Cigarette smoke extract induces aberrant cytochrome-c oxidase subunit II methylation and apoptosis in human umbilical vascular endothelial cells

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Submitted 30 June 2014; accepted in final form 4 December 2014

Cigarette smoke extract induces aberrant cytochrome-c oxidase subunit II methylation and apoptosis in human umbilical vascular endothelial cells. Am J Physiol Cell Physiol 308: C378–C384, 2015. First published December 10, 2014; doi:10.1152/ajpcell.00197.2014.—Cigarette smoke-induced apoptosis of vascular endothelial cells contributes to the pathogenesis of chronic obstructive pulmonary disease. However, the mechanisms responsible for endothelial apoptosis remain poorly understood. We conducted an in vitro study to investigate whether DNA methylation is involved in smoking-induced endothelial apoptosis. Human umbilical vascular endothelial cells (HUVECs) were exposed to cigarette smoke extract (CSE) at a range of concentrations (0–10%). HUVECs were also incubated with a demethylating reagent, 5-aza-2′-deoxycytidinem (AZA), with and without CSE. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay and flow cytometry using annexin V-FITC/propidium iodide staining. We found that CSE treatment significantly increased HUVEC apoptosis in a dose- and time-dependent manner. Quantitative real-time RT-PCR and immunoblot revealed that CSE treatment decreased cytochrome-c oxidase subunit II (COX II) mRNA and protein levels and decreased COX activity. Methylation-specific PCR and direct bisulfite sequencing revealed positive COX II gene methylation. AZA administration partly increased mRNA and protein expressions of COX II, and COX activity decreased by CSE and attenuated the toxic effects of CSE. Our results showed that CSE induced aberrant COX II methylation and apoptosis in HUVECs.

apoptosis; cytochrome-c oxidase subunit II; DNA methylation; endothelial cells; cigarette smoke; apoptosis

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is a growing cause of chronic morbidity and mortality and is expected to be the third worldwide leading cause of death by 2020 (22). This disease is characterized by progressive and poorly reversible airflow restriction, associated with an abnormal inflammatory response of the lungs to noxious particles, particularly cigarette smoke. Recently, a great deal of attention has been paid to comorbidities of COPD, such as cardiovascular disease, diabetes mellitus, body weight, and osteoporosis. Although it has long been established that cigarette smoke induces apoptosis (2), the underlying mechanisms are still poorly understood. The importance of vascular changes, especially endothelial apoptosis within both the pulmonary and systemic circulation, has recently received significant attention (5, 19, 20). Endothelial apoptosis is positively associated with smoking status and intensity (5, 10, 31, 32). However, endothelial apoptosis persists after smoking behavior ceases (17), and the mechanisms responsible for this cellular damage remain undefined. Alternative studies implicated cigarette smoke extract (CSE)-mediated necrosis rather than apoptosis (7, 32).

Mitochondrial changes are involved in the process of apoptosis. Cytochrome-c oxidase (COX), or mitochondrial complex IV, is the last key enzyme in the mitochondrial respiratory chain (MRC), catalyzing the transfer of electrons from cytochrome-c to molecular oxygen (9). COX dysfunction directly affects MRC function and subsequently leads to increased mitochondrial electron leakage. Mitochondrial proapoptotic factors such as cytochrome-c and apoptosis-inducing factors are released into the cytoplasm, hindering cellular energy supply and disrupting cellular oxidation/reduction reactions. These events constitute important steps toward apoptosis and underline the pivotal role of COX (21). COX is composed of 13 subunits, and the second subunit (COX II) contains the cytochrome-c-binding site, a key component of its catalytic core, responsible for the major functions of COX (26). COX dysfunction is very common in COPD (23). COX activity is decreased in both locomotor and respiratory muscles of patients with COPD (24). Decreased COX II levels in the lungs of patients with COPD are associated with pulmonary endothelial apoptosis (8). Some substances in cigarette smoke were found to inhibit COX II expression and to subsequently induce COX dysfunction and apoptosis; however, the specific mechanisms responsible for this activity remain unclear (1, 38).

