Original Article

Tobacco smoke induces MMP-2 activation in rat aortic vascular smooth muscle cells via Egr1

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Abstract: Objective: The current study aimed to investigate whether tobacco smoke activates MMP-2 in rat aortic vascular smooth muscle cells via Egr1. Methods: Cigarette smoke extract (CSE) was prepared by dissolving cigarettes in 10 mL DMEM. About 2,000 cells were planted in each well of 96-well plates. They were divided into 8 groups, with 8 wells in each group. Each group was treated with different concentrations of CSE for 24 hours. Expression of MMP-2, Egr-1 mRNA, and MT1-MMP was compared. Results: Compared with that in the control group, 5% CSE slightly increased MMP-2 expression, while 10% CSE significantly increased MMP-2 expression. MMP-2 expression was the highest under the influence of 10% CSE for 24 hours. Reverse transcription PCR showed that CSE and Egr-1 mRNA expression exhibited time- and concentration-dependent relationships. Western blotting was performed with 10% CSE concentrations. Protein expression of Egr-1 reached its peak at 4 hours, decreasing significantly at 16 hours. Under the action of 10% CSE, Egr-1 mRNA expression significantly decreased, comparing cells transfected with Egr-1 siRNA with those transfected with controls. Moreover, mRNA levels of MMP-2 decreased and the gelatin activity of MMP-2 decreased. The current study also analyzed protein expression of MT1-MMP in A10 cells treated with 10% CSE using Western blotting. Expression increased rapidly. Expression of mRNA and protein expression of MT1-MMP decreased, comparing A10 cells transfected with Egr-1 siRNAs with cells transfected with negative control siRNAs. Conclusion: CSE induces expression and activation of MMP-2 in rat vascular smooth muscle cells. Regulation of MT1-MMP and MMP-2 function by Egr-1 also suggests that Egr-1 plays an important role in tobacco smoke-induced atherosclerosis.

Keywords: MMP-2 activation, Egr1, aortic vascular smooth muscle cells, tobacco smoke

Introduction

Smoking is an independent risk factor for atherosclerosis (As) [1]. However, the mechanisms of As caused by smoking remain unclear. MMP-2 plays an important role in migration, proliferation, and vascular remodeling of vascular smooth muscle cells (VSMC) [2]. The current study aimed to investigate the cell signaling mechanisms of tobacco smoke-induced changes in VSMC MMP-2 expression. Results demonstrated that tobacco smoke extract (cigarette smoke extract, CSE) action on rat VSMC induces protein expression of MMP-2. In addition, CSE induced Egr-1 mRNA and protein expression in time- and concentration-dependent manners [3]. Application of Egr-1 siRNA inhibited CSE-induced Egr-1 and MMP-2 expression. Changes in MT1-MMP expression were also observed. Egr-1 has a specific binding site on the MT1-MMP promoter. This binding can regulate MT1-MMP expression [4]. Results suggest that Egr-1 plays an important role in tobacco smoke-induced VSMC MMP-2 and MT1-MMP expression in rats. This pathway could be a potential mechanism of As induction by smoking.

Causes of vascular diseases include multiple genetic and environmental factors. It is known that smoking is an independent risk factor for cardiovascular disease. Most studies have focused on oxidative stress injuries caused by reactive oxygen species induced by tobacco smoke. However, the specific molecular mechanisms remain unclear. Migration and proliferation of VSMC plays an important role in occurrence and development of As [5]. Di Luzzo et al. found that, as the main component of tobacco smoke, nicotine has potential stimulation effects on the migration of VSMC [6]. Carty et al. reported that a certain concentration of nicotine can promote the proliferation of VSMC [7]. Matrix metalloproteinases (MMPs) are an
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**Table 1. Egr-1 siRNAs sequences used in the present study**

