DUOX1 and 2 to oxidize PTEN and Trx-1 resulting in Akt activation, eventually cause the occurrence of COPD, suggesting that PTEN is a potential target for new therapies in COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a devastating pulmonary disease with progressive increasing morbidity, mortality and high cost to health systems. COPD is the third leading cause of death worldwide by now. Exposure to toxic particles and gases, including cigarette smoke (CS), is considered as the major risk for COPD (Brandsma et al., 2020). Many efforts at understanding how CS causes the development of COPD have been done, yet the exactly pathogenic mechanisms remain unclear. Oxidative stress induced by CS has been recognized as the key mechanism that drives early development of COPD (Barnes, 2020). The airway epithelium is the first defensive barrier that prevents invasion by toxic particles, gases and pathogens. It maintains lung tissue homeostasis, and the first cellular interface that directly interacts with smoke and predisposes after inhalation of CS. So, CS-induced the epithelial dysfunction might be the major contributing factor and early event to the occurrence of COPD (Gao et al., 2015).

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) known as a key tumor suppressor, is a dual-specificity protein and lipid phosphatase. It belongs to the super-family of protein tyrosine phosphatase (PTP), and plays a crucial role in cellular processes including cell cycle progression, proliferation, survival, and energy metabolism (Luo et al., 2003; Maehama et al., 2001; Stambolic et al., 1998). PTEN preferentially dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP_3), a lipid second-messenger, into PIP_2, and as a result, it antagonizes phosphatidylinositol-3-kinase (PI_3K)-Akt signaling activities (Finkel, 1998; Rhee, 1999; Rhee et al., 2000; Thannickal and Fanburg, 2000). The catalytic activity of PTEN is modulated by protein–protein interactions, transcriptional regulations, posttranslational modifications including phosphorylation, ubiquitination, oxidation and carbonylation, as well as microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and antisense RNA (asRNA) (Worby and Dixon, 2014). Among them, PTEN is susceptible for oxidation-mediated inactivation at its active site cysteine residue in oxidative conditions, leading to a

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significant decrease in its catalytic activity. Reactive oxygen species (ROS) in form of H_2O_2 are overproduced endogenously under various stimuli. H_2O_2 oxidizes and inactivates PTEN by forming a disulfide bond between catalytic Cys^{124} and Cys^{71}, which could be reversibly reduced by various cellular antioxidant enzyme systems, such as the thioredoxin (Trx) and glutaredoxin (Grx) systems (Lee et al., 2002, 2015; Schwertassek et al., 2014a).

Thioredoxin (Trx) is an evolutionary conserved and ubiquitously expressed oxidoredeuctase in most living organisms, which plays an important role in maintaining the intracellular redox homeostasis (Han et al., 2017; Koharyova and Kollarova, 2015). The main function of Trx is to reduce disulfide bonds of proteins formed in response to oxidative stress, for example, Trx-1 is a key enzyme to reduce the oxidized PTEN (Zhang et al., 2020). Also, Trx-1 scavenges the excess of hydroxyl radicals and superoxide anion. Thus, Trx-1 is not only a radical scavenger but reactivates the oxidized enzymes by reducing the disulfide bonds, while Trx-1 itself becomes oxidized, which is generally reduced by thioredoxin reductase (TrxR) with electrons donated from reduced nicotinamide adenine dinucleotide phosphate (NADPH). However, the excessive ROS could also induce the dimerization of Trx-1 causing impairment of Trx system and imbalance of intracellular redox homeostasis (Han et al., 2017; Watson et al., 2003).

Recently, the constant increase in literature has emphasized the importance of PTEN in many other human diseases and disorders such as neurodegenerative disorders, diabetes mellitus, asthma, lung fibrosis and COPD (Boosani et al., 2019; Hosgood et al., 2009). In spite of several studies reporting that PTEN was a key risk factor in the development of COPD (Hoffmann et al., 2013; Hosgood et al., 2009; Yanagisawa et al., 2017). However, the redox regulation of PTEN in CS-induced COPD has been studied rarely, especially in epithelial cells. Therefore, we investigated the effect of CSE exposure on the redox status of PTEN using human bronchial epithelial BEAS-2B cells as a model.

2. Materials and methods

2.1. Materials and reagents

Hydrogen peroxide (H_2O_2), *N*-ethyl malebutenimide (NEM), diphenyliodonium chloride (DPI) apocynin, rotenone, *N*-acetylcysteine (NAC), 1,4-dithiothreitol (DTT) were purchased from Aladdin Reagent (Shanghai, China). RIPA lysis buffer kit, BCA protein assay kit, RPMI 1640 medium, phosphate buffered saline (PBS), 5 × non-reducing and reducing loading buffer were purchased from Solarbio Science & Technology Co., Ltd. (Beijing China). Fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trypsin-EDTA and fetal bovine serum (FBS) were purchased from InvitroGen (Carlsbad, CA, USA). PTEN, Trx-1, phosphorylated Akt, and GADPH antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-HA, β-actin antibodies and anti-rabbit IgG horseradish peroxidase-conjugated antibody were purchased from Proteintech (Wuhan, China).

2.2. Preparation of CSE

CSE was prepared according to a method previously reported with minor modification (Slebos et al., 2007). Briefly, one HongqiQu Filter cigarette (10 mg tar, 1.0 mg nicotine content, and 11 mg CO) from Henan Tobacco Industry Co., Ltd. (Zhengzhou, China) was smoked by a modified syringe-driven apparatus after cutting the filter. One hundred percent CSE was prepared by bubbling smoke from one cigarette into 5 mL of serum-free RPMI medium within 5 min and the optical density at 320 nm was measured and adjusted the absorbance to 2.0 with free medium. The resulting solution was sterile-filtered with a 0.22 μm filter as a stock solution (be as 100%) and diluted with cell culture medium for further experiment. CSE was always prepared fresh on the day of each experiment and used within 30 min. We prepared the control medium by bubbling air instead of smoke through 2 mL of serum-free RPMI 1640, and sterile filtering as described above.

