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RESEARCH ARTICLE

Up-regulation of contractile endothelin receptors by airborne fine particulate matter in rat mesenteric arteries via activation of MAPK pathway

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Abstract

Fine particle matters ($PM_{2.5}$) is a well-known risk factor for cardiovascular diseases. However, the underlying molecular mechanisms are largely unknown. Vascular hyper-reactivity plays an important roles in the pathogenesis of cardiovascular diseases. The present study was designed to investigate a hypothesis that $PM_{2.5}$ up-regulated endothelin receptors in mesenteric artery and the potential underlying mechanisms. Rat mesenteric arteries were cultured with $PM_{2.5}$. The artery contractile responses were recorded by a sensitive myograph. ET_B and ET_A receptor expressions of mRNA and protein were assessed by quantitative real-time PCR, Western blotting, and immunohistochemistry, respectively. Results showed that ET_B receptor agonist, sarafotoxin 6c induced a negligible contraction in fresh artery segments, while ET_A receptor agonist, ET-1 induced an obvious contraction. After organ culture, the contraction curve mediated by ET_B and ET_A receptors were shifted toward the left. $PM_{2.5}$ 1.0 μg/ml cultured for 16 h further enhanced ET_B and ET_A receptor-mediated contractile responses with a markedly increased maximal contraction. The organ culture enhanced ET_B and ET_A receptor mRNA and protein levels from fresh arteries, which were further increased by $PM_{2.5}$. The U0126 (MEK/ERK1/2 inhibitor) and SB203580 (p38 inhibitor) significantly attenuated both organ cultured-induced and $PM_{2.5}$-induced up-regulation of ET_B receptor. U0126 also suppressed organ culture-increased and $PM_{2.5}$-increased expressions of ET_A receptor. SB203580 only suppressed $PM_{2.5}$-induced enhanced expressions of ET_A receptor In conclusion, airborne $PM_{2.5}$ up-regulates ET_B and ET_A receptors of mesenteric artery via p38 MAPK and MEK/ERK1/2 MAPK pathways.

Keywords Fine particulate matter · Mesenteric artery · ET_B and ET_A receptors · MAPK pathway

Introduction

Fine particle matters ($PM_{2.5}$) refers to particulate matter in the air that with aerodynamic diameter ≤ 2.5 μm. Rapid industrialization and urbanization have led to an increase in particulate pollution, especially $PM_{2.5}$ pollution (Kodavanti et al. 2011). Due to their small particle sizes, $PM_{2.5}$ is deposited throughout respiratory tract and even enter the vascular microcirculation, causing lung diseases and cardiovascular diseases (MohanKumar et al. 2008; Schwarze et al. 2006). WHO reported that in 2016, around 3 million premature deaths linked to air pollution. Previous studies reported that air pollution risks on cardiovascular diseases are far greater than that on respiratory diseases. Numerous epidemiological studies suggested that exposure to $PM_{2.5}$ have been linked with cardiovascular morbidity and mortality (Hystad et al. 2013; Kodavanti et al. 2013; Pope et al. 2015). Accordingly, $PM_{2.5}$ might be regarded as a risk factor for hypertension. Lin et al. estimated that airborne $PM_{2.5}$ has been significant burden in hypertension in Chinese adults and about 12% of hypertension causes could be attributable to airborne $PM_{2.5}$ exposure (Lin et al. 2017). For each 10 μg/m^3 increase in $PM_{2.5}$, the odds

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ratio of hypertension was 1.084, which was the highest effect estimate observed among the morbidity studies (Pui et al. 2014). However, the cellular mechanism to explain how $PM_{2.5}$ induces enhanced risk of hypertension is still unknown.