Recently, the gene-regulating function of DNA methylation has attracted great interest. Changes in DNA methylation patterns, particularly in the promoter region of genes, have profound effects on gene expression. Aberrant DNA methylation in multiple genes was observed in smokers (29). Therefore, we conducted this study to investigate whether DNA methylation plays a role in smoking-induced endothelial apoptosis in COPD.

MATERIALS AND METHODS

Cell culture and treatment. Commercial human umbilical vascular endothelial cells (HUVECs) were obtained from the American Type Cell Culture Collection (ATCC, lot no.: CRL-1730) and cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (Invitrogen) and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO₂. After serum starvation for 24 h, HUVEC medium was supplemented with CSE at the indicated concentrations and for the indicated time periods. To investigate the role of DNA methylation in CSE-
induced apoptosis, after serum starvation, HUVECs were pretreated with 1 μM 5-aza-2'-deoxycytidin (AZA), a demethylating agent, for 48 h, followed by cotreatment with 1 μM AZA and 2.5% CSE for 24 h. During this exposure, culture medium was replaced every 24 h to prevent the depletion of essential nutrients. Cells were harvested for the determination of apoptosis, COX II expression, and caspase-3 and COX activity.

Preparation of CSE. CSE was prepared by a modification of a previously published method (31). Smoke of one unfiltered cigarette (Furong; China Tobacco Hunan Industrial, Changsha, Hunan, China) was bubbled through 25 ml of PBS using a vacuum pump. Tar content was 12 mg/cigarette, smoke nicotine was 10 mg/cigarette, and smoke carbon monoxide was 14 mg/cigarette. This 100% CSE solution was adjusted to pH 7.4 and filtered through a 0.22-μm-pore filter to remove bacteria and large particles. CSE was freshly prepared within the 30 min preceding each experiment.

Apoptosis determined by annexin V and propidium iodide staining. Apoptosis was determined using an annexin V-FITC apoptosis detection kit (Biossea Biotechnology, Beijing, China) according to the manufacturer’s instructions. Cells were harvested by centrifugation, resuspended in binding buffer, and successively incubated with 10 μl annexin V-FITC and 5 μl of propidium iodide (PI) at room temperature for 15 min protected from light. Apoptosis was determined by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA). Annexin V was set as the horizontal axis, and PI was set for the vertical axis. Cells staining positive for PI and negative for annexin V were considered mechanically damaged. Cells staining negative for PI and positive for annexin V were considered early apoptotic. Cells staining positive for PI and annexin V were considered late apoptotic or necrotic cells. Cells staining negative for PI and positive for annexin V were considered normal.

TUNEL assay. To assess apoptosis, a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed with an in situ Cell Death Detection Kit POD (Roche Molecular Systems, Pleasanton, CA), according to the manufacturer’s instructions. FITC-labeled nucleotides were incorporated into sites of DNA breakage by terminal deoxynucleotidyl transferase, detected by a horseradish peroxidase (HRP)-conjugated anti-FITC antibody, and visualized with a peroxidase-substrate reaction. The TUNEL assay was also performed without terminal deoxynucleotidyl transferase as a negative control. Counterstaining was performed with hematoxylin and eosin. TUNEL-positive cells, considered to be apoptotic, were counted under Zeiss Axioskop 40 Trinocular Microscope with fluorescence (Germany). Four slides were prepared for each condition under analysis. Five fields with similar cell density were randomly selected within each slide, and apoptotic cells were counted by a colleague blinded to the study at a magnification of ×200. The apoptosis index (AI) was calculated as follows: AI = (number of apoptotic cells/number of total cells) × 100%.

Detection of COX and caspase-3 activity. COX activity assay kit was purchased from Genmed (Shanghai, China). Total soluble proteins extracted from HUVECs were used for the measurement of COX activity with a spectrophotometer (DU-70 spectrophotometer; Beckman Coulter, Fullerton, CA) detecting the extinction coefficient of the oxidation of reduced cytochrome-c at 550 nm. COX-specific activity was determined by calculating the absolute value of the difference between OD at 0 and 60 s (OD60s - OD0s).