2.3. Cell culture and treatment

Human bronchial epithelial BEAS-2B cells were cultured with RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37 °C. Cells were seeded into 35 mm cell culture dish in complete medium with 10% FBS to reach 80% confluence. After PBS rinsing for three times, cells were additionally incubated in serum-free RPMI 1640 for 2 h, followed by stimulation with various concentrations of H_2O_2 or CSE for the indicated experiments. The reactions were terminated with 5 mM NEM. NEM was used to prevent artificial redox reactions by blocking free thiol groups.

2.4. Cell viability assay

To measure the cytotoxic effects of CSE exposed in BEAS-2B cells, cell viability assay was performed using CCK-8 kit from Zeta life, (Menlo Park, CA, USA) according to the instruction. Briefly, 3.0×10^4 cells BEAS-2B cells were seeded in 96-well plate overnight. Cells were serum-starved over 2 h and exposed under various concentrations of CSE. The culture medium was removed, washed with pre-warmed PBS once, and replaced with 100 μL of serum-free medium containing CCK-8 solution for additional 2 h incubation at 37 °C, directly measured the absorbance at 450 nm. Three independent experiments in triplicates have been performed, and the obtained data was analyzed using Graph Pad Prism v6 software.

2.5. Measurement of redox status of PTEN and Trx-1

The redox status of PTEN and Trx-1 in response to CSE or H_2O_2 exposure were analyzed using non-reducing SDS-PAGE gel as reported previously (Han et al., 2015, 2017). In brief, cells were washed with ice-cold PBS twice and lysed with RAPI buffer containing protease inhibitor cocktail and 5 mM NEM. Protein concentrations were quantified by the BCA protein assay kit and denatured by non-reducing loading buffer, followed by immunobloting for PTEN aor Trx-1 antibody.

2.6. Transfection of PTEN cDNAs

To identify the oxidative site of sulfydryl groups of PTEN under CSE exposure, cells were transfectedwith cDNAs encoding *N*-terminal HA-tagged wild-type or mutant PTEN (C71S, C124S and C71S/C124S) using calcium phosphate precipitation method once reaching 70% confluence as previously reported (Lee et al., 2002). After 24 h transfection, cells were washed by PBS and stimulated with 2.5% CSE for 5 h after serum starvation, washed again with ice-cold PBS, followed by protein extraction and non-reducing SDS-PAGE gel analysis against HA antibody.

2.7. Measurement of intracellular ROS

To measure the effect of CSE exposure on the total ROS level, H_2DCF-DA fluorescent probe was utilized according as the manufacturer's instruction. Briefly, cells were grown in 35 mm cell culture dish overnight and serum-starved with RPMI1640 medium supplemented with 0.1% FBS for an additional 2 h. Cells were pretreated with various inhibitors for 1 h prior to CSE stimulation. After CSE exposure for 5 h, the medium containing CSE and chemicals was discarded, washed with PBS and digested by the diluted Trypsin-EDTA for 1 min. Cells were resuspended in 1 mL PBS containing fluorescent probe H_2DCF-DA (10 μM) and incubated at 37 °C for 20 min, followed by dilution with ice-cold PBS (5 mL). Cells were washed with ice-cold PBS twice, suspended in 1 mL PBS again and measured the fluorescent intensity at 488 nm on a FACS CantoTM II (BD Biosciences, San Jose, CA, USA), the obtained results

were analyzed with the FlowJo7.6.1 software (TreeStar, USA).

2.8. Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using RNAiso plus reagent (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. 1 μg of total RNA was utilized for first-strand complementary DNA synthesis by HiScript qRT Supermix II (Vazyme, Nanjing, China). The cDNA was diluted and subjected to quantitative PCR (Applied Biosystems, CA, USA) amplification with SYBR Green master mix (Vazyme, Nanjing, China). The specific primers involved in this study were present in Table 1 (Chang et al., 2017; Gaurav et al., 2015). GAPDH was used as a house-keeping gene. The samples in PCR tubes were reacted at 95 °C for 30 s, cycled at 95 °C for 10 s, 60 °C for 30s, and extension at 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s.

2.9. Statistics analysis

All data were analyzed by a Student's *t*-test using Graph Pad Prism v6 software and all the experiments were repeated three times or more. Data were presented as mean ± SD. $*p < 0.05$ verse control was considered statistically significant.

3. Results

3.1. CSE induced PTEN oxidation in BEAS-2B cells

To investigate the effects of CSE exposure on PTEN redox regulation, we first studied the effect of various concentrations of CSE on cell viability using CCK-8 assay. It showed that there was noticeable increase in cell viability under 1.25–5% CSE exposure, whereas significant decrease was observed when CSE concentration was higher than 10% (data not shown). Considering that chronic low dose exposure of CS in human probably induces the development of COPD, 2.5% CSE was chosen in the following study. Cells were rinsed with PBS, serum-starved for 2 h, and then exposed to 2.5% CSE for increasing periods of time (0, 0.5, 1, 2, 4, 8, 12 and 24 h). The redox status of PTEN was tested in non-reducing SDS-PAGE gel. Surprisingly, we found that 2.5% CSE exposure caused PTEN oxidation in a time-dependent manner. PTEN started to be oxidized at 1 h and its oxidation extended to 24 h (Fig. 1A). Next, we investigated the oxidation of PTEN in a series of concentrations of CSE for 5 h, and it was found that PTEN oxidation wasn't in a dose-dependent fashion (Fig. 1B).

We further investigated H_2O_2-induecd PTEN oxidation in BEAS-2B cells. Interestingly, it showed that oxidation of PTEN in H_2O_2 treatment had distinct difference from CSE exposure. First, PTEN oxidation occurred within 30 minute and the oxidized PTEN could be almost reduced after 2 h compared to that of CSE exposure. Second, H_2O_2 -induced PTEN oxidization was in a dose- and time-dependent manner (Fig. 1C and D). Interestingly, the total PTEN expression (reducing condition) after CSE exposure has no significant differences compared with untreated group (Fig. 1A–D).

Table 1
Primers used in this study.