<table>
<thead>
<tr>
<th>Egr-1 siRNA</th>
<th>The sequences of the siRNAs</th>
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<tr>
<td>1637400</td>
<td>Sense 5’-(CUCACUCCACUCCACUA) d (TT)-3’&lt;br&gt;Antisense 5’-(UAGUGGAUAGUGGAUGUAU) d (TT)-3’</td>
</tr>
<tr>
<td>1637401</td>
<td>Sense 5’-(CUCAUAUGCUCCUGUAAUA) d (TT)-3’&lt;br&gt;Antisense 5’-(UUAUUCAGAGCGAUGUCAG) d (TT)-3’</td>
</tr>
<tr>
<td>1637402</td>
<td>Sense 5’-(CCAGACUUAAAGCGUCUU) d (TT)-3’&lt;br&gt;Antisense 5’-(AAGAGCUUUAAAGCUCCUG) d (TT)-3’</td>
</tr>
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</table>

important family of zinc ion-related endopeptinases. They play an important role in vascular remodeling. Recent studies have found that membrane type 1 matrix metalloproteinase (MT1-MMP) can activate matrix metalloproteinase-2 (MMP-2) on the cell surface [8, 9]. It has been reported that increased expression of MMP-2 can promote migration and proliferation in a variety of cells (e.g., in fibroblasts, VSMC, and tumor cells) [10-12]. Early growth response factor-1 (Egr-1) is a member of the zinc finger protein transcription factor family [13]. Several extracellular signals can upregulate Egr-1 expression [14]. In turn, Egr-1 can regulate expression of multiple genes. Studies have shown that vascular injuries can stimulate Egr-1 and promote the migration and proliferation of VSMC [15]. It is currently believed that MMP-2 plays an important role in tobacco smoke action on VSMC. Wen Ning et al. [16] reported that tobacco smoke can increase MMP-2 levels by upregulating Egr-1 in human lung fibroblasts. It was speculated that there is a similar mechanism in rat VSMC. Unraveling the molecular pathways that regulate aortic VSMC migration and proliferation induced by tobacco smoke via Egr1 can lead to the development of drug therapies proving to be remarkably effective in reducing clinical As incidence.

**Materials and methods**

**Materials**

Polyclonal EGR-1 (sc-110), MMP-2 (sc-13594), and MT1-MMP (sc-12366) antibodies were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA).

**Methods**

**Cell culturing:** Rat A10 aorta VSMC cell lines were purchased from ATCC cell bank. DMEM medium containing 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100 μg/ml streptomycin was used to culture the cells in an incubator at 37°C with 5% CO₂ containing saturated humidity. The cells were digested and passaged with 0.25% pancreatin. The medium was changed every other day. Moreover, 18-24th generation of cells were used for the experiments. For cell infusion, the cells were incubated in serum-free medium 24 hours before the experiment. Every group was separated with about 2,000 cells in each well. Cells were separated the same in every group before the experiment. They were divided into 8 groups, with 8 wells in each group. Different CSE concentrations were used (0, 5%, 10%, 20%) or CSE of treatment for different time concentrations (0, 0.5, 1, 4, 24 hours).

**MTT analysis:** Cytotoxicity and proliferation were evaluated using the MTT method (kits were purchased from Amresco (Solon, OH)). MTT was conducted as follows. About 2,000 cells were seeded in each well of 96-well plates. They were divided into 8 groups, with 8 wells in each group. Each group was treated with different concentrations of CSE for 24 hours. Moreover, 5 mg/mL MTT was added to the wells for 4 hours. Dimethyl sulfoxide (150 μL) was added to each well. Observations were made at 570 nm wavelength using a microplate reader.

**Preparation of CSE:** The 3R4F cigarettes were purchased from the University of Kentucky in the United States. The protocol followed the same methods described by Ishii and colleagues [17]. CSE was prepared by dissolving cigarettes in 10 ml DMEM. It was defined as 100% concentration. Also, pH was adjusted to 7.4 before use. The solution was filtered with a 0.22 micron filter.

**Synthesis of Egr-1 siRNA and transient transfection of A10 cells:** Egr-1 siRNA with 21-base pairs was synthesized with negative control oligonucleotide sequence by BIONEER (Korea) (Table 1). The negative control oligonucleotide sequence was as follows: [sense 5’-(CCUACGCCAACAAUUUGCU) d (TT)-3’] [antisense 5’-(ACGAAAUUGGUGGCGUAGG) d (TT)-3’]. The target was its coding region. When the A10 cells were grown and reached 80-90% confluence, they were placed in serum-free medium to culture for 6 hours. Egr-1 siRNA (100 nM) and its negative control siRNA were transfected in cells.
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using Lipofectamine™ 2000. The transfection protocol was as follows: 100 nM Egr-1 siRNA and its negative control siRNA were incubated with 250 μL serum-free DMEM (containing 10 μL lipofectamine™ 2000) for 20 minutes. Next, A10 cells (1 mL DMEM medium) were added at 37°C for 24 hours.