Caspase-3 activity was assessed using caspase-3 assay kit (KeyGEN, Nanjing, China), according to the manufacturer’s instructions. HUVECs were harvested by centrifugation and resuspended in 50 μl cold lysis buffer containing 0.5 μl diithiothreitol (DTT) and incubated on ice for 30 min. After centrifugation, the supernatant containing total protein (50 μl) was incubated with 2× reaction buffer containing 0.5 μl DTT and 5 μl caspase-3 substrate at 37°C for 4 h protected from light. Caspase-3 activity was determined by spectrophotometer at 400 nm. Lysis buffer and reaction buffer were used as blank controls. Caspase-3 activity induced by CSE was calculated as the OD of treated cells divided by the OD of control samples.

Immunoblot. Cell lysates were prepared using RIPA lysis buffer (Solarbio, Beijing, China), according to the manufacturer’s instructions. Proteins were quantified using a BCA Protein Assay Kit. Protein (50 μg) was separated on 12% SDS-PAGE and then transferred to a PVDF membrane (Pall Life Sciences, Ann Arbor, MI). After being blocked overnight at 4°C with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween, membranes were incubated with monoclonal mouse anti-human COX II (Molecular Probes, Eugene, OR) dissolved in 1% BSA at a concentration of 1 μg/ml, at room temperature for 2 h, followed by HRP-conjugated rabbit anti-mouse (KPL, Gaithersburg, MD). Blots were developed with the BeyoECL Plus substrates (Beyotime Bio), according to the manufacturer’s instructions, and the membrane was exposed to a Kodak film. Band intensity was quantified using Quality One software (Bio-Rad, Hercules, CA). The expression of COX II protein was normalized to the corresponding expression of β-actin as an internal control (Beyotime Bio).

Quantitative real-time RT-PCR. Total RNA was extracted using Trizol purchased from Invitrogen according to standard procedures, and real-time PCR was performed with a random hexamer primer and RevertAid M-MuLV reverse transcriptase (RevertAid First Strand cDNA Synthesis Kit; Fermentas China, Shenzhen, China). Primers were obtained from Invitrogen. COX II cDNA was amplified using the forward primer 5'-ACCACCTTTCACCGCTACAGCA-3' and the reverse primer 5'-TAATTCTAGGACGATGGGCATG-3'. β-Actin amplification used the forward primer 5'-CCAACCGGAAGATGAGA-3' and the reverse primer 5'-CCAGAGGCTACAGGATAG-3'. Total RNA solution (2 μl) was added to 18 μl of DyNaMo SYBR Green qPCR Master Mix from Finnzymes (Espoo, Finland) and primers for amplification. Real-time PCR was performed using an ABI 7500 Real-Time PCR System (ABI, Foster City, CA) using SYBR Green I as the detection dye. Each sample was run in triplicate, and threshold cycle (Ct) values were averaged from each reaction. First, \( \Delta Ct = Ct_{\text{Gene}} - Ct_{\beta-actin} \). Then, \( \Delta Ct = \Delta Ct_{\text{Treated}} - \Delta Ct_{\text{Control}} \). Last, 2\(^{-\Delta Ct}\) was calculated to represent the relative mRNA expression of target genes. β-Actin was used as an internal control.

Methylation-specific PCR and bisulfite sequencing. Total DNA was extracted from HUVECs using a conventional phenol/chloroform/isoamyl alcohol and ethanol precipitation method and subsequently converted by bisulfite using EZ DNA Methylation kit (Zymo, Beijing, China), according to the manufacturer’s instructions. Two pairs of primers were purchased from Invitrogen with one pair specific for methylated (M) and the other for unmethylated (U) COX II as follows: M forward primer: 5'-GGTTAAATTTATATTTATATTTAT-3'; M reverse primer: 5'-AAATATAATTCTCTTCTATCTTTAAATTTA-3'; anticipated product size was 752 bp; U forward primer: 5'-GTGAAAGTTTTATATGTTATGATGAT-3'; U reverse primer: 5'-AGTTATGCTTTACAGTGGGCTCTAGA-3'; (anticipated product size was 789 bp). PCR was conducted using a PCR kit (Fermentas, Burlington, Ontario, Canada). Water was substituted for DNA as a negative control, and SsI methylase-treated DNA (New England Biolaboratories, Beverly, MA) was used as a positive control. After amplification, each PCR product was visualized using a 2% agarose gel. PCR products of methylation-specific PCR (MSP)-positive groups were sent for direct bisulfite sequencing. The 789-bp fragment contained 23 CpG dinucleotide sites, which can be methylated. Five samples randomly selected from each group were sent for direct bisulfite sequencing. The rate of methylation was calculated as follows: number of methylated sites/total possible methylation sites.