Endothelin-1 (ET-1) is the most potent endogenous vasoconstrictor participating in the regulation of vascular functions (Yanagisawa et al. 1988), such as controlling the systemic blood pressure and/or local blood flow, played an important role in the pathogenesis of hypertension (Yanagisawa et al. 1988). Vascular contractile responses to ET-1 is mediated through two G-protein coupled receptors (GPCRs), endothelin type A (ET_A), and endothelin type B (ET_B). The ET_B receptors are further divided into two subtypes, ET_{B1} and ET_{B2} (Zhang et al. 2010). ET_A and ET_{B2} receptors are located on vascular smooth muscle cells (VSMCs) and induce vascular contraction. ET_{B1} receptors are located on epithelium cells and mediate relaxation via nitric oxide and prostacyclin release (Naline et al. 1999; Xu et al. 2014). The ET receptors especially ET_B receptors demonstrate plasticity in SMC contractility and are vulnerable to environmental influences (Adner et al. 1996). Previous studies reported that cigarette smoke induced ET_A (Cao et al. 2011; Zhang et al. 2017) and ET_B (Huang et al. 2013; Xu et al. 2008) receptor-mediated contractions in resistance arteries of rats. The major component of cigarette smoke is $PM_{2.5}$ (Garza et al., 2016). Therefore, we hypothesize that expose to airborne $PM_{2.5}$ may connect with up-regulation of ET receptors in vascular smooth muscles cells. The present study was designed, using an organ culture model, to demonstrate that $PM_{2.5}$ may up-regulate artery vascular ET receptors and to examine the intracellular signal mechanisms of $PM_{2.5}$-induced enhanced ET receptor expressions.

Material and methods

Chemicals

ET-1 and sarafotoxin 6c (S6c) were obtained from NeoMPS (NeoMPS SA. Strasbourg, France) and dissolved in 0.1% bovine serum albumin (Sigma. St. Louis, USA). c-Jun N-terminal kinase (JNK) inhibitor (SP600125), p38 inhibitor (SB203580), and extracellular signal-regulated protein kinase 1and 2 (ERK1/2) inhibitor (U0126) were obtained from Sigma and dissolved in DMSO. BQ-788 was obtained from Med chemexpress (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) were purchased from Thermo Fisher.

Animals

Male Sprague-Dawley rats (280 ± 10 g) were purchased from Experimental Animal Center of Xi'an Jiaotong University Health Science Center, China. All of the rats were fed a normal diet. All the experimental procedures were approved by the Ethics Committee of Xi'an Jiaotong University.

$PM_{2.5}$ sources and characterization

$PM_{2.5}$ samples were supplied by Prof. Zhen-Xing Shen. The $PM_{2.5}$ samples were collected during 2012 winter in Xi'an, China from the roof of a 15 m high building on the campus of Xi'an Jiaotong University using a mini volume sampler (BGI Inc., Waltham, MA, USA) operating at 47 mm quartz microfiber filters (Whatman Ltd., Maidstone, UK) and 5 L/min of flow rate. The concentrations of organic carbon (OC), elemental carbon (EC), and carbonaceous fractions were analyzed using a thermal and optical carbon analyzer. The concentrations of inorganic ions were analyzed using an ion chromatography. Metal elements were determined by inductively coupled plasma-atomic emission spectrometry. The concentrations of saccharides and polycyclic aromatic hydrocarbons (PAHs) were analyzed by gas chromatography-mass spectrometry. The detailed methods of sample collection and analyses were generated from previous laboratory studies (Wang et al. 2016; Wang et al. 2017). $PM_{2.5}$ was isolated from filters, weighed, and dissolved in DMSO to create a 10 mg/ml $PM_{2.5}$ suspension. The $PM_{2.5}$ suspension was further diluted in DMSO to obtain a series of concentrations (0.3, 1.0, and 3.0 mg/ml).

Preparation and organ culture of mesenteric artery

SD rats were anesthetized with CO_2 and decapitated. The mesentery dissected free and immersed in ice-cold 3-Morpholine propanesulfonic anid (MOPS) solution (mM: $Na_2HPO_4 \cdot 12H_2O$ 1.2,MOPS 2.0,EDTA 0.02,NaCl 140, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4 \cdot 7H_2O$ 1.2 and glucose 5.6) (Ping et al. 2012). The mesenteric arteries were carefully removed under a dissection microscope and cut into 1 mm long segments. Individual mesenteric artery segment was placed into well of 24-well plate with 1 ml of serum-free DMEM supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The arteries segments were randomly assigned and divided fresh group (without culture), control group (DMSO culture), $PM_{2.5}$ group, $PM_{2.5}$ + signal pathway inhibitor groups and DMSO + inhibitor groups. One microliter $PM_{2.5}$ samples were added to 1 ml culture medium and the final concentrations of inhibitors were 10^{-5} M in the wells. The plates were incubated at 37 °C in humidified 5% CO_2 and 95% air for 16 h. Some artery segments were used for functional myograph studies. In addition, some artery segments were snap-frozen at − 80 °C for quantitative real-time PCR for mRNA and Western blotting. Some artery segments were fixed in paraformaldehyde for immunohistochemistry.