Gene	Primer sequence	
	Forward	Reverse
NOX1	TGCGCTGGACAAATGTTCCATTCC	TCAGGAAGGCATCCACAAACAGGA
NOX2	AGACTTTGTATGGACGGCCCAACT	AAGGCTTCAGGTCCACAGAGGAAA
NOX3	ACCGTGGAGGAGGCAATTAGACAA	TTCCAGGTTGAAGAAATGCGCCAC
NOX4	AGCAGAGCCTCAGCATCTGTTCTT	TGGTTCTCCTGCTTGGAACCTTCT
NOX5	TGCTGCTCCTCCTCATGTTCATCT	TCCAGAAGTTGGGCCCATGAAAGA
DUOX1	AACAATTTGTGCGGCTACGGGATG	TCCTGCAGGGTGGTATTTCGGATT
DUOX2	AGTACAAGCGCTTCGTGGAGAACT	TCTGCAAACACGCCAACACAGATG
GAPDH	TCGACAGTCAGCCGCATCTTCTTT	ACCAAATCCGTTGACTCCGACCTT

3.2. CSE stimulated overproduction of intracellular ROS resulting in PTEN oxidation

We presumed that the observed differences on PTEN oxidation was probably due to the gradual accumulation of intracellular ROS in CSE-stimulated BEAS-2B cells, instead of H_2O_2 in CSE solution. To substantiate this guess, we first measured the contents of H_2O_2 in the CSE solution and our results showed that H_2O_2 in the fresh prepared CSE solution was undetectable (data not shown). Moreover, we tested the effect of CSE on the intracellular ROS production in CSE-induced BEAS-2B cells by a fluorescent probe H_2DCFDA. Cells were exposed in various concentrations of CSE for 5 h, and then incubated with 10 μM fluorescent probe H_2DCFDA for 20 min. As shown in Fig. 2, ROS were evaluated after CSE exposure for 5 h compared to untreated group, but not in a dose-dependently manner.

3.3. NADPH oxidases are the major source of ROS production in CSE stimulated BEAS-2B cells

The increased production of ROS is probably responsible for PTEN oxidation, and it is well-known that mitochondria are the major source of ROS production. To examine the source of ROS for PTEN oxidation, we first pre-treated cells by various inhibitors including a mitochondrial complex I inhibitor rotenone, a mitochondrial complex III inhibitor antimycin A (Anti-A). As shown in Fig. 3A, PTEN oxidation was not affected by pretreatment of rotenone and Anti-A, indicating that mitochondrial ROS was not the primary source for PTEN oxidation. We next investigated the effect of NADPH oxidase inhibitors including apocynin (Apo) and diphenyleneiodonium chloride (DPI) on PTEN oxidation in CSE-induced BEAS-2B cells. As shown in Fig. 3B, we found that pre-treatment with 5 μM DPI could very significantly reduce the oxidized PTEN, almost close to the untreated group, suggesting that NADPH oxidase-derived ROS are the key triggers to induce PTEN oxidation. But, pre-treatment with Apo was unable to suppress the oxidation of PTEN. In addition, we investigated the effects of antioxidants ascorbate, D-dithiothreitol (DTT) and *N*-acetyl-L-cysteine (NAC) on PTEN oxidation. Ascorbate and DTT could reduce the oxidized PTEN, but NAC failed to inhibit PTEN oxidation (Fig. 3C).

3.4. CSE induced PTEN oxidation by formation of a disulfide bond between Cys^{71} and Cys^{124} in BEAS-2B cells

It is well established that the posttranslational modification of PTEN upon H_2O_2 exposure is prone to form an intramolecular disulfide bond between Cys^{71} and Cys^{124} residues. To examine whether an identical mechanism exists for PTEN oxidation upon CSE exposure, cells were transfected with the cDNAs of PTEN wild-type and mutants for cysteines at C71S, C124S, and C71S/C124S. Cells were treated with 2.5% CSE, followed by analyzing the extracted protein lysates on non-reducing SDS-PAGE gel with HA antibody. As depicted in Fig. 4, there were two bands (oxidized and reduced PTEN) for wild-type group, whereas the C71S, C124S and C71S/C124S only presented one reduced band. This result is the same as the previous studies that H_2O_2 oxidized PTEN by forming a disulfide bond between Cys^{71} and Cys^{124} (Han et al., 2015, 2017; Lee et al., 2002; Zhang et al., 2017). Our result suggested that CSE-induced ROS and H_2O_2 shared same mechanism on the redox regulation of PTEN by forming a disulfide bond.

3.5. CSE modulates the mRNA expression level of DUOX-1 and 2 in BEAS-2B cells

Given that NADPH oxidase (NOX) family was identified as the major source of ROS implicated in PTEN oxidation in CSE-induced BEAS-2B cells, and NOX consists in seven enzyme isoforms including NOX1-5, Dual oxidase 1 and 2 (DUOX1, DUOX2) in this family. So, the specific NOXs to generate ROS under CSE exposure were further measured by

