Cigarette Smoke Extract Changes Expression of Endothelial Nitric Oxide Synthase (eNOS) and p16(INK4a) and is Related to Endothelial Progenitor Cell Dysfunction

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Background: Endothelial dysfunction is an important pathophysiologic feature in many smoke-related diseases. Endothelial progenitor cells (EPCs) are the precursors of endothelial cells and play a fundamental role in the maintenance of endothelial integrity and function. Endothelial nitric oxide synthase (eNOS) is the dominant NOS isoform in the vasculature and plays a central role in the maintenance of endothelial homeostasis. p16(INK4a) is a cyclin-dependent kinase inhibitor and could be regarded as a major dominant senescence gene. The present study aimed to determine whether the expression of eNOS and p16(INK4a) in EPCs is related to EPCs function and the possible epigenetic mechanism, if any.

Material/Methods: We investigated EPCs capacity for proliferation, adhesion, and secretion, and the expression of eNOS and p16(INK4a) in EPCs which were altered by cigarette smoke extract (CSE) in vitro. Furthermore, Decitabine (Dec), an agent of demethylation, was used to examine whether it could alter the changes induced by CSE.

Results: The present study demonstrated that EPCs altered by CSE in vitro displayed decreased capacities of proliferation, adhesion, and secretion, which was accompanied by decreased eNOS expression and increased p16(INK4a) expression in EPCs. Furthermore, Dec could alleviate the changes in the expression of eNOS and p16(INK4a), and protect against the EPCs dysfunction caused by CSE.

Conclusions: The decreased eNOS expression and increased p16(INK4a) expression was associated with dysfunction of EPCs caused by CSE. The mechanism of methylation, one of the most common epigenetic mechanism, may be involved in the EPCs dysfunction caused by CSE.

MeSH Keywords: Cyclin-Dependent Kinase Inhibitor p16 • Endothelial Cells • Methylation • Nitric Oxide Synthase Type III • Smoking

Abbreviations: acLDL – acetylated low-density lipoprotein; CSE – cigarette smoke extract; DAPI – 4',6-diamidino-2-phenylindole; Dec – Decitabine; Dil – 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; EGM-2 – endothelial growth medium-2; eNOS – endothelial nitric oxide synthase; EPC – endothelial progenitor cell; FBS – fetal bovine serum; FITC – fluorescein isothiocyanate; LSCM – laser scanning confocal microscope; NO – nitric oxide; OD – optical density; PBS – phosphate-buffered saline; ROS – reactive oxygen species; UEA-1 – Ulex europaeus agglutinin-1

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The EPCs were derived from healthy 4–6 weeks old male Cells

Material and Methods

Expression of eNOS and p16(INK4a) relate to EPCs dysfunction 
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Background

Cigarette smoking is a major risk factor of cardiovascular and pulmonary diseases. Cigarette smoke extract (CSE) contains most of the compounds inhaled by cigarette smokers and is usually used as a surrogate for cigarette smoke [1]. The method of stimulating isolated cells with CSE in vitro has been explored and applied to determine the direct causes in the relationships between cigarette smoking and cellular functions [2]. Endothelial progenitor cells (EPCs) were isolated in 1997 by Asahara et al. [3]. Bone-marrow-derived EPCs provide an alternative source of endothelial cells and play a fundamental role in the maintenance of endothelial integrity and function, vascular homeostasis, postnatal vasculogenesis, and tissue regeneration and repair through pivotal bioactivities, including differentiating into endothelial cells, secretion of vasoactive substances, proliferation, homing, and migration [4,5]. The normal function of EPCs is required for neo-angiogenesis and tissue repair [6]. However, the mechanism by which smoke influences EPCs function remains unclear.