Mesenteric artery contractile studies

A wire myograph (Danish Myograph Technology A/S, Aarhus Denmark) was used for recording the vessels contraction. Each artery segment was mounted through two thin wires (40 μm in diameter). One wire was attached to a force displacement transducer and attached to an analog-to-digital converter unit (AD Instruments, Hastings, UK).The other wire was attached to a movable displacement device that allowed adjustment of the distance between the two parallel wires. Data were recorded by the software program Chart™ (AD Instruments, Hastings, UK). The artery segments were immersed in temperature-controlled (37 °C) baths containing 5 ml MOPS solution. The O_2 was continuously gassed into baths. The segments were allowed to stabilize for 1.5 h at an optimal resting tension of 3.0 mN. The contractile capacity of each segment was checked by exposure to a K^+-rich MOPS solution. The contraction induced by K^+ was used as a reference for the contractile capacity. The segments were used only if K^+ elicited reproducible responses > 2 mN with a variation < 10%.

Before receptor agonist was administered, 3 μm indomethacin and 100 μm of L-NG-monometylarginin were incubated with segments for 30 min to inhibit the prostaglandin and NO release. Concentration-contraction responses were obtained by cumulative administration of receptor agonists S6c and ET-1. The ET_B receptor-mediated contractile response was obtained by using a selective ET_B receptor agonist S6c. ET-1 is an ET_A and ET_B receptor agonist. To specifically measure ET_A receptor-mediated responses, S6c ($10^{-7} \sim 10^{-11}$ M) was added firstly and cumulatively.

The segments were maintained with the highest concentration of S6c for an additional 1 h until the contractile curves fell to a baseline level, which was considered as a total desensitization. The selective ET_B receptor antagonist BQ-788 (10^{-6} M) was added to confirm the desensitization procedure. After the ET_B receptors were desensitized, ET-1 ($10^{-7} \sim 10^{-11}$ M) was added cumulatively and the response curve was exclusively generated by ET_A receptors.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from fresh or cultured arteries using an E.Z.N.A.® MicroElute Total RNA kit (Omega Biotech, USA) according to the manufacturer's protocols. The cDNA was synthesized from mRNA using reverse transcription-PCR kits (Thermo Fisher Scientific, USA) in a 20 μl reaction volume. RT-PCR was performed using the SYBR® PreMix Ex Taq™ kits (Takara, Japan) in an Agilent M × 3005P detection system (Agilent Technologies). RT-PCR was performed in a 25-μl reaction volume. The following RT-PCR amplification procedures were used: 94 °C for 3 min, 40 thermal cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s. Dissociation curves were identified for the specific PCR products, and the comparative cycle threshold (C_T) method was used for mRNA content analyses. The relative quantification of the expression of the target genes were measured using β-actin mRNA as an internal control. The specific primers were designed as follows: ET_B receptor: forward:TGACGCCACCCACTAAGACC, reverse: GGCACGGAGGAGGGAAGG.

ET_A receptor: forward: GACTGGTGGCTCTTTGGATTCTAC, reverse: GACGCTGCTTGAGGTGTTCG. β-actin, forward: CTATCGGCAATGAGCGGTTCC, reverse: TGTGTTGGCATAGAGGTCTTTACG.

Western blotting

The arteries were harvested as mentioned above. The artery segments were homogenized in 150 μl RIPA lysis buffer (Upstate USA, Inc., Charlottesville, VA, USA) with 1% phenylmethylsulfonyl fluoride. Total protein was quantified using a BSA protein assay kit (Pierce Biotechnology) according to the manufacturer's instructions. After gel electrophoresis, the proteins were transferred to a nitrocellulose membranes, the membranes were then blocked in 5% non-fat dried milk at room temperature for 2 h and incubated with primary antibodies at 4 °C overnight. The antibodies used were shown as follows: anti-ET_B (diluted 1:500, GeneTex, 39,603), anti-ET_A (diluted 1:300, Abcam, 85,163), and anti-β-actin (diluted 1:1500; Santa Cruz, sc-32,537). Then, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies (diluted 1:4000, Cell Signaling Technology) for 1 h at room temperature. Finally, proteins were visualized using a GeneTex Image Analyzer (Fujifilm, Stamford, USA). Band intensity quantification was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

The arteries were fixed, dehydrated, and cut into 10 μm sections. The sections were blocked with 10% goat serum solution for 30 min at 37 °C and incubated with primary antibodies at 4 °C overnight. The antibodies used were shown as follows: anti-ET_B receptor or anti-ET_A receptor antibody (diluted 1: 50, goat polyclonal, Santa Cruz, sc-21196 or sc-21194). Finally, the sections were washed with PBS and incubated with a rabbit anti-goat IgG conjugated to fluorescent isothiocyanate (FIFC, diluted 1: 100, Cwbio, CW0115) for 1 h. Immunoreactivity was visualized with a fluorescence microscope (Olympus, Japan), and images were analyzed using Image-Pro Plus 5.0 (Media Cybernetics, USA).