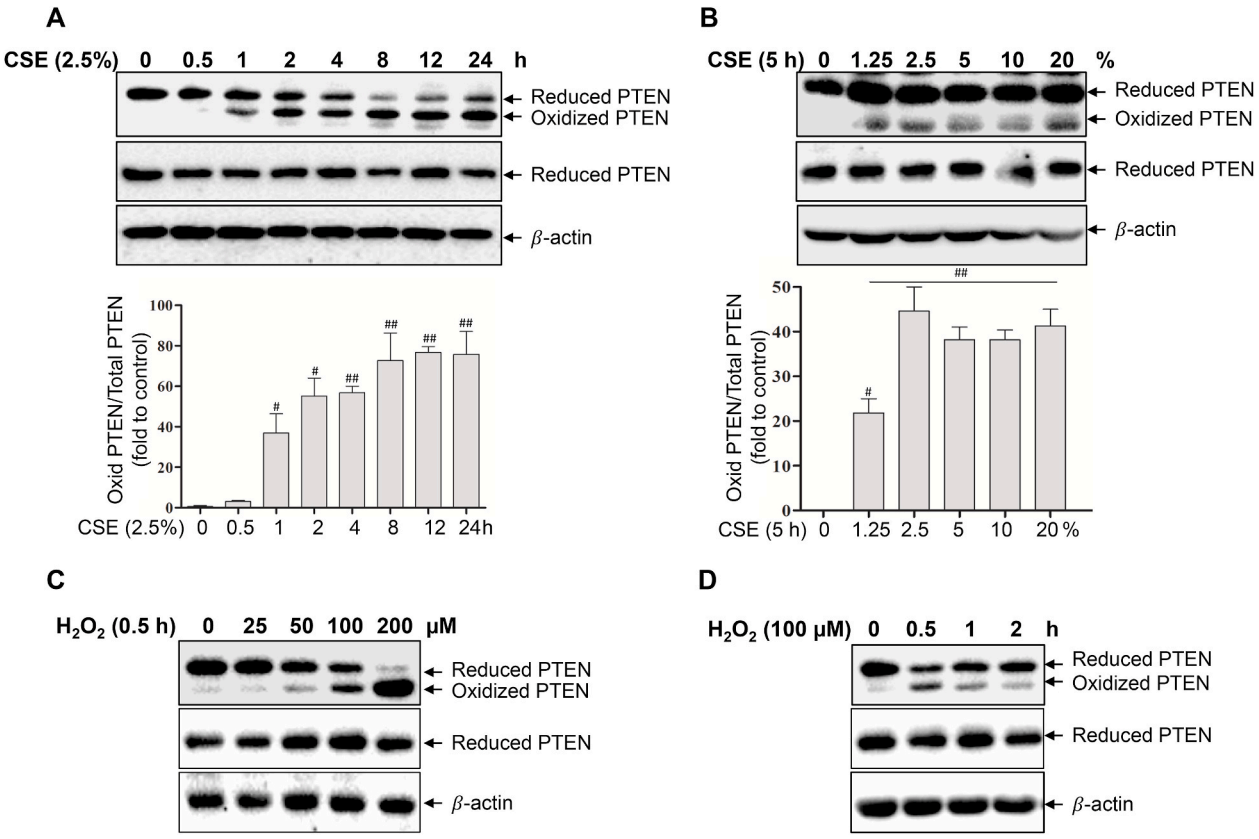

Fig. 1. Effects of CSE exposure on redox status of PTEN in BEAS-2B cells. Cells were challenged by a series of concentrations of CSE and H_2O_2 for the indicated times. Protein was extracted by lysis buffer containing 5 mM NEM to block free thiol groups, and analyzed by non-reducing SDS-PAGE gel for PTEN. For the reduced PTEN, sample was denatured by reducing loading buffer and analyzed by SDS-PAGE gel. (A) Effect of 2.5% CSE exposure on the PTEN oxidation in BEAS-2B cells for the indicated times. (B) Effect of various concentrations of CSE exposure on the PTEN oxidation in BEAS-2B cells for 5 h. (C) Treatment of H_2O_2 in the concentration range from 0 to 200 μM for 30 min. (D) 100 μM H_2O_2 treatment for the indicated times. Data are representative of three independent experiments and the intensity of oxidized PTEN was determined using Image J software. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs 0 time point.

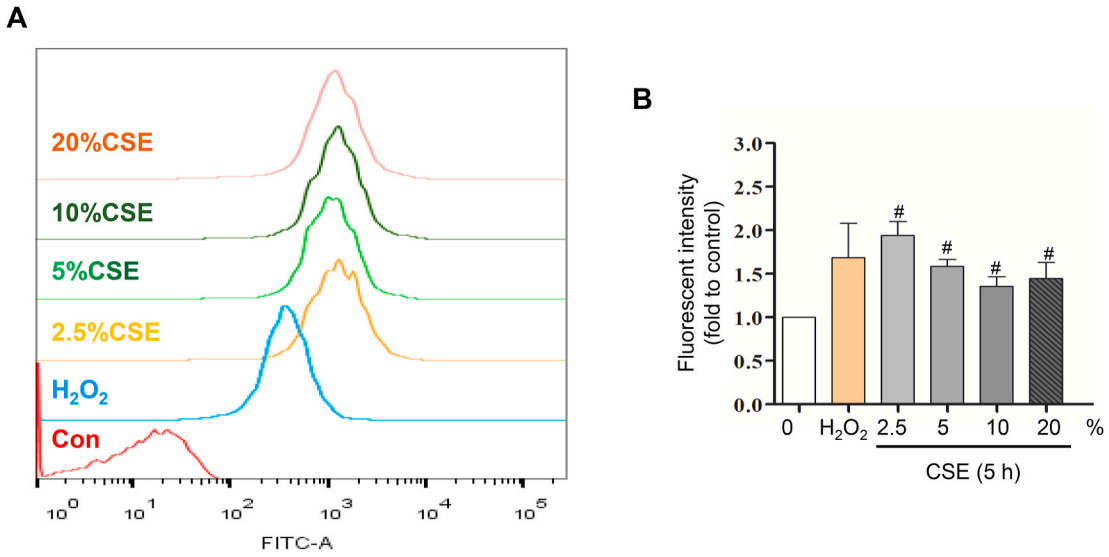

Fig. 2. Effects of CSE exposure on the ROS production in BEAS-2B cells. The cells were treated with 0–20% CSE for 5 h or 100 μM H_2O_2 for 30 min. Cells were washed, trypsinized, centrifuged, incubated with 10 μM H_2DCFDA fluorescent probe for 20 min and analyzed by FACS. Data are representative of three independent experiments. $^{*}P < 0.05$ vs vehicle.

qPCR. As shown in Fig. 5, the mRNA levels of NOX1, 2, 3 and 5 had no significant difference compared to the untreated cells in CSE-exposed BEAS-2B cells at 5, 12 and 24 h, but NOX4 was not detectable. We found that DUOX1 was significantly augmented in CSE-exposed BEAS-2B cells at all tested time points, an obvious increase in mRNA expression of DUOX2 was also observed for 12 and 24 h exposure of 2.5% CSE.