Reverse transcription PCR: Total RNA was extracted using TRIzol Reagent (Invitrogen, CA, USA), according to manufacturer instructions. Reverse transcription PCR was performed using the TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa Dalian, China), according to manufacturer instructions. PCR conditions were: 94°C for 4 minutes; 94°C for 1 minute, annealing at 56°C (Egr-1), 58°C (MMP-2), 52°C (MT1-MMP), or 55°C (GAPDH) for 30 seconds, 72°C for 30 seconds, 35 cycles; 72°C for 10 minutes. The product was resolved by 1.5% agarose gel electrophoresis (100 V, 30 minutes), followed by EB staining and automated imaging analysis of the gel. Average optical density levels and the illuminating area of the electrophoretic band were measured using a fully automated image analysis system (Table 2).

Western blot analysis: Protein samples (80 μg, extracted from A10 cells) were subjected to 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was washed and blocked with 5% skim milk powder and incubated with a primary antibody (anti-Egr-1 (1:300), anti-MMP-2 (1:300), anti-MT1-MMP (1:300), and β-actin (1:500)) at 4°C overnight. HRP-IgG was used as a secondary antibody with which the membrane was incubated. ECL was used to visualize protein bands (Thermo Fisher Scientific Inc, Fremont, CA, USA). If necessary, the strip was stripped with antibody stripping liquid (KeyGen, Nanjing, China). Incubation with other antibodies was done if required. The experiment was repeated three times.

Chromatin immunoprecipitation (CHIP): A ChIP assay kit (Upstate USA) was used, according to manufacturer instructions. The process mainly involved crosslinking of chromosomal DNA and protein. The DNA was sheared into small fragments. Co-immunoprecipitation with Egr-1 antibody and PCR were used to determine relevant DNA sequences. PCR primers for MT1-MMP promoter sequence were: sense: 5'-TCTTGGCGTGTAATTGGATTC-3', anti-sense: 5'-TCTTTCTTCTGTCTTAGTCGG-3'. PCR conditions: 94°C for 4 minutes; 94°C for 1 minute, annealing at 48°C for 30 seconds, 72°C for 30 seconds, 35 cycles; 72°C for 10 minutes. Input was used for positive control.

Gelatin zymography: Gelatin zymography was used to analyze the activity of MMP-2 in the cell culture medium. The conditioned medium was dissolved in sodium dodecyl sulfate gel loading buffer (containing 0.01% sodium dodecyl sulfate) without boiling. After loading, the gel was electrophoresed with 10% sodium dodecyl sulfate (containing 0.1% gelatin) and polypropylene gel at 125 volts for 2 hours. The gel was washed twice with 100 mM distilled water (containing 2% triton) by shaking at room temperature for 30 minutes. The gel was then incubated in 100 mM reaction buffer (40 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 0.02% NaN₃) at 37°C for 12 hours. Staining was done with Coomassie brilliant blue R-250, then the gel was decolorized. The experiment was repeated three times.

**Statistical analysis**

Data are expressed as mean ± standard deviation (X ± s). The experiments were repeated three times. One-way ANOVA was used to analyze and compare parameters between different groups, using SPSS V17.0 software. The LSD method was used for pairwise comparisons. P < 0.05 indicates statistical significance.

**Results**

**Increased MMP-2 protein and activity induced by CSE in A10 cells**

Like other members of the MMP family, MMP-2 is secreted as a zymogen. It is then activated extracellularly by matriptase. To investigate the

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence 5’→3’</th>
<th>Size (bp)</th>
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<tr>
<td>Egr-1</td>
<td>ACTCCACTATCCACTATCAAAGCC</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>GTGGTCACTACGACTGAAATTACG</td>
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</tr>
<tr>
<td>MMP-2</td>
<td>GTCTGACCAAGGATATAGCC</td>
<td>465</td>
</tr>
<tr>
<td></td>
<td>AGACCGACTCATCTCCGTGG</td>
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</tr>
<tr>
<td>MT1-MMP</td>
<td>TGGTACGAGTGAACTCTTC</td>
<td>319</td>
</tr>
<tr>
<td></td>
<td>AGTAAAGCAGTCGCTGGGT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTGTGAGTATGTCGCTGGG</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>TCTTCTGAGTGGGCGATG</td>
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**Table 2. PCR primers used in the present study**
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Figure 1. Effects of CSE on MMP-2 protein expression and activation in A10 cells. CSE at various concentrations and treatment times was used on A10 cells. Western blotting (A, B) and gelatin zymography were adopted (C). (A) MMP-2 protein expression for CSE at different concentrations (0, 5%, 10%, 20%) for 24 hours; (B) MMP-2 protein expression for 10% CSE with different treatment times (0, 8 h, 16 h, 24 h, 48 h); (C) MMP-2 protein gelatin activity for 10% CSE with different treatment times (0, 16 h, 24 h, 48 h). *P < 0.05.