Statistical analysis

Data are presented as the means ± SE, and *n* refers to the number of rats. Contractile responses to K^+, S6c and ET-1

were recorded as mN. The E_{max} values represent maximal contraction. The pEC_{50} values represent the negative logarithm of the concentration that produced 50% E_{max}. Differences between means were calculated using SPSS version 13.0. Statistical analyses were performed using analysis of variance (ANOVA) or unpaired Student's *t*-test. Statistical significance was set at $P < 0.05$.

Results

Measurement of components of $PM_{2.5}$

Metals, PAHs, organic, and inorganic carbon were the most components in $PM_{2.5}$ samples. A total of 14 metals in $PM_{2.5}$ samples were measured. The results showed that Fe, K, and Zn were the major metals found in $PM_{2.5}$, which accounted for 90.2% in total metal mass concentration. Benzo(a)pyrene, Benzo (b) fluoranthene, Dibenzo (a,h) anthracene, Acenaphthene, Benzo (g,h,i) perylene, Indeno (1,2,3-cd) pyrene and Naphthalene were the main constituents of PAHs found in $PM_{2.5}$ samples, which accounted for 66.4% in total PAHs concentration. SO_4^{2-} and NO_3^- were the main ions, which accounted for 54.8% in total ions mass concentration. The concentrations of organic carbon and inorganic carbon were 37.31 ± 2.56 and 10.70 ± 0.44 mg/m^3, respectively. The specific data were reported as previously described (Wang et al. 2016; Wang et al. 2017).

$PM_{2.5}$ increased mesenteric arteries contractility mediated by ET_B and ET_A receptors

The contraction induced by K^+ was used as a reference for the contractile capacity. Organ culture in vitro did not affect the ability of the smooth muscle to contract in response to 63.5 mm K^+. The ET_B receptor-mediated contraction was induced by specific ET_B receptor agonist S6c. After desensitization of ET_B receptor, the ET_A-mediated contraction was induced by ET receptor agonist ET-1.

The mesenteric artery segments were cultured in the presence of $PM_{2.5}$ or DMSO for 8, 16, or 24 h in order to study the time-effect relationship. Firstly, the effect of DMSO cultured on artery contraction was examined. S6c induced a negligible contraction in mesenteric arteries of fresh group and DMSO cultured for 8 h group. While the contractile response to S6c was enhanced with an increased E_{max} in 16 h and 24 h cultured arteries ($P < 0.05$), compared to that in the fresh segments (Fig. 1a, Table 1). Compared with fresh group, mesenteric artery segments cultured with 1.0 μg/ml $PM_{2.5}$ for 8, 16 or 24 h markedly increased contractile responses ($P < 0.05$, Fig. 1b, Table 1). S6c induced contractile response was in a concentration-dependent manner. However, there was no significant difference of contraction induced by S6c between $PM_{2.5}$ cultured 16 and 24 h ($P > 0.05$). The results showed that exposure to 1.0 μg/ml $PM_{2.5}$ for 16 or 24 h resulted in stronger vasoconstriction and there was not statistically significant for 16 h and 24 h. The mesenteric artery segments were cultured with 0.3, 1.0, and 3.0 μg/ml $PM_{2.5}$ for 16 h to evaluate the concentration-effect relationship (Fig. 2). Compared with DMSO group, cultured with 0.3 or 1.0 μg/ml $PM_{2.5}$ enhanced the S6c-induced artery contractile responses ($P < 0.05$, Fig. 2a, Table 2). While the concentration of $PM_{2.5}$ increased to 3.0 μg/ml, S6c-induced artery contraction did not enhance ($P > 0.05$, Fig. 2a, Table 2).