Nitric oxide (NO) is a major regulator of blood vessel homeostasis and is synthesized by endothelial nitric oxide synthase (eNOS) [7]. eNOS is the dominant NOS isoform in the vasculature, rather than being a constitutive enzyme as was first suggested [8]. In the guinea pig, exposure to cigarette smoke induces endothelial dysfunction in pulmonary arteries and reduced lung expression of eNOS. These changes appear to precede the development of pulmonary emphysema [9]. p16(INK4a), initially discovered as a tumor suppressor gene, is a member of the INK4 family and a cyclin-dependent kinase inhibitor [10]. It has the ability to block cell progression from G1 to S phase and is regarded as a major dominant senescence gene [11,12]. It has been demonstrated that cord blood EPCs in premature neonates exhibit increased p16(INK4a) expression, contributing to accelerated senescence of EPCs [13].

It has been demonstrated that a demethylation agent can alleviate emphysema in an animal model [14,15]. Decitabine (Dec) is the most widely used agent of demethylation; it triggers de novo transcription of epigenetically silenced genes in vitro and in vivo [16]. The present study aimed to investigate whether expression of eNOS and p16(INK4a) in EPCs can be altered by CSE in vitro, and capacities of EPCs for proliferation, adhesion, and secretion. We also examined whether Dec can alleviate the damages caused by CSE.

Material and Methods

Cells

The EPCs were derived from healthy 4-6 weeks old male C57BL/6J mice. A total of 36 male C57BL/6J mice aged 4–6-week-old were randomly enrolled in the study. All animals were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (SLACAS, Shanghai, China) and fed in a specific pathogen free (SPF) animal facility at 23–25°C, 50–60% humidity and 12 h cycles of night and day. They were provided free access to water and food. The study was approved by the Institutional Review Board of Central South University and followed internationally recognized guidelines for the Research Involving Animals and Human beings [17]: every effort was made to minimize the animals’ suffering.

Preparation of CSE and Decitabine solutions

The CSE solution was prepared according to a previously published method [18]. Briefly, the smoke of 1 non-filtered Fu-Rong cigarette (Tar: 13 mg, Nicotine: 1.0 mg, Carbon Monoxide: 14 mg/cigarette, China Tobacco Hunan Industrial Co., Ltd., Changsha, China) was passed through 20 ml of endothelial growth medium-2 (EGM-2) free of fetal bovine serum (FBS) (SingleQuots, Lonza, Basel, Switzerland) connected to a vacuum pump with a constant pressure of ~0.1 Kpa. After being adjusted with 1 mmol/L NaOH to 7.4 pH, the product was subsequently filtered through a device with 0.22-μm pores (Fisher, Hampton, NH, USA) to remove particles and bacteria. This mother CSE solution was further diluted with the vehicle control to 1.0% CSE solution. The solution was freshly prepared for each experiment.

Decitabine solution was prepared as follows: 5 grams of Decitabine (Dec, Sigma, Santa Clara, CA, USA) was dissolved in 2 ml of FBS-free EGM-2 and diluted to a concentration of 2.0 μmol/l before being repackaged and stored at –80°C.

Isolation, culture, and identification of EPCs

Mononuclear cells (MNCs) from the bone marrow of C57BL/6J mice were isolated via Ficoll density gradient centrifugation (Histopaque-1083, Sigma, Santa Clara, CA, USA) before being inoculated into culture flasks at a density of (3–5)×10^6/ml and cultured with EGM-2 in 5% FBS (SingleQuots®, Lonza, Basel, Switzerland) under an atmosphere of 95% humidity and 5% CO2 at 37°C [15]. On day 4 of the culture, the culture fluid was replaced with fresh culture medium to remove the unattached cells. Cell harvesting was performed on day 7 of the culture. Double-positive staining of Ulex europaeus agglutinin-1 (UEA-1) lectin and acetylated low-density lipoprotein (acLDL) was used for EPCs identification [15]. Briefly, the harvested cells were incubated with 7.5 μg/ml 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate(Dil)-labeled acLDL, tetramethylrhodamine isothiocyanate (TRITC)-labeled UEA-1 (Molecular Probes, Eugene, OR, USA) at 37°C for 4 h and fixed with 4% paraformaldehyde for 10 min. After being washed, the cells were treated with 10.0 μg/ml fluorescein...
isothiocyanate (FITC)-labeled UEA-1 (FITC-UEA-1, Sigma, Santa Clara, CA, USA) for 30 min. Finally, the cells were treated with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min before identification was performed using a laser scanning confocal microscope (LSCM 510, Carl Zeiss, Jena, Germany).