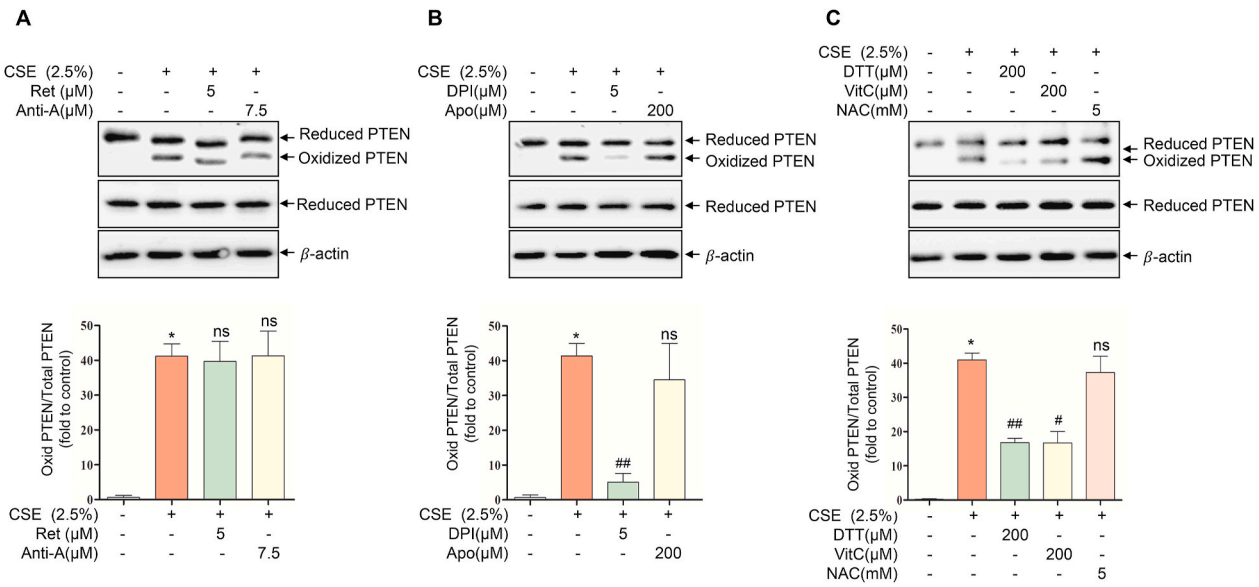

Fig. 3. Major sources of ROS responsible for PTEN oxidation in CSE-exposed BEAS-2B cells. (A) Effects of mitochondrial respiratory complex inhibitors on PTEN oxidation. (B) Effects of NADPH oxidase inhibitors on PTEN oxidation. (C) Pretreatment of antioxidants prevented PTEN oxidation. Cellular protein extracts were alkylated with 5 mM NEM, electrophoresed by either non-reducing or reducing SDS-PAGE gel, and then analyzed with antibodies of PTEN or β-actin. Data are representative of three independent experiments and the intensity of oxidized PTEN was determined using Image J software. $^{*}P < 0.01$ vs vehicle, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs CSE. "ns" means no significant difference compared to CSE exposure only.

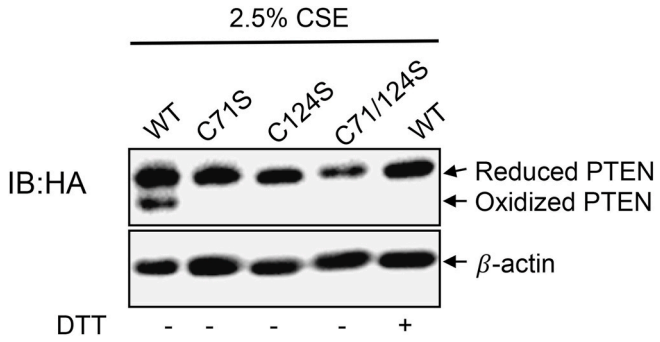

Fig. 4. CSE-induced PTEN oxidation was mediated by forming a disulfide bond between Cys^{71} and Cys^{124}. Cells were transfected with cDNAs encoding *N*-terminal HA-tagged wild-type or mutant PTEN (C71S, C124S and C71S/C124S) using calcium phosphate precipitation, followed by CSE exposure for 5 h. Cellular protein extracts were alkylated with 5 mM NEM and electrophoresed by either non-reducing or reducing SDS-PAGE gel, and then analyzed with HA or β-actin antibody.

3.6. Trx-1 was involved in the redox regulation of PTEN in CSE-exposed BEAS-2B cells

Previous studies reported that H_2O_2-induced PTEN oxidization was reversibly reduced by cellular reducing systems, preferentially by Trx system (Schwertassek et al., 2014b). To examine whether Trx-1 was involved in the redox regulation of PTEN induced by CSE, BEAS-2B cells were challenged by 2.5% CSE for the indicated time points, and the redox status of Trx-1 was determined by reducing and non-reducing SDS-PAGE gel. As showed in Fig. 6A, Trx-1dimers appeared after 1 h exposure of 2.5% CSE and gradually increased along with times. Besides Trx-1 dimers and its oligomers were also observed in CSE-exposed BEAS-2B cells, whereas the dimeric and oligomeric Trx-1 disappeared in the reducing condition (Fig. 6B). It was reported that Trx is active as a monomer in redox reactions (Weichsel et al., 1996), indicating that CSE exposure led to Trx-1 inactivation and impairment of Trx-1 reducing system because of the formation of the dimeric and oligomeric Trx-1. It could explain why the oxidation of PTEN was increased along with time and the oxidized PTEN failed to be reduced.

3.7. Inactivation of PTEN elevated the phosphorylation of Akt in CSE-exposed BEAS-2B cells

CSE exposure led to oxidation of PTEN and impairment of Trx-1, the oxidized PTEN has reduced catalytic activity to antagonize PI_3K-Akt signaling pathway. We further examined the phosphorylation level of Akt in CSE-exposed BEAS-2B cells. As shown in Fig. 7, CSE exposure resulted in a significant increase in the phosphorylated Akt compared to the untreated group, which was abolished by pretreatment of DPI.

Based on the results above, it could be concluded that CSE exposure induced augmentation of ROS mainly from DUOX1 and 2 of NADPH oxidases, the increased ROS led to PTEN oxidative inactivation and simultaneous inhibition of Trx-1 activity mainly by dimerization. Consequently, the inactivation of PTEN failed to antagonize the activation of Akt (Fig. 8).

