PRMT6 mediates CSE induced inflammation and apoptosis

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Abstract

Cigarette smoke extract (CSE) induces apoptosis and inflammation, but the mechanism is unknown. Arginine methyltransferase (PRMT6) catalyzes the asymmetric di-methylation of histone H3 arginine 2 (H3R2me2a) to control global level transcription. We hypothesized that PRMT6 mediates CSE induced apoptosis and inflammation through H3R2me2a. The apoptosis after CSE treatment in human umbilical vein endothelial cells (HUVECs) was fully measured with real-time reverse transcription PCR, western blotting and Annexin-V staining. Meanwhile, the inflammation in HUVECs after CSE exposure was detected with real-time reverse transcription PCR, western blotting and ELISA. CSE treatment promoted apoptosis and inflammation in HUVECs, coinciding with the decreased protein abundance of PRMT6. Meanwhile, HUVECs transfected with PRMT6 expressing plasmid inhibited the CSE-induced apoptosis and inflammation. Also, the inhibition of PRMT6 promoted the apoptosis and inflammation in HUVECs induced by CSE. Notably, H3R2me2a was associated with the modulation of PRMT6 in CSE induced apoptosis and inflammation in HUVECs. In conclusion, PRMT6 mediates CSE induced apoptosis and inflammation through H3R2me2a in HUVECs.

1. Introduction

Chronic obstructive pulmonary disease (COPD), which is characterized by airflow limitation, is an important global health problem because of its high prevalence and growing cause of morbidity and mortality worldwide [1]. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung [2]. Although only 20% of smokers develop COPD, the major triggering factor is cigarette smoking, which accounts for 80–90% of the COPD cases [3]. The cigarette smoke causes airway inflammation by activating epithelial cells and macrophages, releasing inflammatory mediators, such as tumor necrosis factor alpha (TNF-α), cyclooxygenase-2 (COX2) and matrix metallopeptidases (MMPs), leading to inflammatory process in airway and progressive airflow limitation [4–6]. Besides the inflammation, an abnormal protease/anti-protease balance, leading to apoptosis, is also a feature of COPD [7–10]. Although, COPD has received more attention by research community, and the mechanism by which cigarette smoke extracts (CSE) induced inflammation and apoptosis in the pathogenesis of COPD is not fully known, and more information associated with CSE induced inflammation and apoptosis in COPD continue to uncover.

Arginine methyltransferase (PRMT6) catalyzes the asymmetric di-methylation of histone H3 arginine 2 (H3R2me2a) and controls global levels of H3R2me2a in vivo [11]. Meanwhile, H3R2me2a obviously prevents the methylation of di- and tri-methylation of H3 lysine 4 (H3K4me2, H3K4me3) [11,12]. Thus, the outcome of overexpression of PRMT6 could be responsible for the global repression of gene expression, like MMPs [13,14]. Interestingly, other investigations have indicated that PRMT6 associates with the apoptosis in vivo [15,16]. These compelling results are suggesting the possible role of PRMT6 in CSE induced inflammation and apoptosis in COPD.

In a current study, the role of PRMT6 in CSE induced inflammation and apoptosis was explored.

2. Materials and methods

2.1. Antibodies

The specific antibodies against PRMT6 (15295-1-AP) were purchased from Proteintech Group, Inc. (Chicago, IL, USA). The specific antibodies against H3R2me2a (NB21-1002) were purchased from Novus Biologicals (Littleton, CO, USA). Specific antibodies against H3K4me3 (9727S) and caspase-3 (9662) were purchased from Cell Signaling Technology (Beverly, MA, USA), Antibodies against IL-1 beta (sc-7884), TNF-alpha (sc-8301), Bcl-2 (sc-7382) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, U.S.A.).

2.2. Cell culture

The human kidney 2 (HK2), human pulmonary microvascular endothelial cells (HPMVE), and human umbilical vein endothelial cells (HUVECs) were purchased from the China Center for Type Culture
Collection (CCTCC, Wuhan, China). Cells were cultured in DMEM medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS, at 37 °C in humidified air containing 5% CO2; cells were passaged when 80% confluent. Cells were grown to confluence for the experiments. All experiments were performed triplicate and repeated at least 3 times. At the end of the incubation period, cell lysate and culture supernatants were harvested and stored at −80 °C until further analysis.