**Detection of the proliferation, adhesion and secretion of EPCs**

The EPCs on day 7 of the culture were trypsinized (0.25% trypsin, Amresco, Cleveland, OH, USA), resuspended with FBS-free EGM-2 and transplanted to a 96-well plate (1×10^4 in 200 μl volume per well). The wells were divided into 3 groups: control group, CSE group, and CSE+Dec group. We added 200 μl FBS-free EGM-2 per well to the control group and CSE group, and 200 μl Dec solution per well was added to the CSE+Dec group. After being incubated for 48 h, the culture media of the cells were removed and replaced by 200 μl FBS-free EGM-2 per well in the control group, and by 200 μl CSE solution per well in the CSE group and CSE+Dec group. After being incubated for 24 h, the EPCs were used for the following assays.

**Proliferation**

To each well we added with 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Santa Clara, CA, USA) (5 mg/ml) and incubated for 4 h. The supernatant was removed and replaced by 150 μl dimethyl sulfoxide (DMSO) (Sigma, Santa Clara, California, USA). Finally, the EPCs were shaken for 10 min to dissolve crystals before optical density (OD) measurement at 490 nm (ELX800, Bio-Tek, Burlington, VT, USA).

**Adhesion**

To each well we added 20 μl of 1:4 DRAQ5 (1:20, Santa Cruz Biotechnology, Santa Cruz, TX, USA) and 200 μl of 5'-TAATGTCACGCACGATTTCC-3' (reverse); 5'-CATCCTGCGTCTGGACCTGG-3' (forward), 5'-CTATGGCAGACAGAGACAT-3' (eNOS: 1:1000, Proteintech, Chicago, IL, USA; p16(INK4a): 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min. The EPCs on day 7 of the culture were trypsinized (0.25% trypsin, Amresco, Cleveland, OH, USA), resuspended with FBS-free EGM-2 and transplanted to a 96-well plate (1×10^4 in 200 μl volume per well). The wells were divided into 3 groups: control group, CSE group, and CSE+Dec group. We added 200 μl FBS-free EGM-2 per well to the control group and CSE group, and 200 μl Dec solution per well was added to the CSE+Dec group. After being incubated for 48 h, the culture media of the cells were removed and replaced by 200 μl FBS-free EGM-2 per well in the control group, and by 200 μl CSE solution per well in the CSE group and CSE+Dec group. After being incubated for 24 h, the EPCs were used for the following assays.

**Secretion**

The EPCs secretion was assessed via the detection of nitric oxide (NO) level in the cell culture supernatants. The level of NO in the cell culture supernatant was measured by chemical method according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). OD was measured at 550 nm. NO_\text{sample} = \frac{(\text{OD}_{\text{sample}}-\text{OD}_{\text{blank}})}{(\text{OD}_{\text{standard}}-\text{OD}_{\text{blank}})} \times \text{NO}_{\text{standard}}.

**Western blotting**

Briefly, EPCs were washed 3 times with ice-cold phosphate-buffered saline (PBS), then lysed in RIPA lysis (Applygen Technologies Inc, Beijing, China) for 30 min on ice. The solution of EPCs was centrifuged at 4°C and 12000×g for 5 min. BCA protein quantitation kit (W ellbio Inc, Changsha, China) was used for protein measurement. Protein was mixed 1:1 with 2×SDS loading buffer (20% glycerol, 4% sodium dodecyl sulfate, 3.12% dithiothreitol, 0.2% bromphenol blue, and 0.1 mol/l Tris HCl, pH 6.8, all from Sigma, Santa Clara, CA, USA), and incubated at 100°C for 4 min. Equal amounts of protein for each sample were separated by 10–12% SDS-polyacrylamide gel run at 120V for 90 min and blotted onto a polyvinylidene difluoride microporous membrane (Millipore, Billerica, MA, USA). Membranes were incubated overnight with primary antibody (eNOS: 1:1000, Proteintech, Chicago, IL, USA; p16(INK4a): 1:200, Santa Cruz Biotechnology, Santa Cruz, TX, USA), and subsequently washed 3 times with Tris-buffered-saline with Tween (TBS-T) and visualized following incubation with horse-radish peroxidase-conjugated secondary antibody (1:3000) for 1 h, followed by washing with TBS-T again. Immunoreactive bands were developed using an enhanced chemiluminescent substrate (Thermo, Waltham, MA, USA). After exposure, the films were scanned and analyzed using Quantity One (Bio-Rad, CA, USA).