Statistical analysis. Statistical analysis was conducted using SPSS software 16.0 (SPSS, Chicago, IL). Data are expressed as means ± SD. Groups were compared using a one-way ANOVA. The correla-
tion between apoptosis and COX activity was assessed using Pearson correlation. A $P$ value $\leq 0.05$ was considered significant (two tailed).

RESULTS

CSE induced apoptosis of HUVECs. The TUNEL assay indicated that CSE induced HUVEC apoptosis in a dose- and time-dependent manner. After 24 h of treatment, apoptosis was detected at 2.5% CSE ($P < 0.05$) (Fig. 1A). The apoptosis rates determined by annexin V/PI were similar to those achieved with the TUNEL assay (Fig. 1B). After incubation with 2.5% CSE, a time-related increase of CSE-induced apoptosis was observed from 6 h ($P < 0.05$ at 6 h; $P < 0.01$ at 12 and 24 h) (Fig. 2B). Furthermore, Pearson correlation analysis suggested that CSE-mediated HUVEC apoptosis was negatively correlated with COX activity ($r = -0.88, P < 0.01$).

Effects of AZA pretreatment on apoptosis, COX activity, and COX II expressions in HUVECs. As shown in Fig. 3A, apoptosis was significantly increased in CSE-treated HUVECs ($P < 0.01$ vs. controls), whereas AZA treatment significantly reduced the apoptosis caused by CSE ($P < 0.01$ vs. CSE-treated HUVECs). COX activity was decreased in CSE-treated HUVECs ($P < 0.05$ vs. controls), and AZA treatment reversed CSE inhibition of COX ($P < 0.01$) (Fig. 3B). COX II mRNA and protein levels were also significantly decreased in CSE-treated HUVECs ($P < 0.01$ vs. controls), and, again, AZA reversed this reduction in COX II mRNA and protein ($P < 0.01$ vs. CSE-treated HUVECs) (Fig. 3, C and D). AZA alone had no effect on these parameters compared with the control group. Figure 3E presents a typical immunoblot of COX II protein expression.

Fig. 1. Effect of cigarette smoke extract (CSE) on apoptosis in human umbilical vascular endothelial cells (HUVECs). HUVECs were cultured with 0–10% CSE for 24 h, and apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (A) and annexin V/propidium iodide (PI) staining by flow cytometry (B). HUVECs were cultured with and without 2.5% CSE for the indicated times, and apoptosis was detected by TUNEL (C). Caspase-3 activity was assessed in HUVECs treated with 0–10% CSE for 24 h (D). Data shown represents means ± SD from 3 or 4 independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. negative control (HUVECs without CSE); # $P < 0.05$ vs. 2.5% CSE-treated HUVECs, HLog, homolog.

Fig. 2. Effect of CSE on cytochrome-c oxidase (COX) activity in HUVECs. A: HUVECs were cultured with 0–10% CSE for 24 h, and COX activity was detected by spectrophotometry. B: HUVECs were cultured with 2.5% CSE for the indicated time periods, and COX activity was detected by spectrophotometry. Data shown represent means ± SD from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. negative control (HUVECs treated without CSE).
Effect of AZA pretreatment on COX II methylation in HUVECs. With the use of MSP, methylated COX II was detected in HUVECs treated with CSE and cotreated with AZA and CSE but not in control HUVECs (Fig. 4A). Direct bisulfite sequencing was performed to validate MSP reliability and indicated that no CpG site was methylated in control (Fig. 4B) and AZA-treated HUVECs. However, several CpG sites were methylated in the COX II gene in CSE-treated and CSE plus AZA-cotreated HUVECs (Fig. 4C and D). The average rates of methylation in CSE-treated and AZA plus CSE-cotreated HUVECs were 26.3 ± 2.4% and 15.6 ± 1.9%, respectively.