2.3. CSE preparation

CSE was prepared by a modification of the method of Richter et al. [17]. One commercial Marlboro Red cigarette (0.8 mg nicotine; 10 mg Tar; 10 mg carbon monoxide) was combusted with a modified syringe-driven apparatus. The smoke was bubbled through 25 ml of media over 5 min by drawing 35-ml volume of smoke every 15 s. The resulting suspension was filtered through a 0.2 μm pore-size filter to remove large particles and bacteria. This solution was regarded as “100% CSE” and freshly generated for each experiment, and subsequently serially diluted with culture medium to obtain a final 1%, 5%, 10% and 20% working concentration. The optical density was constant when comparing a series of CSE solutions prepared in this manner.

2.4. Annexin-PI staining

Apoptosis was analyzed using Annexin V (Av) and Propidium Iodide (PI) staining (eBioscience, UK) [18]. Cells which were negative for Av and PI were considered to represent viable cells, while those positive for Av, but negative for PI represented early apoptosis and events positive for both Av and PI were regarded as late apoptosis. To eliminate debris from the analysis, the discrimination level was set to 100. Cells, adjusted to 1 × 10^5 cells per 100 μl, were suspended in binding buffer and incubated with the fluorochrome-conjugated Av for 15 min. Subsequently, after washing, cells were re-suspended in fresh binding buffer and stained with PI. In order to set gates and establish appropriate compensation settings, cells were stained with PI alone, Av alone, and both PI and Av.

2.5. Immunoblotting

Western blot analysis was conducted according to a previous study [19]. Briefly, equal amounts of proteins obtained from cytoplasmic fractions were separated by a reducing SDS-PAGE electrophoresis. The proteins were transferred onto PVDF membranes (Millipore, MA, USA) and blocked with 5% non-fat milk in Tris–TWEEN buffered saline buffer (20 mM Tris, pH 7.5150 mM NaCl, and 0.1% Tween-20) for 3 h. The primary antibodies were incubated overnight at 4 °C; the HRP-conjugated secondary antibodies were subsequently incubated for 1 h at 25 °C before development of the blots using the Alpha Imager 2200 software (Alpha Innotech Corporation, CA, USA). We quantified the resultant signals and normalized the data to the abundance of actin. Actin was used as an indicator of cytoplasmic protein fractions.

2.6. Real-time quantitative (RT-PCR)

Total RNA was isolated from cells using TRIZOL reagent (Invitrogen, USA) and then treated with DNase I (Invitrogen, USA) according to the manufacturer’s instructions. Primers used in this study were synthesized according to either previous protocols or designed with Primer 5.0. Sequences of all primers used were:

- COX2-F: 5-GTTCCACCCGCAGTACAGAA-3,
- COX2-R: 5-AGGGCTTCAGCATAAAGCGT-3,
- PRMT6-F: 5-ACTGTAGAGTTGCCGGAACA-3,
- PRMT6-R: 5-TCCTTACGACACTTGTTCG-3,
- IL-6-F: 5-CGCCCTCGGTCCAGTGC-3,
- IL-6-R: 5-TGGAATCTTCTCCTGGGGGT-3,
- MMP2-F: 5′-CCCAAAACGGACAAAGTT-3′,
- MMP2-R: 5′-GGTATTCTGGTCAAGATCACC-3′,
- MMP9-F: 5′-CAAACTACTCGGAAGACTTGC-3′,
- MMP9-R: 5′-GTCCCGGCTGTACACGCGA-3′,
- Caspase-1-F: 5′-TTATTCCGAAAGGGGCACAG-3′,
- Caspase-1-R: 5′-GGGCATAGCTGGGTTGTCCTGC-3′,
- Caspase-3-F: 5′-CAGAGGGATCGTTGTAGAAG-3′,
- Caspase-3-R: 5′-CATACAGAGTCGGCTCCA-3′.