**RNA extraction and quantitative real time-PCR (RT-PCR)**

p16(INK4a) and eNOS mRNA expression in bone marrow-derived EPCs were detected by quantitative RT-PCR. Total RNA was extracted from the EPCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using the RevertAid™ First-Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA) according to the manufacturer’s instructions, and used as the template for quantitative RT-PCR analysis. DNase-treated samples were subjected to RT-PCR using SYBR Green qPCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) on the Bio-Rad CFX96 Real Time system (Bio-Rad, Hercules, CA, USA) with β-actin used as an internal control. The PCR amplification conditions consisted of 10 min at 95°C followed by 40 cycles of denaturation step at 95°C for 15 s and annealing and extension for 1 min at 60°C. Data were analyzed using the comparative Ct (ΔΔct) method [15]. The relative expression levels of p16(INK4a) were calculated by determining a ratio between the amount of p16(INK4a) or eNOS and that of internal control. Melting curve analysis (65–95°C) was used to determine the melting temperature of specific amplification products and primer dimers. Each experiment was repeated 2 times in triplicates. The primers sequences synthesized by Invitrogen Co., Ltd (Shanghai, China) were as follows: β-actin, 5'-CATCTGCCGTGACCTGG-3' (forward), 5'-TAATGTACAGCAGATTTC-3' (reverse);
eNOS, 5'-GGGCTCGGGCTGGGTTTAGG-3' (forward), and 5'-CCTGGGCACTGAGGGTGTCG-3' (reverse); p16(INK4a), 5'-CCGCCTCAGCCCGCCTTTTT-3' (forward), and 5'-CCGCCGCCTTCGCTCAGTTT-3' (reverse).

Statistical analysis

Analyses were performed using SPSS for Windows 16.0 (SPSS Inc., Chicago, IL, USA). All data are expressed as means±standard deviation (SD). Analysis of differences among groups were performed using analysis of variance (one-way ANOVA), followed by post hoc analysis, as appropriate. Values of \( P<0.05 \) were considered statistically significant.

Results

Culture and identification of EPCs

On day 1 of the culture, the MNCs isolated from bone marrow were round, with similar sizes, and were suspended in the culture media (Figure 1A). On day 4 of the culture, the cells became larger, with oval or spindle shape (Figure 1B). On day 7 of the culture, the cells attempted to attach to one another and follow fusiform or polygonal patterns (Figure 1C). The scale bar represents 50 μm. EPCs – endothelial progenitor cells.