The same to ET_B receptor-mediated contractile responses, the effect of $PM_{2.5}$ time- and concentration-effect relationship on ET_A receptor were detected. ET-1-elicited significant contraction in arteries of fresh group. There was no significant change in ET-1-induced contractile response between the fresh group and the artery segments cultured for 8 h ($P > 0.05$, Fig. 1c, Table 1). While the artery contractile response to ET-1 was obviously increased in 16 or 24 h culture groups ($P < 0.05$, Fig. 1c, Table 1). Compared with DMSO group, cultured with 1.0 μg/ml $PM_{2.5}$ for 8 and 24 h did not affect artery contractile responses ($P > 0.05$, Fig. 1d, Table 1). Compared to 16 h culture of DMSO alone, artery contractile responses were markedly enhanced with $PM_{2.5}$ cultured for 16 h ($P < 0.05$, Fig. 1d, Table 1). The results showed that the artery segments were cultured with $PM_{2.5}$ for 16 h, the contractile responses mediated by ET_A receptor reached the maximal contractile effects. $PM_{2.5}$ 0.3, 1.0 and 3.0 μg/ml cultured with mesenteric artery segments to examine the concentration-effect relationship on ET_A receptor. Compared to DMSO group, 0.3 μg/ml $PM_{2.5}$ cultured for 16 h did not increase artery contractile responses ($P > 0.05$, Fig. 1d, Table 2). 1.0 μg/ml $PM_{2.5}$ significantly shifted ET_A receptor-mediated contraction response curves toward the left with increased E_{max} of $344.4 \pm 30.1\%$ ($P < 0.05$). However, 3.0 μg/ml $PM_{2.5}$ did not further increased ET_A receptor-mediated contraction response compared to DMSO group ($P > 0.05$, Fig. 1d, Table 2).

These results showed that exposure to 1.0 μg/ml $PM_{2.5}$ for 16 h resulted in stronger vasoconstriction mediated by ET_B and ET_A receptors. Therefore, we chose 1.0 μg/ml $PM_{2.5}$ for 16 h as the culture concentration and period in subsequent experiments.

The MAPK pathway was involved the $PM_{2.5}$-increased vasoconstriction mediated by ET_B and ET_A receptors

The mitogen-activated protein kinase (MAPK) signal pathways can regulate the gene expression on transcriptional and post-transcriptional levels (Pearson et al. 2001). In order to investigate the mechanisms involved in the $PM_{2.5}$-induced ET_B and ET_A receptors up-regulation, MAPK pathway inhibitors were used.