DISCUSSION

Our study demonstrated that CSE exposure induced apoptosis and a reduction of COX activity in HUVECs in a dose- and time-dependent manner, along with a decrease in COX II mRNA and protein expression. The effects of CSE were partly abolished by AZA, a demethylating reagent. With the use of MSP and bisulfite sequencing, methylated COX II was detected in CSE-treated HUVECs but not in controls and AZA-treated HUVECs.

Products of cigarette smoke absorbed into the systemic circulation can injure the arterial endothelial cells lining the pulmonary vessels. Endothelial apoptosis is markedly increased in smokers with COPD compared with nonsmokers or smokers without airway obstruction (5, 19, 20). Meanwhile, endothelial apoptosis induced either by directly targeting these cells (28) or by indirectly blocking the VEGF-VEGFR pathway (2) contributes to COPD pathogenesis. Moreover, interventions against endothelial apoptosis effectively delay emphysema development (4, 37), highlighting a crucial role of endothelial apoptosis in COPD pathogenesis. We found that CSE induced HUVEC apoptosis in a dose- and time-dependent manner, which was consistent with previous studies. Wang et al. (31) observed this dose-dependent relationship between CSE and HUVEC apoptosis and detected similar changes in caspase-3 activity that we detected in our study. This effect has also been reported elsewhere (11, 36). Various mechanisms of action have been proposed as responsible for CSE-mediated HUVEC apoptosis, including accumulation of misfolded proteins in the endoplasmic reticulum (7) or alterations in the JNK pathway (13). Although some studies proposed that CSE induces HUVEC apoptosis or necrosis directly, the associated process may depend on the concentration of CSE used. Low concentrations of CSE may induce HUVEC apoptosis, whereas high levels of CSE may induce necrosis (7, 18, 32). In the present study, caspase-3 activity was increased in the 0.5–2.5% CSE range but was decreased in the 1.0–2.5% range. Two factors could partly explain this observation. First, HUVECs exposed to 0.5–10% of CSE may show late apoptosis and varying degrees of necrosis, which can be shown by annexin V/PI. Indeed, results showed that early apoptotic cells in the lower right quadrant of the flow cytometric dot plot were sharply increased from 1.0% to 2.5% of CSE. In addition, late apoptotic or necrotic cells in the upper right quadrant were increased over time. Secondly, a noncaspase-3-dependent apoptosis pathway might be involved but was not assessed in the present study (6, 12, 25, 32, 33).

The specific mechanisms underlying CSE-induced endothelial apoptosis are still unclear, and mitochondria may play an important role. In fact, several key events in apoptosis are related to mitochondria (30). Signals converge on the mito-
chondria to trigger or inhibit these events and/or their downstream factors, delineating several major apoptotic pathways. The MRC is recognized as the basis of mitochondrial functions. Alonso et al. (1) demonstrated that the MRC was susceptible to CSE at multiple sites and that COX is more sensitive than other components. We have previously reported (8, 35) a negative correlation between COX activity and pulmonary endothelial apoptosis and suggested that COX-mediated endothelial apoptosis was associated with smoking-associated pulmonary endothelial apoptosis in COPD. In this study, we demonstrated that CSE dose, COX activity, and apoptosis were correlated. Furthermore, we found a decrease in COX II mRNA and protein levels induced by cigarette smoke, indicating a potential downregulation of COX II expression at the translation level (DNA mutation, elimination, rearrangement, methylation, etc.), supported by a previous study by Masayesva et al. (17). Chemical substances in cigarette smoke, such as CO and NO, were proposed to cause COX II suppression. Alonso et al. (1) described a detrimental effect of CO on COX activity. Zhang et al. (38) reported that NO might be associated with COX suppression in smokers. However, both studies failed to identify that the mechanism by CSE reduces COX expression and induces endothelial apoptosis even decades after smoking cessation (17). Individuals with a smoking history (either ex-smokers or current smokers) are susceptible to abnormal DNA methylation (29). Moreover, DNA methylation patterns are inheritable. Once DNA methylation develops, it will stably propagate, leading to a persistent downregulation of gene expression (15). Therefore, we speculated that DNA methylation might account for long-term abnormalities in COX expression and endothelial apoptosis in ex-smokers. We employed standard MSP and bisulfite sequencing to demonstrate that COX II methylation was present in CSE-treated HUVECs. AZA, a demethylation agent, effectively upregulated CSE-suppressed COX II expression, supporting our hypothesis. Clinical trials have previously demonstrated that AZA treatment is effective in myelodysplastic syndrome, leukemia, and thoracic malignancies (27). Potential involvement of abnormal DNA methylation in COPD pathogenesis may prompt the use of AZA in the treatment of this disease.