The expression of eNOS and p16(INK4a) in EPCs

The expression of eNOS protein and mRNA in EPCs were significantly lower in the CSE+Dec group (0.39±0.05 and 0.36±0.05) and CSE group (0.27±0.04 and 0.20±0.04) compared with the controls (0.95±0.11 and 0.97±0.08) (p<0.01). However, the expression of eNOS protein and mRNA in EPCs was significantly higher in the CSE+Dec group compared with the CSE group (p<0.05) (Figure 4A, 4B). The expression of p16(INK4a) protein and mRNA in EPCs was significantly higher in the CSE+Dec group (0.61±0.08 and 2.25±0.23) and CSE group (0.92±0.09 and 5.46±0.57) compared with the controls (0.35±0.05 and 1.17±0.10) (p<0.01 or p<0.05). However, the expression of p16(INK4a) protein and mRNA in EPCs was significantly lower in the CSE+Dec group compared with the CSE group (p<0.01) (Figure 4A, 4C). In summary, the expression of eNOS protein and mRNA in descending order were controls, CSE+Dec group, and CSE group. The expression of p16(INK4a) protein and mRNA in descending order were CSE group, CSE+Dec group, and controls.
The most important finding of this study was that the EPCs in CSE intervened by CSE in vitro displayed significantly decreased proliferation, adhesion, and secretion capacities, decreased expression levels of eNOS protein and mRNA, and increased expression levels of p16(INK4a) protein and mRNA. Furthermore, Dec alleviated the changes in expression of eNOS and p16(INK4a), and partly protected against the EPCs dysfunction caused by CSE. These results suggest that the decreased eNOS expression and increased p16(INK4a) expression may contribute to EPCs dysfunction, and the mechanism of methylation may be involved in the EPCs dysfunction caused by CSE.

EPCs are the precursors of endothelial cells, and it is thought that EPCs with normal self-renew, proliferation, secretion, differentiative potential are essential for their own transendothelial shift, homing to injured location, dividing into mature

**Discussion**

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**Figure 2.** The identification of EPCs by double positive staining with Dil-acLDL and FITC-UEA-1. The laser scanning confocal microscope (LSCM) demonstrated that the cells displayed red cytoplasm while taking up Dil-acLDL on day 7 of the culture (A), green cytomembrane when binding FITC-UEA-1 (B), and orange when positively stained with Dil-acLDL and FITC-UEA-1 (C), and blue when staining with DAPI in nuclear localization (D). The scale bar represents 10 μm. EPCs – endothelial progenitor cells; Dil – 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; acLDL – acetylated low density lipoprotein; FITC – fluorescein isothiocyanate; UEA-1 – ulex europaeus agglutinin-1; DAPI – 4',6-diamidino-2-phenylindole.
Figure 3. The comparisons of EPCs functions. The proliferation (A), adhesion and secretion (B) of EPCs were significantly lower in the CSE+Dec group and CSE group compared with the controls. However, the proliferation, adhesion and secretion of EPCs were significantly higher in the CSE+Dec group compared with the CSE group. Data are represented as mean ±SD. EPCs – endothelial progenitor cells; Dec – Decitabine. * P<0.01 compared with controls; @ P<0.05 compared with CSE+Dec group; # P<0.01 compared with CSE+Dec group.

Figure 4. The expression levels of eNOS and p16(INK4a) in EPCs. There were pictures of the expression levels of the eNOS protein and mRNA (A, B). The expression levels of eNOS protein and mRNA in EPCs were significantly lower in the CSE+Dec group and CSE group compared with the controls. However, the expression levels of eNOS protein and mRNA in EPCs were significantly higher in the CSE+Dec group compared with the CSE group. The pictures of the expression levels of the p16(INK4a) protein and mRNA (A, C) showed that the expression levels of p16(INK4a) protein and mRNA in EPCs were significantly higher in the CSE+Dec group and CSE group compared with the controls. However, the expression levels of p16(INK4a) protein and mRNA in EPCs were significantly lower in the CSE+Dec group compared with the CSE group. Data are represented as mean ±SD. eNOS – endothelial nitric.
endothelial cells, keeping the integrity of the endothelial monolayer, and repair and regeneration of various tissues and organs [4,6,20]. Accumulating evidence indicates that EPCs derived from bone marrow contribute to re-endothelialization of injured vessels, as well as neo-vascularization of ischemic lesions, in either direct or indirect pathways under physiological or pathological conditions [21,22]. Normal proliferation of EPCs is essential to maintain sufficient numbers of EPCs in postnatal vasculogenesis. Normal migration, adhesion, and secretion are helpful for transendothelial motivation, homing to impaired location, and effective repair [23]. As the surrogate of cigarette smoke, CSE can directly induce superoxide generation [24]. The dysfunction of EPCs observed in the present study is consistent with what seen in chronic obstructive pulmonary disease patients [25]. EPCs demonstrate impairment due to CSE.