**Fig. 1.** The protein abundance of PRMT6. The protein abundance of PRMT6 were analyzed in human kidney 2 (A), human pulmonary microvascular endothelial cells (B), and human umbilical vein endothelial cells (C) after graded dose of CSE treatment at different time points. Data are from one experiment representative of six experiments (biological replicates). CSE: cigarette smoke extract; PRMT6: arginine methyltransferase.
Caspase-9-F: 5′-CCAGTGGACATTGGTTCTGG-3′.
Caspase-9-R: 5′-TCACGGCAGAAGTTCACATT-3′.
Actin-F: 5′-CATCCTGGCTGGACAGCGG-3′.
Actin-R: 5′-TAATGTACCGACAGATTTCC-3′.

Real-time PCR was performed according to a previous study [20,21]. Relative expression of genes in the treatment group was normalized to the values for the control group.

2.7. Silencing of PRMT-6 in HUVEC cells

siRNA targeting PRMT-6 was transiently transfected into cells using lip2000 transfection reagent. siRNA sequences were PRMT6-siRNA, with sequence as 5′-TTGTGCTTGGTACTACATGCT-3′ (Well-Biology Biotechnology, Changsha, China). Scrambled siRNA (non-silencing sequence) (Ambion, Texas, USA) was used as a control with sequence as 5′-TTCTCCGAACGTGTCACGT-3′ (Well-Biology Biotechnology, Changsha, China). The experiment was conducted according the manufacturer’s protocol of transfection reagent. Briefly, cells were seeded in 6-well plates at a density of 2.5 × 10^5 cells/well. Cells were transfected with different concentrations of siRNA ranging from 10 to 50 nM for 48 h or 72 h, using lip2000 transfection reagent. The ratio of siRNA to transfection reagent was maintained as 1:0.5 for efficient silencing without toxicity according to the manufacturer’s protocol. The final concentrations of siRNA were chosen based on dose–response studies. Forty-eight hours after the transfection, cells were used for studies.

2.8. Transfection with PRMT-6-Flag plasmid

Plasmid pcDNA3-PRMT-6-Flag was used as the mammalian expression vector. This plasmid was from Well-Biology Biotechnology (Changsha, China). PRMT-6 was cloned to pcDNA3-Flag plasmid (Well-Biology Biotechnology, Changsha, China) with following primers: F: GGAATTCCATGTCGCAGCCCAAG and R: CCAAGCTTTCAGTCCTCCATGGC (Well-Biology Biotechnology, Changsha, China). Cells were transiently transfected with PRMT-6-Flag plasmids using Lipofectamine and Plus Reagent (Invitrogen). PcDNA3-Flag was also constructed and transfected as control. PRMT-6-Flag expression was confirmed by immunoblotting.

2.9. Statistical analysis

The results are expressed as means ± SEM. Analysis among groups was performed with a one-way ANOVA. For each analysis, P values less than 5% were considered statistically significant.

<table>
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<th>Control</th>
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<th>Flag</th>
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<th>PRMT6-Flag+CSE</th>
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<td>2</td>
<td>2.5</td>
<td>3</td>
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<td>1.5</td>
<td>2</td>
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<tr>
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Fig. 2. mRNA expression of PRMT6 and caspase1, 3 and 9. A: mRNA expression of PRMT6 was analyzed after different treatments. B: mRNA expression of caspase1 was analyzed after different treatments. C: mRNA expression of caspase9 was analyzed after different treatments. D: mRNA expression of caspase3 was analyzed after different treatments. Control: HUVEC received no treatment; CSE: HUVEC was treated with 20% CSE for 24 h; Flag: HUVEC transfected with Flag and no CSE treatment; Flag + CSE: HUVEC transfected with Flag and treated with 20% CSE for 24 h; PRMT6-Flag: HUVEC transfected with PRMT6-Flag and no CSE treatment; PRMT6-Flag + CSE: HUVEC transfected with PRMT6-Flag and treated with 20% CSE for 24 h; S-siRNA: HUVEC transfected with scrambled siRNA and no CSE treatment; siRNA: HUVEC transfected with siRNA targeting PRMT-6; CSE: cigarette smoke extract, PRMT6: arginine methyltransferase. Data are presented as mean ± SEM, n = 6, with A–D used to indicate a statistically significant difference (P < 0.05, one way ANOVA method).
3. Results