relationship between CSE and MMP-2, this study treated A10 cells with CSE, observing expression levels of MMP-2 protein by Western blotting. CSE increased expression of MMP-2 in a concentration-dependent manner. Compared with that in the control group, 5% CSE slightly increased MMP-2 expression, while 10% CSE significantly increased MMP-2 expression (Figure 1A). In addition, CSE exhibited time-dependent effects on MMP-2 expression. Compared with that in the control group, MMP-2 expression was the highest under the action of 10% CSE for 24 hours (Figure 1B). The current study evaluated the effects of 10% CSE on A10 cells via the MTT method. There were no significant differences in cell viability, compared with the control group, after 24 hours of CSE treatment. However, treatment with 40% CSE caused a decrease in cell viability. This study further analyzed MMP-2 gelatin activity via gelatin zymography. MMP-2 gelatin activity significantly increased, compared with that in the control group, after CSE treatment for 24 hours (Figure 1C). Current data indicates that CSE treatment on A10 cells can increase MMP-2 protein expression and gelatin activity.

mRNA and protein expression of Egr-1 induced by CSE

The current study observed the relationship between CSE and Egr-1. Reverse transcription PCR analysis revealed that CSE had time-dependent (Figure 2A) and concentration-dependent (Figure 2B) effects on Egr-1 mRNA expression. When Western blotting was used with 10% CSE concentrations, protein expression of Egr-1 reached its peak at 4 hours, decreasing significantly at 16 hours (Figure 1C).
Egr-1 siRNA inhibits MMP-2 activation induced by CSE

Aiming to evaluate the roles of Egr-1 in CSE-induced MMP-2 expression for A10 cells, this study used Egr-1 siRNA to inhibit Egr-1 protein expression. Under the action of 10% CSE, Egr-1 mRNA levels significantly decreased in cells transfected with Egr-1 siRNA, compared with that in the cells transfected with negative control siRNA (Figure 3A). Protein (Figure 3B) and mRNA expression levels of MMP-2 (Figure 3C), as well as gelatin activity, decreased (Figure 3D).

Egr-1 regulates MT1-MMP expression induced by CSE

Present results showed that Egr-1 is involved in CSE-induced MMP-2 activation. However, there is no regulatory element for Egr-1 in the promoter region of MMP-2. It has been reported that activation of MMP-2 depends on MT1-MMP zymogen and TIMP2. Moreover, the promoter region of MT1-MMP had an Egr-1-specific binding site. First, this study observed protein expression of MT1-MMP after 10% CSE’s treatment on A10 cells by Western blotting (Figure 4A). Protein expression of MT1-MMP increased rapidly after CSE treatment. It reached its peak at 16 hours, then decreased. Moreover, mRNA expression (Figure 4B) and protein expression (Figure 4C) of MT1-MMP decreased in A10 cells transfected with Egr-1 siRNA, compared with that in cells transfected with negative control siRNA.

The current study hypothesized that Egr-1 can regulate MT1-MMP expression by binding to a specific site in the latter’s promoter region. ChIP analysis was used for validation. When the A10 cells were grown and reached 80-90%
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confluence, the serum medium was replaced with a serum-free medium for 24 hours. ChIP assay was performed after 10 hours of stimulation with 10% CSE. Cross-linked purified

Figure 3. Egr-1 siRNA inhibits Egr-1 mRNA and protein expression and MMP-2 protein activation induced by CSE. EGR-1 siRNA or negative control EGR-1 siRNA was used to transfect A10 cells. After 24 hours, cells were treated with 10% CSE. When cells transfected with EGR-1 siRNA were compared with those transfected with negative control EGR-1 siRNA, (A) Egr-1 mRNA (B) Egr-1 protein, and (C) MMP-2 mRNA were significantly reduced; (D) MMP-2 gelatin activity comparison in both cells.
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Genomic DNA was used as an input control. Co-immunoprecipitation was conducted with Egr-1 antibodies. Related DNA sequences were identified by the PCR method. Present experiments showed that a specific MT1-MMP promoter sequence can be amplified after CSE treatment on A10 cells. Results also indicated that Egr-1 can bind directly to the MT1-MMP promoter. MT1-MMP in A10 cells was induced by CSE and MMP-2 activation was regulated by Egr-1 (Figure 5).