4. Discussion

Cigarette smoking is recognized as a primary cause in the development and progression of COPD, and the main mechanisms involve in the onset of the amplified local inflammation and oxidative stress in epithelium triggered by CS, which might subsequently cause global destruction of lung tissue and a progressive decline in lung function, consequently induce occurrence of various lung diseases including COPD and fibrosis (van der Toorn et al., 2007). Although many efforts on the identification of various cellular factors and the major cellular pathways associated with CS-induced COPD have been done. However, the decisive trigger or precise target molecules in the development of CS-induced COPD remains an open question. So, it is necessary to find out the potential targets for drug development or treatment methods to combat CS-induced lung diseases.

PTEN known as a tumor suppressor gene plays an important role in the regulation of various cellular processes in variety of other diseases including COPD and lung fibrosis. Several studies focused on the expression level of PTEN and the response of PI_3K, its major downstream

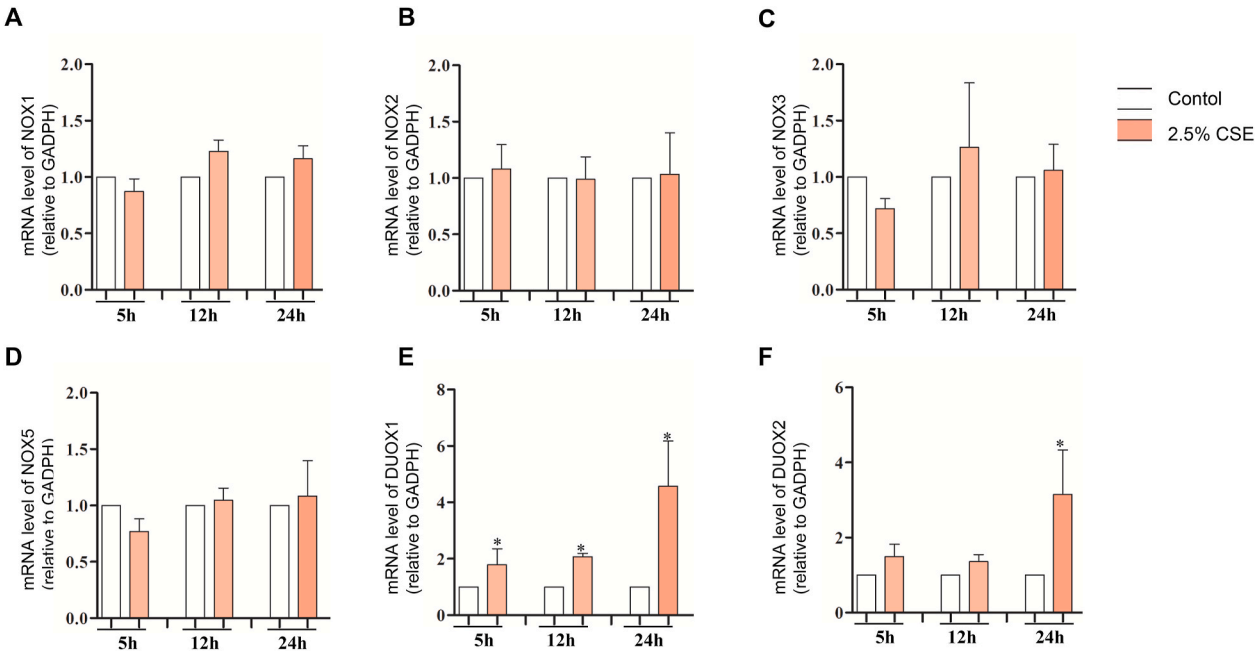

Fig. 5. Effects of 2.5% CSE on the mRNA expression of NADPH oxidase in BEAS-2B cells. Total RNA was extracted after CSE exposure at the indicated time and subjected to cDNA synthesis and qPCR analysis with various primers. Data are representative of at least three independent experiments. *$P < 0.01$ vs vehicle.

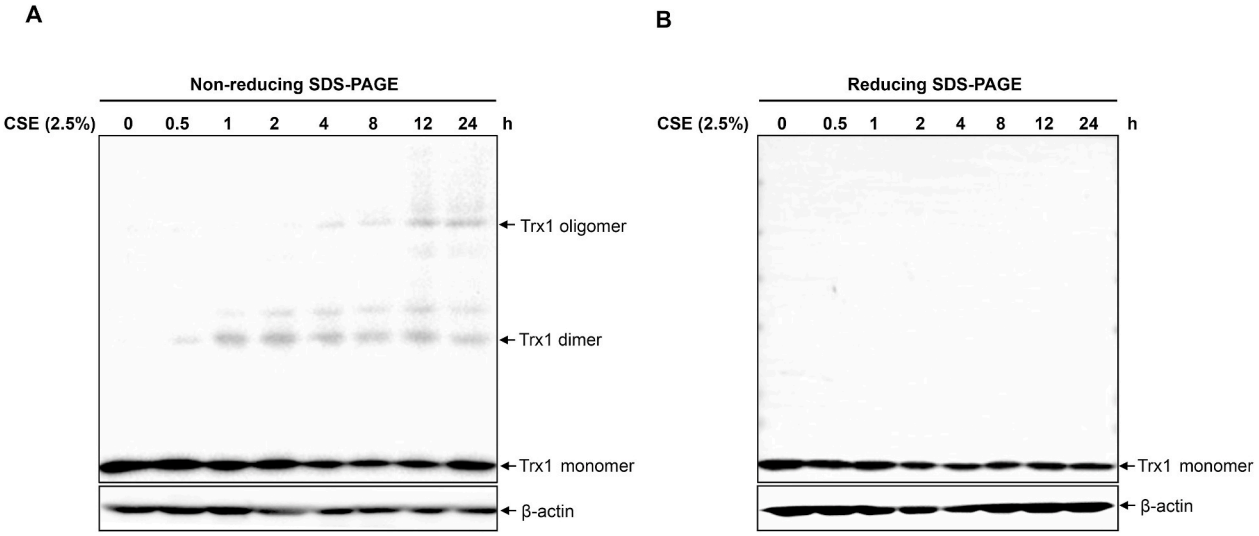

Fig. 6. Effects of CSE on the redox status of Trx-1 in BEAS-2B cells. Cells were challenged by 2.5% CSE for the indicated times. Cellular protein was extracted with lysis buffer containing 5 mM NEM and analyzed by reducing or non-reducing SDS-PAGE gel against Trx-1or β-actin antibody.

signaling pathway in COPD patients as well as CSE-induced epithelium (Yanagisawa et al., 2017). It is well-known that PTEN is vulnerable to oxidation under oxidative stress, resulting in completely blocking its catalytic activity. However, little is to know regarding PTEN oxidative modification and the sources of such mediators in the presence of CS. In present study, we would like to provide evidence that the redox regulation of PTEN and the involvement of Trx-1 in CSE-induced BEAS-2B cells.