3.1. CSE inhibits the expression of PRMT6

To explore the effects of CSE on the expression of PRMT6, the protein abundance of PRMT6 in three kinds of cell lines was measured after different dosages of CSE treatment in different time points. As indicated in Fig. 1A, 20% CSE treatment significantly $(P < 0.05)$ decreased the protein abundance of PRMT6 at 6 and 24 h post-treatment in HK-2 cell lines (Fig. 1A). Likewise, the protein abundance of PRMT6 in 20% CSE treatment at 6 and 24 h post-treatment was significantly lower $(P < 0.05)$ than those with lower dose of CSE treatment in the HPMEC and HUVEC cell lines (Fig. 1B and C). Collectively, 20% CSE treatment significantly decreases the protein abundance of PRMT6 at 6 and 24 h post-treatment. Thus, following experiment was conducted with 20% CSE treatment for 24 h in HUVECs.

3.2. PRMT6 mediates CSE induced apoptosis

As shown in Fig. 2, CSE treatment significantly $(P < 0.05)$ induced the mRNA expression of caspase-1, 3 and 9, compared to the control group. Also, CSE treatment significantly $(P < 0.05)$ lowered the protein abundance of Bcl-2, while it increased the abundance of cleaved caspase-3, compared with those in control group (Fig. 4). These results

![Fig. 3](image-url)

Fig. 3. mRNA expressions of pro-inflammatory factors. A: mRNA expression of COX-2 was analyzed after different treatments. B: mRNA expression of IL-6 was analyzed after different treatments. C: mRNA expression of mmp9 was analyzed after different treatments. D: mRNA expression of mmp2 was analyzed after different treatments. Control: HUVEC received no treatment; CSE: HUVEC was treated with 20% CSE for 24 h; Flag: HUVEC transfected with Flag and no CSE treatment; Flag + CSE: HUVEC transfected with Flag and treated with 20% CSE for 24 h; PRMT6-Flag: HUVEC transfected with PRMT6-Flag and no CSE treatment; PRMT6-Flag + CSE: HUVEC transfected with PRMT6-Flag and treated with 20% CSE for 24 h; S-siRNA: HUVEC transfected with scrambled siRNA and no CSE treatment; siRNA: HUVEC transfected with siRNA targeting PRMT-6. CSE: cigarette smoke extract, PRMT6: arginine methyltransferase, COX-2: cyclooxygenase-2, IL-6: interleukin 6, MMP: matrix metalloproteinase. Data are presented as mean ± SEM, $n = 6$, with A–D used to indicate a statistically significant difference $(P < 0.05$, one way ANOVA method).
suggested that CSE promoted the apoptosis, which was confirmed with FITC analysis to show increased positive cells for Av and PI (Fig. 5A and B). Although there was little change on the mRNA expression of PRMT6 after CSE treatment (Fig. 2A), a significant decrease in protein abundance of PRMT6 was observed after CSE treatment, compared to controls (P < 0.05) (Figs. 1 and 4).

To explore the functions of PRMT6 in CSE induced apoptosis, a PRMT6-Flag expressing plasmid was transfected to cells, resulting in increased (P < 0.05) expression of PRMT6 in cells from mRNA and protein levels (Figs. 2A and 4), compared with the non-transfected cells. The increased expression of PRMT6 had little effects on cell apoptosis without CSE treatment (Figs. 2, 4 and 5). As the control, Flag expressing plasmid transfection showed little effects on the expression of PRMT6 in cells and cell apoptosis (Figs. 2, 4 and 5). However, the PRMT6-Flag transfected cells showed lower (P < 0.05) mRNA expression of caspase-1 (Fig. 2B) and 3 (Fig. 2D), as well as protein abundance of cleaved caspase-3 (Fig. 4), leading to lower percentage of positive cells for Av and PI after CSE treatment (Fig. 5F), compared with the non-transfected cells after CSE treatment. As the control, flag tag plasmid transfection had little effect on the CSE induced apoptosis in cells (Figs. 2, 4 and 5).