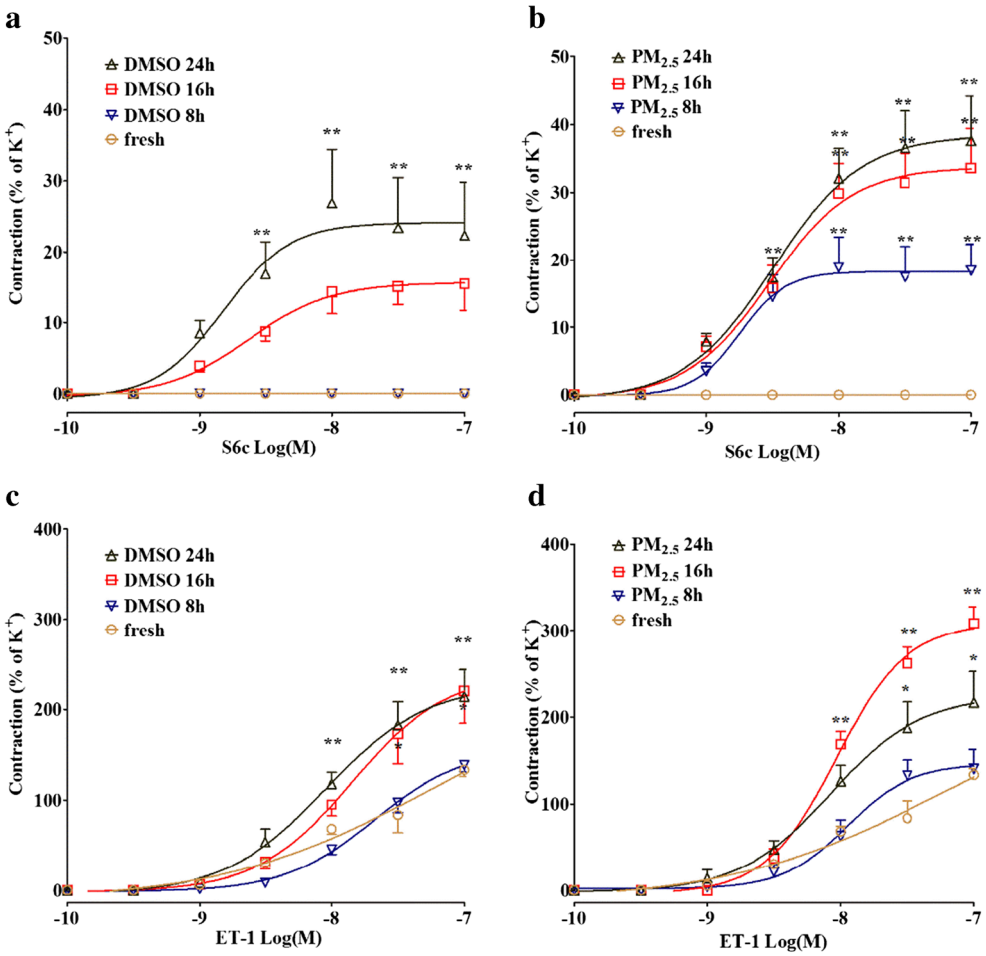

Fig. 1 Culturing time-effect relationship of $PM_{2.5}$ on the contractile responses to cumulative application of S6c and ET-1. The isolated rat mesenteric artery segments were cultured with DMSO or $PM_{2.5}$ for 0, 8, 16, and 24 h. Only DMSO or $PM_{2.5}$ culture for 0 to 24 h (**a**, **c**). Cultured with $PM_{2.5}$ for 0 to 24 h (**b**, **d**). Values are presented as mean ± SE, $n = 6 \sim 8$. $^{*}P < 0.05$ vs. DMSO group

For the ET_B receptor, the p38 inhibitor SB203580 or ERK1/2 inhibitor U0126 almost wholly abolished both organ culture-enhanced and $PM_{2.5}$-enhanced contraction. The decreasing range for $PM_{2.5}$ groups were higher than those for DMSO groups, SB203580 and U0126 significantly decreased the E_{max} from 35.0 ± 4.6% ($PM_{2.5}$ group) to negligible contraction ($PM_{2.5}$ + SB203580 group and $PM_{2.5}$ + U0126 group, Fig. 3b, c, Table 3), which suggested that SB203580 and U0126 on $PM_{2.5}$-increased contraction were more potent than that on culture alone group. However, the JNK inhibitor SP600125 did not affect the $PM_{2.5}$-increased contraction, and the E_{max} of contractile response did not show statistic difference between $PM_{2.5}$ group and $PM_{2.5}$ + SP600125 group (Fig. 3a, Table 3).

For the ET_A receptor, SB203580 significantly reduced the $PM_{2.5}$-enhanced contractile response with a decreased E_{max} ($P < 0.05$, Fig. 3e, Table 3). While SB203580 did not affect DMSO culture-enhanced contraction, U0126 suppressed both organ culture-enhanced and $PM_{2.5}$-enhanced contraction. U0126 significantly decreased $PM_{2.5}$- and DMSO-enhanced contractile response with a decreased E_{max} of 146.5 ± 43.1%, 166.8 ± 18.4% from 344.4 ± 30.1%, 263.4 ± 43.9% ($P < 0.05$, Fig. 3f, Table 3). There were significant differences in the inhibition rate for $PM_{2.5}$ group or DMSO group (57.4 vs. 36.6%). SP600125 did not affect the contractile response in $PM_{2.5}$ and DMSO group. These results showed that SB203580 and U0126 significantly inhibited the $PM_{2.5}$-induced enhanced contraction mediated by ET_B and ET_A receptors.

The ET_B and ET_A receptor mRNA expressions and MAPK pathway involvement

RT-PCR was used to examine the ET_B and ET_A receptor mRNA expressions to study the transcriptional mechanism. Compared to fresh group, organ culturing for 16 h significantly increased the ET_B and ET_A receptor mRNA expressions ($P < 0.05$). After cultured with 1.0 μg/ml $PM_{2.5}$ further enhanced ET_B and ET_A receptor mRNA expressions, which were significantly different compared with that in control groups ($P < 0.05$, Fig. 4a, b). Co-culture for 16 h with SP600125 did not diminish culture-alone-induced increase in mRNA levels of

Table 1 Time course of DMSO and $PM_{2.5}$ culture on the contractile responses induced by S6c and ET-1 in rat mesenteric artery segments