Nitric oxide (NO) is a major regulator of blood vessel homeostasis and is synthesized by eNOS [7]. Endothelial nitric oxide synthase (eNOS) was first found in vascular endothelial cells [26], eNOS-derived NO serves as an endogenous vasodilator, platelet inhibitor, antioxidant, and regulator of vascular endothelium [8]. A deficiency of eNOS-derived NO exacerbates the early stage of non-alcoholic steatohepatitis pathogenesis in a mouse model via the regulation of hepatic tissue blood flow [27]. eNOS is important in the progress of neovasculogenesis [28] and may be involved in various disorders by various signaling pathways [29–31]. eNOS plays a central role in the maintenance of endothelial homeostasis [32]. eNOS(-/-) mice developed increased pulmonary arterial pressure after 6-month cigarette smoke exposure, and this was associated with vascular remodeling [33]. Promoted eNOS expression and NO synthesis in/around the regenerated tissues were observed in diabetic skin ulcers, leading to accelerated vascularization, elevated blood supply, and rapid wound healing [34]. In the present study, the NO level was decreased in CSE-induced EPCs culture supernatant, and the change in NO level, together with the changes of EPCs proliferation and adhesion, was consistent with the change in the expression of eNOS in EPCs, suggesting that the reduced level of eNOS may contribute to the dysfunction of EPCs induced by CSE.

p16(INK4a) has the capacity to arrest cells in the G1-phase of the cell cycle, which is often considered to be critical for establishing a senescence-like growth arrest [35]. There was evidence [36] that the expression of p16(INK4a) in aged cells may be more than 10 times that of the young cells. Inserting p16(INK4a) cDNA in normal fibroblasts slowed cell growth, aggravated nonenzymatic glycosylation, increased senescence-associated β-galactosidase positivity, and shortened telomere length. On the contrary, by inserting antisense p16(INK4a), a significant delay of several senescent features could be observed and the life span of fibroblasts was significantly extended [36]. It has been also demonstrated that low expression of p16(INK4a) was a predictor of greater in vitro lifespan and growth potential of mesenchymal stem cells [37]. Senescence is always accompanied by decreased function. In our study, decreased function of EPCs, decreased expression of eNOS, and increased expressions of p16(INK4a) were observed simultaneously in EPCs induced by CSE. This finding may be explained by the accelerated senescence of EPCs due to the oxidative stress and the increased expression of p16(INK4a), leading to the arrest of the cell cycle at G1 phase.

The advent of epigenetic studies makes it possible to gradually elucidate pathophysiological mechanisms of some diseases. Dec is a deoxynucleoside analog of cytidine, in which the carbon 5 position of the pyrimidine ring is substituted by nitrogen [38]. It is an inhibitor of DNA methyltransferase, which triggers demethylation, leading to changes in gene reactivation [16], sparking interest in its use as a potential therapeutic agent. It has been demonstrated that the demethylation treatment can protect against CSE-induced emphysema in an animal model [14,15]. In our study, the dysfunction of EPCs, decreased expression of eNOS, and increased expression of p16(INK4a) caused by CSE could be partly reversed by Dec. This finding suggested that the mechanism of DNA methylation may be involved in the dysfunction of EPCs. The molecular mechanism of active demethylation in mammalian cells is not clear, but seems to be linked to the DNA repair mechanism [39].

Conclusions

We demonstrated that CSE can induce EPCs dysfunction with decreased eNOS expression and increased p16(INK4a) expression. Furthermore, Dec can alleviate the changes in the expression of eNOS and p16(INK4a), and protect against the EPCs dysfunction caused by CSE. These results suggest that the decreased eNOS expression and increased p16(INK4a) expression may contribute to EPC dysfunction caused by CSE, and the mechanism of methylation, one of the most common epigenetic mechanisms, may be involved in EPC dysfunction caused by CSE. The exact mechanism by which eNOS and p16(INK4a) regulate EPC function should be further investigated.
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