Furthermore, siRNA targeting PRMT-6 treatment significantly (P < 0.05) decreased the expression of PRMT-6 in cells from both mRNA and protein levels (Figs. 2 and 4). The inhibition of PRMT-6 significantly (P < 0.05) promoted the mRNA expression of caspase-1 and 9, as well as the protein abundance of cleaved caspase-3, while it lowered the protein abundance of Bcl-2, compared to the control group (Figs. 2 and 4). Consequently, the inhibition of PRMT-6 significantly (P < 0.05) increased the percentage of cells in early and late apoptosis (Fig. 5). As the control, scrambled siRNA treatment had little effects on cell apoptosis (Figs. 2, 4 and 5).

Collectively, the PRMT6 mediates the CSE induced apoptosis in cells, and the overexpression of PRMT6 inhibits the CSE-induced apoptosis.

3.3. PRMT6 mediates CSE induced inflammation

Similar to cell apoptosis, CSE treatment induced obvious inflammation in cells from the enhanced mRNA expression of COX2, IL-6, mmp2 and 9, as well as the protein abundance of IL-1 beta and TNF-alpha, compared to the controls (P < 0.05) (Figs. 3 and 4). PRMT6-Flag or Flag expressing plasmid transfection had little effects on inflammation in cells without CSE treatment (Figs. 3 and 4). However, cells transfected with PRMT6-Flag showed lower (P < 0.05) mRNA expression of IL-6, mmp2 and 9, as well as the protein abundance of IL-1 beta and TNF-alpha after CSE treatment, than the non-transfected cells after CSE treatment (Figs. 3 and 4). As the control, Flag tag plasmid transfection had little effects on the CSE induced inflammation in cells (Figs. 3 and 4). Similarly, inhibition of PRMT6 significantly (P < 0.05) promoted the inflammation from the increased mRNA expression of COX2, IL-6, mmp2, as well as the protein abundance of IL-1 beta and TNF-alpha, compared to the controls (P < 0.05) (Figs. 3 and 4). As the control, scrambled siRNA treatment had little effects on pro-inflammation in HUVECs (Figs. 3 and 4).

Summarily, the PRMT6 mediates the CSE induced inflammation in cells, and the overexpression of PRMT6 inhibits the CSE-induced inflammation.

3.4. Expression of H3R2me2a and H3K4me3

As shown in Fig. 4, CSE treatment significantly (P < 0.05) promoted the protein abundance of H3K4me3, while it decreased the protein abundance of H3R2me2a. Cells transfected with PRMT6-Flag expressing plasmid significantly (P < 0.05) promoted the protein abundance of H3R2me2a, while it decreased the protein abundance of H3K4me3, compared with the non-transfected cells (Fig. 4). As the control, cells transfected with Flag expressing plasmid had little effects on the expression of H3R2me2a and H3K4me3 (Fig. 4). Likewise, cells transfected with PRMT6-Flag expressing plasmid significantly (P < 0.05) promoted the protein abundance of H3R2me2a, while it decreased the protein abundance of H3K4me3 after CSE treatment, compared with the non-transfected cells after CSE treatment (Fig. 4). As the control, cells transfected with Flag expressing plasmid had little effects on the expression of H3R2me2a and H3K4me3 after CSE treatment (Fig. 4). Furthermore, inhibition of PRMT6 CSE treatment significantly (P < 0.05) promoted the protein abundance of H3K4me3, while it decreased the protein abundance of H3R2me2a (Fig. 4). As the control, scrambled siRNA treatment had little effects on the expression of H3R2me2a and H3K4me3 (Fig. 4).

4. Discussion

This study has found that CSE treatment affects the expression and function of some regulators in apoptosis pathway, like caspase-3, suggesting that CSE treatment induces cell apoptosis. In fact, further analysis from FITC has also demonstrated this suggestion with more positive cells for Av and PI. In line with our observation, previous study has also reported that CSE treatment will induce cell apoptosis in different cells, including primary nasal epithelial cell cultures (PNECs) [18], well-differentiated human bronchial epithelial cells (WD-HBECs) [22], and normal human bronchial epithelial (NHBE) cells [23], as well as in vivo [24]. Mechanically, CSE may induce apoptosis through intrinsic and extrinsic pathways. For example, Damico et al. have reported that CSE triggers the death of human pulmonary macrovascular endothelial cells (HPAECs) through caspase9-dependent apoptotic pathway [25]. Meanwhile, other investigations have also found that CSE induces apoptosis through caspase8 in human lung fibroblast (MRC-5) cells [26,27] and human aortic endothelial cells (HAEC) [28]. Indeed, we have also observed the change of caspase9 pathway after CSE treatment in this study. This study has found that CSE treatment triggers the inflammation from the expression of pro-inflammatory cytokines. Likewise, numerous well-designed investigations have shown that CSE exposure induces the inflammation in various cells, like human bronchial