	Incubation time (h)	*n*	Contraction by K^+(mN)	Contraction by S6c		Contraction by ET-1	
				E_{max} (% of K^+)	pEC_{50}	E_{max} (% of K^+)	pEC_{50}
D-MS-O	0	6	4.59 ± 1.10	–	–	150.5 ± 11.8	7.92 ± 0.092
	8 h	6	4.62 ± 1.02	–	–	162.3 ± 15.3	7.30 ± 0.41
16 h	8	4.59 ± 0.75	16.8 ± 3.1*	8.60 ± 0.13	263.4 ± 4-3.9*	7.90 ± 0.-071	
24 h	6	5.25 ± 0.85	25.5 ± 7.6*	8.82 ± 0.11	225.1 ± 3-0.1*	8.07 ± 0.-062	
$PM_{2.5}$	0	6	4.59 ± 1.10	–	–	162.1 ± 15.7	7.89 ± 0.092
	8 h	7	4.73 ± 0.83	23.3 ± 3.2#	8.48 ± 0.28	157.4 ± 17.1	7.91 ± 0.13
16 h	6	4.05 ± 1.05	35.0 ± 4.6#	8.47 ± 0.16	344.4 ± 3-0.1#	7.87 ± 0.-074	
24 h	6	4.24 ± 1.07	39.1 ± 5.8#	8.52 ± 0.13	223.1 ± 34.1	8.17 ± 0.-091	

The rat mesenteric artery segments were isolated and cultured with DMSO or 1.0 μg/ml of $PM_{2.5}$ for different time. K^+-induced contractions were represented as absolute values of contraction (mN). ET_B and ET_A receptors-mediated contractions were recorded and expressed as percentage of K^+-induced contraction. The E_{max} values represent maximal contraction; the pEC_{50} values represent the negative logarithm of the concentrations that produces 50% of E_{max}; values are presented as mean ± SE. n denotes the number of rats. * $P < 0.05$ vs. DMSO group. # $P < 0.05$ vs. $PM_{2.5}$ group

ET_B and ET_A receptors. SB203580 obviously diminished DMSO-cultured increase in mRNA expression levels of ET_B receptor and did not affect the mRNA expression levels of ET_A receptor after DMSO cultured. While, U0126 strongly attenuated the mRNA levels of ET_B and ET_A receptors by DMSO. Co-culture for 16 h with SP600125 or SB203580 did not diminish culture-alone-induced increase in mRNA levels of ET_B and ET_A receptors. However, U0126 strongly attenuated the mRNA levels of ET_B and ET_A receptors by DMSO. SP600125 did not affect the mRNA levels of ET_B and ET_A receptors of $PM_{2.5}$-cultured group. SB203580 and U0126 significantly suppressed the increase in mRNA expression levels of ET_B and ET_A receptors induced by $PM_{2.5}$. While, the decreasing range of U0126 on ET_B and ET_A receptors mRNA levels for $PM_{2.5}$ groups were higher than those for DMSO groups ($P < 0.05$, Fig. 4c, d).

The ET_B and ET_A receptor protein expressions and MAPK pathway involvement

Western blotting and immunofluorescence were used to examine the protein expressions of ET_B and ET_A receptors. Western blotting showed that artery segments cultured with DMSO for 16 h obviously enhanced the ET_B and ET_A receptor protein expression levels ($P < 0.05$, Fig. 5)a, b. Artery segments cultured with 1.0 μg/ml $PM_{2.5}$ for 16 h further enhanced ET_B and ET_A receptor protein expressions ($P < 0.05$, Fig. 5a, b). Co-culture with SP600125 did not affect culture-alone-induced increase in protein levels of ET_B and ET_A receptors and that in $PM_{2.5}$-cultured group. SB203580 and U0126 significantly decreased the ET_B and ET_A receptor protein expressions induced by $PM_{2.5}$. SB203580 only decreased ET_B receptor protein expressions induced by organ culturing,