Given that chronic low-dose exposure of CS resulted in COPD, and CS directly interacts with lung epithelia during smoking, it is more rational to use epithelial cells to investigate the effect of CS on redox regulation of PTEN. Thus, we examined the effect of 2.5% CSE exposure on the redox status of PTEN in bronchial human epithelial BEAS-2B cells. Results showed that 2.5% CSE could induce oxidation of PTEN in a time-dependent manner, but independent of dose. Interestingly, PTEN oxidation in the presence of CSE was obviously delayed compared to that of H_2O_2 treatment. Previous studies reported that the extract of CS contains stable oxidants that could directly oxidize proteins such as BSA, and a substantial protein oxidation was found in the mixture of CSE and pure proteins or proteins in microsomal suspension after 2 h incubation, which was similar to our results (Panda et al., 2001). However, the tested proteins were directly exposed to CSE in their study, which is a little different from our study because PTEN is an intracellular protein. So, we suspected that the oxidants in CSE have low reactivity to directly react with proteins or poor permeability in the cell membrane, for instance lipid peroxides failed to oxidize PTEN, whereas its oxidation occurs after 5 min if treated with Lipofectamine loaded lipid peroxides, the poor cell membrane permeability of lipid peroxides results from the negative charge endowed by the carboxylate group and the phospholipid-like structure (Zhang et al., 2019). Additionally, the

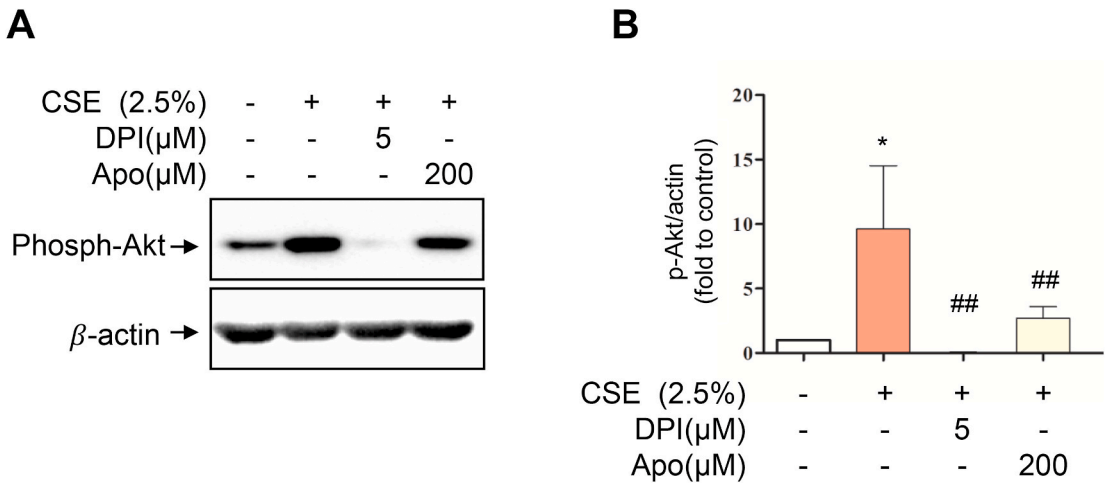

Fig. 7. Effect of CSE on the phosphorylation of Akt in BEAS-2B cells. Cells were pretreated by DPI or apocynin before 2.5% CSE exposure. Cellular protein was extracted with lysis buffer containing phosphostase inhibitor and analyzed with SDS-PAGE gel, against phosphorylated-Akt antibody. Data are representative of at least three independent experiments. $^{*}P < 0.01$ vs vehicle, $^{\#\#}P < 0.01$ vs CSE only.