Fig. 4. Effect of AZA pretreatment on COX II methylation in CSE-treated HUVECs. After serum starvation, HUVECs were pretreated with 1 μM AZA for 48 h and further treated with 2.5% CSE and 1 μM AZA for 24 h. A: total DNA was extracted, converted by bisulfate, and subsequently subjected to PCR. PCR products were separated using a 2% agarose gel. PC, positive control, DNA treated by SssI methylase; NC, negative control; lanes M, amplified product with primers recognizing methylated COX II; lanes U, amplified product with primers recognizing unmethylated COX II. PCR products of methylation-specific PCR-positive groups were sent for direct bisulfite sequencing. No CpG site was methylated in control (HUVECs treated without CSE and AZA) (B) and AZA-treated HUVECs, whereas several CpG sites were methylated in COX II DNA in CSE-treated HUVECs (C) and CSE- and AZA-treated HUVECs (D).
these amounts could influence methylation patterns in specific cells in humans, such as HUVECs. This issue is complicated by the wide variety of tobacco brands and modifications. However, the present study used the CSE from a single cigarette as the 100% starting solution, which was diluted for studies. According to the content of a single cigarette used in the present study, this 100% CSE solution should contain about 12 mg of tar, 10 mg of nicotine, and 14 mg of carbon monoxide. It is reasonable to assume that the lungs and circulatory system will be exposed to the highest levels of these compounds, but the exact amount of CSE reaching distant organs is unknown. Nevertheless, some studies in animals showed that CSE exposure affected the methylation status in mice tests (14, 34). Whole human genome studies showed that CSE affected the methylation status of the whole human genome (29). Of course, the first organs being reached by CSE, the lungs, show significant changes in methylation after exposure to CSE (3, 16). These studies showed that physiological levels of CSE reaching different target organs induced changes in methylation status that participated in pathological processes. However, the present study was performed in cultured cells. Further experiments should be conducted in animals to determine the exact amount of CSE compounds reaching specific organs.

Nevertheless, there are still several limitations in our study. Although in vitro CSE exposure is a simple, reproducible, and widely used method, it cannot reflect the in vivo effects of cigarette smoking, therefore limiting the immediate translation of our results to humans. In addition, because CSE composition is very complex and varies between tobacco brands, trying to extrapolate the identity and amount of the chemicals that reach specific target organs is nearly impossible without studies designed specifically to answer this issue. Furthermore, different legislation between countries and the continuous drive for new tobacco modifications according to new laws alleviate the necessity of determining the exact composition of the CSE, and we considered CSE as a single entity irrespective of tobacco brands. The TUNEL assay and flow cytometry using annexin V-FITC/PI staining does not discriminate precisely between necrosis and late apoptosis, and further clarification of these processes will require additional methods.

In conclusion, the present study highlighted the potential role of DNA methylation in CSE-induced COX II expression and COX activity suppression and endothelial apoptosis.

ACKNOWLEDGMENTS

We thank Dr. Cheng Zhang for language help and writing assistance.

GRANTS

This work was supported by grants from the National Natural Science Foundation of China (no. 30770931, no. 81100301, and no. 30800503) and the Hunan Province University Innovation Platform Open Fund (09K004).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