![Fig. 4. Protein abundance. Control: HUVEC received no treatment; CSE: HUVEC was treated with 20% CSE for 24 h; Flag: HUVEC transfected with Flag and no CSE treatment; Flag + CSE: HUVEC transfected with Flag and treated with 20% CSE for 24 h; PRMT6-Flag: HUVEC transfected with PRMT6-Flag and no CSE treatment; PRMT6-Flag + CSE: HUVEC transfected with PRMT6-Flag and treated with 20% CSE for 24 h; s-siRNA: HUVEC transfected with scrambled siRNA and no CSE treatment; siRNA: HUVEC transfected with siRNA targeting PRMT-6; CSE: cigarette smoke extract, PRMT6: arginine methyltransferase, H3R2me2a: histone H3 arginine 2, H3K4me3: trimethylation of H3 lysine 4. Data are from one experiment representative of six experiments (biological replicates).]
epithelial BEAS-2B cells [29] and primary nasal epithelial cell cultures (PNECs) [18], and in vivo [29,30]. Detailed analysis indicates that CSE functions in various signaling pathways, like the nuclear factor-kappaB (NF-κB) [18], mitogen-activated protein kinases (MAPKs) [31], and signal transducer and activator of transcription proteins 3 (STAT3) [31], which are associated with inflammation [32].

Intriguingly, this study has found that PRMT6 mediates the CSE-induced apoptosis and inflammation. This conclusion comes from three levels of evidence: 1) CSE treatment decreases the protein abundance of PRMT6, coinciding with the increased apoptosis and inflammation; 2) cells transfected with PRMT6 expressing plasmid inhibits the CSE-induced apoptosis and inflammation; and 3) inhibition of PRMT6 promotes the cell apoptosis and inflammation. Although, no difference is found about the mRNA expression of PRMT6, CSE treatment decreases the protein abundance of PRMT6. The mechanism by which CSE exposure decreases the protein abundance of PRMT6 is missing. The possible reason relates to the activation of the ubiquitin-proteasome pathway after CSE treatment. Indeed, Ottenheijm et al. have demonstrated that the increased activity of the ubiquitin-proteasome pathway accounts for the loss of contractile protein content in patients with mild to moderate COPD [33]. Meanwhile, growing evidence is showing that the ubiquitin-proteasome system is playing a crucial role in degradation of contractile proteins, thus promoting the development of muscle atrophy in patients with COPD [34]. Furthermore, Kim et al. have found that CSE treatment decreases the protein abundance of total and phosphorylated Akt (p-Akt) through the ubiquitin-proteasome system [35]. However, whether CSE decreases the protein abundance of PRMT6 by enhanced PRMT6 degradation via the ubiquitin-proteasome system is worthy of further study.

Notably, PRMT6 may mediate CSE induced apoptosis and inflammation through H3R2me2a. Firstly, this study has found that the inhibition of PRMT6 is associated with the decreased protein abundance of H3R2me2a and the increased protein level of H4K3me3. Similarly, previous studies have also indicated that PRMT6 catalyzes H3R2 dimethylation and controls global levels of H3R2me2a in vivo, which conversely prevented di- and tri-methylation of H3K4 [11,12]. Secondly, the change of H3R2me2a is coincided with the CSE-induced inflammation and apoptosis. Indeed, previous studies have found that H3R2me2a affects global transcription to function in apoptosis and inflammation [12,24]. However, the detail function of H3R2me2a in apoptosis and inflammation needs further investigation.

In conclusion, CSE treatment induces obvious apoptosis and inflammation in HUVECs. PRMT6 mediates CSE induced apoptosis and inflammation through H3R2me2a. To our knowledge, this is the first report to show that PRMT6 mediates CSE induced apoptosis and inflammation. This compelling finding is fruitful to understand the pathogenesis of COPD, and has important implications on the clinical treatment of COPD.

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References