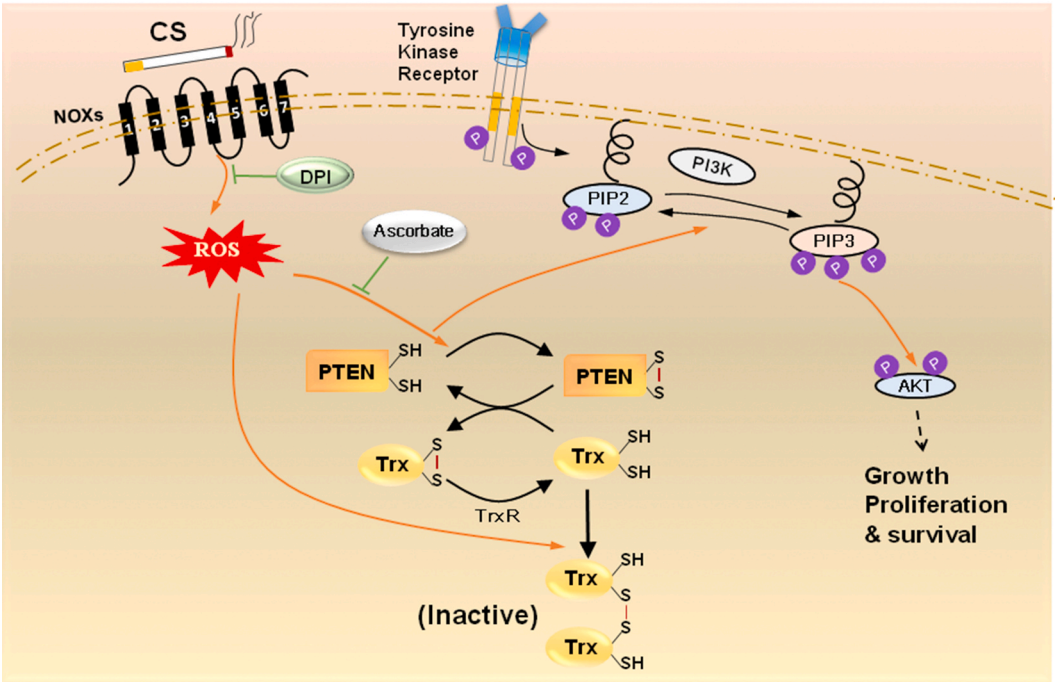

Fig. 8. Proposed mechanism of CSE exposure on the redox regulation of PTEN by the Trx-1 system. H_2O_2 derived from DUOX1 and 2 of NADPH oxidases are the major sources leading to PTEN oxidation. An excess of ROS also led to Trx-1 inactivation by dimerization, resulting in delayed reduction of PTEN and activation of Akt signaling, which was abolished by pretreatment of DPI. TrxR, thioredoxin reductase; Trx-1, thioredoxin-1.

oxidative modification of PTEN might be attributed to the augmentation of endogenous ROS stimulated by CSE exposure. We further determined the intracellular production of ROS by a fluorescent probe H_2DCFDA, consistent with previous studies (Fan et al., 2020), CSE exposure indeed enhanced the intracellular ROS, but a relative decrease in ROS production was observed in the treatment of 10% CSE, which was largely attributed to the decline in cell viability, cell damage or reduced enzyme activities after CSE exposure for 5 h. Moreover, it was reported that CS could irreversibly reacts with glutathione (GSH) causing GSH depletion, GSH plays an important role in the cellular redox balance and the depletion of GSH increases the oxidative stress and endogenous ROS production (van der Toorn et al., 2007).

Since augmentation of the intracellular ROS in CSE exposure at best partially contributes to the PTEN oxidation, we would like to figure out what are the major sources of ROS leading to PTEN oxidation. First, we found that the extent of PTEN oxidation was not affected in the pretreatment of Anti-A and rotenone, suggesting that mitochondria was not the main source of ROS for PTEN oxidation after CSE exposure. Numerous studies also showed that CS exposure induces activation of NADPH oxidases leading to the overproduction of ROS (Ng et al., 2014), we also studied whether NADPH oxidases are the major sources of ROS production to induce PTEN oxidation. Surprisingly, we found that pretreatment of DPI abolished the extent of PTEN oxidation induced by CSE, and the observed results may result from the significant inhibition of intracellular ROS production. It has been reported that DPI is a suitable reference compound for NOX inhibition *in vitro* and it could dose-dependently inhibit all isoforms of NOXs (Augsburger et al., 2019). In spite of numerous studies reporting that apocynin is frequently used

as an NOX2 inhibitor to decelerate the production of superoxide anion (Hougee et al., 2006), several studies also showed that apocynin is inactive for NOX2 or any other NOX isoform, even at high concentration (Augsburger et al., 2019). Our results showed that pretreatment of apocynin failed to inhibit PTEN oxidation in CSE-exposed BEAS-2B cells. Since NADPH oxidases consist of seven isoforms including NOX1-5, DUOX1 and 2, and we further tested their mRNA expression in CSE-exposed BEAS-2B cells. Results exhibited that DUOX1 and 2 were significantly increased, which were consistent with previous results (Nagai et al., 2008; Tian et al., 2017). DUOX1 and 2 were involved in the H_2O_2 production after activation (Augsburger et al., 2019), indicating that the oxidation of PTEN was probably mediated by CSE-induced H_2O_2. Oxidation of PTEN could be prevented by antioxidants including ascorbate and DTT (Kwon et al., 2004; Lim and Clément, 2007), similar results were obtained in our study as well. NAC is a common free radical scavenger, but it was unable to reduce the oxidized PTEN. It is probably due to the insufficient generation of GSH from NAC under oxidative condition. It has been previously reported that PTEN is readily oxidized by H_2O_2 via forming a disulfide bond at the active Cys^{124} residue and Cys^{71} (Lee et al., 2002). We confirmed that oxidation of PTEN upon CSE exposure also followed the same mechanism as H_2O_2. It is possible that oxidative inactivation of PTEN induced by CSE exposure could functionally curtail its catalytic enzyme activity leading to the aberrant cell events due to increased Akt kinase activity by phosphorylation (Kwon et al., 2004; Lee et al., 2002). In our study, we found that CSE exposure significantly elevated the phosphorylation of Akt, which was completely abolished by pretreatment of DPI and it was probably due to the inhibition of DUOX1 and DUOX2-mediated ROS production. Interestingly, apocynin also reduced the phosphorylation level of Akt, and this was contradictory to the result that apocynin failed to inhibit PTEN oxidation for CSE exposure in Fig. 3B. We speculated that the observed difference was probably due to apocynin inhibited the phosphorylation of Akt by multiple ways.

It has been reported that the oxidized PTEN can be reversibly reduced by intracellular reducing systems, preferentially by Trx-1 system (Kim et al., 2011). Trx system consists of Trx, NADPH, and TrxR, which plays a major role in redox homeostasis in cells by catalytic conversion of protein disulfide to dithiol. Trx-1 is generally oxidized to dimers in the excess of ROS (Watson et al., 2003), and its oligomers were also observed in the long-term treatment of organic peroxides and hydroperoxides. The oxidized Trx-1 resulted in the impairment of Trx system and inability to reduce the oxidized PTEN (Han et al., 2017; Zhang et al., 2017). In our study, Trx-1 dimers were obviously appeared upon CSE exposure at 1 h in BEAS-2B cells, consistent with PTEN oxidation.

In the present study, we showed for the first time that CSE could induce oxidative inactivation of PTEN in BEAS-2B cells. ROS derived from DUOX1 and 2 of NADPH oxidases are the major trigger to this event. Trx-1 was involved in CSE-mediated redox regulation of PTEN.

CRediT authorship contribution statement

Bangrong Cai: Conceptualization, Methodology, Investigation, Writing - original draft. **Mengya Liu:** Methodology, Investigation, Writing - review & editing. **Dujuan Xu:** Methodology, Investigation, Writing - review & editing, Formal analysis, Data curation. **Jiansheng Li:** Writing - review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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