Discussion

Epidemiological studies have shown that smoking is a major risk factor for atherosclerosis [18]. Migration and proliferation of VSMC play a major role in the development and occurrence of atherosclerosis [19, 20]. Nicotine, a major component of tobacco smoke, can induce VSMC to migrate. Moreover, MMP-2 may also play a major role in human disease [6, 21]. Pauly et al. [22] found that MMP-2 mediates the vessel of VSMC to pass through the basement membrane. The chemotaxis of human VSMC is also dependent on the activity of MMP-2 [23, 24]. In in vivo experiments of a rat carotid stripping model, knockout of MMP-2 reduced the migration and intimal formation of VSMC [25, 26]. The current study showed that 10% CSE treatment on rat VSMC (A10) induced

![Figure 4](image-url). MT1-MMP expression induced by CSE via Egr-1. (A) Expression of MT1-MMP protein was observed by Western blotting after CSE treatment on A10; (B) mRNA expression; and (C) Protein expression of MT1-MMP after treatment of A10 cells (transfected with EGR-1 siRNA) with CSE.
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expression of MMP-2 protein. In addition, it increased the gelatin activity of MMP-2. Next, the intracellular mechanisms in this process were further elaborated.

Studies have shown increased Egr-1 expression in atherosclerotic plaques, along with some growth factors and extracellular signals, can increase transient Egr-1 protein expression in various blood vessel cells [27, 28]. With Egr-1 antisense technology, migration and proliferation of VSMC can be inhibited after vascular injuries [29]. In animals, fungi, and plants, RNA interference has become a powerful technique specifically inhibiting genetic expression [30, 31]. Present data indicates that CSE can rapidly increase Egr-1 expression. The interference technique of Egr-1 significantly inhibited MMP-2 activity induced by CSE.

MMP-2 is the major downstream effector of Egr-1. However, there is no specific binding site for Egr-1 on the promoter of MMP-2. Studies have shown that MMP-2 activation is associated with MT1-MMP expression [32], while the promoter of MT1-MMP has a binding site for Egr-1 [33]. MMP-2 activation is dependent on the trio of MT1-MMP, TIMP2, and MMP-2 zymogens [34]. The Sho research team reported that blood flow and shear forces increased MT1-MMP and TIMP2 levels, further activating MMP-2 [32]. The current study confirmed that CSE can continuously increase expression of MT1-MMP and MMP-2 in A10 cells. Their increase can be effectively inhibited by Egr-1 siRNA. Additionally, ChIP analysis suggested that the promoter sequence of MT1-MMP can be amplified. This indicates that the transcription factor, Egr-1, can specifically bind to the promoter of MT1-MMP. Expression of MMP-2 can be further regulated.

In summary, the current study showed that CSE induces expression and activation of MMP-2 in rat VSMC. During this process, to some extent, CSE activates transcription factor Egr-1, regulates MT1-MMP, and activates MMP-2. Changes in the extracellular matrix caused by CSE may play a role in the early stages of atherosclerosis. MT1-MMP and MMP-2 expression regulation by Egr-1 also demonstrates that Egr-1 plays an important role in tobacco smoke-induced atherosclerosis. However, these factors require further examination in future research.

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Disclosure of conflict of interest

None.

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References


Figure 5. Immunoprecipitation analysis of the binding of the transcription factor Egr-1 to the MT1-MMP promoter. The blank control group did not receive CSE, while the other group received 10% CSE. The cells were collected according to the ChIP protocol. The purified genomic DNA after cross-linking was used as a control (input). Egr-1 antibody was used for ChIP. Finally, the relevant DNA sequence was amplified by PCR. The experiment was repeated three times.


[29] Santiago FS, Atkins DG and Khachigian LM. Vascular smooth muscle cell proliferation and...
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