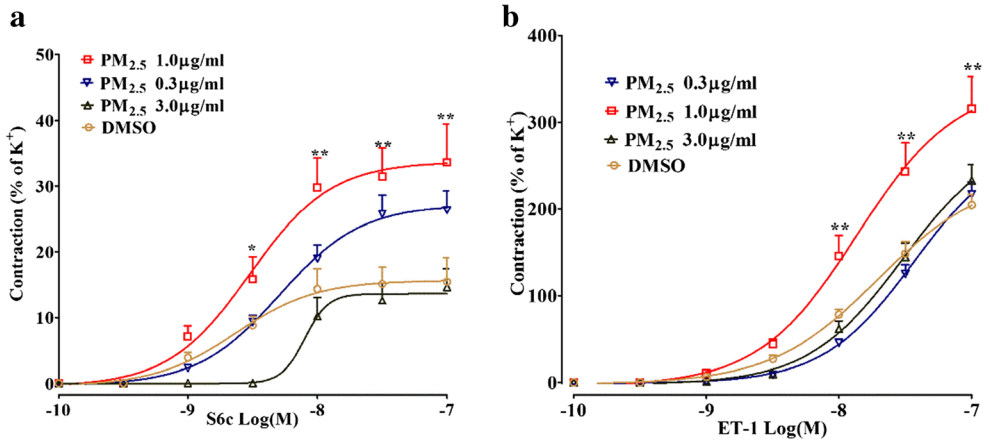

Fig. 2 Concentration-effect relationship of $PM_{2.5}$ on the contractile responses to cumulative application of S6c and ET-1. The isolated rat mesenteric artery segments were cultured with different concentrations of $PM_{2.5}$ (0.3, 1.0, 3.0 μg/ml) for 16 h. ET_B and ET_A receptors-mediated contractions were recorded and expressed as percentage of K^+-induced contraction. The ET_B-mediated contraction was induced by a specific agonist of ET_B receptors S6c (**a**). After desensitization of ET_B receptor, the contraction was induced by ET-1, an agonist for ET_A and ET_B receptors (**b**). Values are presented as mean ± SE, $n = 6$. * $P < 0.05$ vs. DMSO group

ET_B and ET_A receptor protein expressions in culture alone group. However, the inhibition of SB203580 and U0126 on $PM_{2.5}$-increased protein were more remarkable than that on culture group (Fig. 6).

Immunofluorescence was used to further test the ET_B and ET_A receptor protein expressions in the vascular smooth muscle cells. ET_B and ET_A receptor protein were dying green. DMSO cultured elevated the protein levels of ET_B and ET_A receptors compared with fresh group. $PM_{2.5}$ culture further increased the receptor protein expressions compared with DMSO group ($P < 0.05$, Fig. 5a). The effect of MAPK inhibitors demonstrated similar results in receptor protein expressions. SP600125 did not affect receptors protein expression in DMSO group and $PM_{2.5}$-cultured group. SB203580 only decreased ET_B receptor protein expressions induced by organ culturing. U0126 significantly decreased not only $PM_{2.5}$-induced, but also culture-alone-induced increases in protein levels of receptors. There was significant difference of the protein levels between $PM_{2.5}$ + U0126 groups and the culture with U0126 groups (Fig. 7).

Discussion

$PM_{2.5}$ is an independent risk factor of cardiovascular diseases. Up-regulation of ET receptors is implicated in the pathogenesis of vascular disease. In this study, we found that exposure to $PM_{2.5}$ induced the up-regulation of ET_B and ET_A receptors in mesenteric arteries and subsequently results in vascular hyperreactivity. The administration of SB203580 and U0126

Table 2 Contractile responses to S6c and ET-1 in rat mesenteric artery segments cultured with different concentration of $PM_{2.5}$

Groups	*n*	Contraction by K^+(mN)	Contraction by S6c		Contraction by ET-1	
			E_{max} (% of K^+)	pEC_{50}	E_{max} (% of K^+)	pEC_{50}
DMSO	6	4.59 ± 1.10	16.8 ± 3.1	8.60 ± 0.13	263.4 ± 43.9	7.90 ± 0.071
0.3 μg/ml $PM_{2.5}$	6	4.51 ± 0.71	27.1 ± 3.3*	8.30 ± 0.04	287.61 ± 25.3	7.41 ± 0.062
1.0 μg/ml $PM_{2.5}$	6	4.05 ± 1.05	35.0 ± 4.6*	8.47 ± 0.16	344.4 ± 30.1	7.87 ± 0.073
3.0 μg/ml $PM_{2.5}$	6	5.48 ± 0.72	16.2 ± 3.6	8.08 ± 0.02	270.9 ± 45.4	7.30 ± 0.29

The rat mesenteric artery segments were isolated and cultured with DMSO or different concentrations of $PM_{2.5}$ for 16 h. K^+-induced responses were represented as absolute values of contraction (mN). ET_B and ET_A receptors-mediated contractions were recorded and expressed as percentage of K^+-induced contraction. The E_{max} values represent maximal contraction; the pEC_{50} values represent the negative logarithm of the concentration that produces 50% of E_{max}; values are presented as mean ± SE. *n* denotes the number of rats. * $P < 0.05$ vs. DMSO group

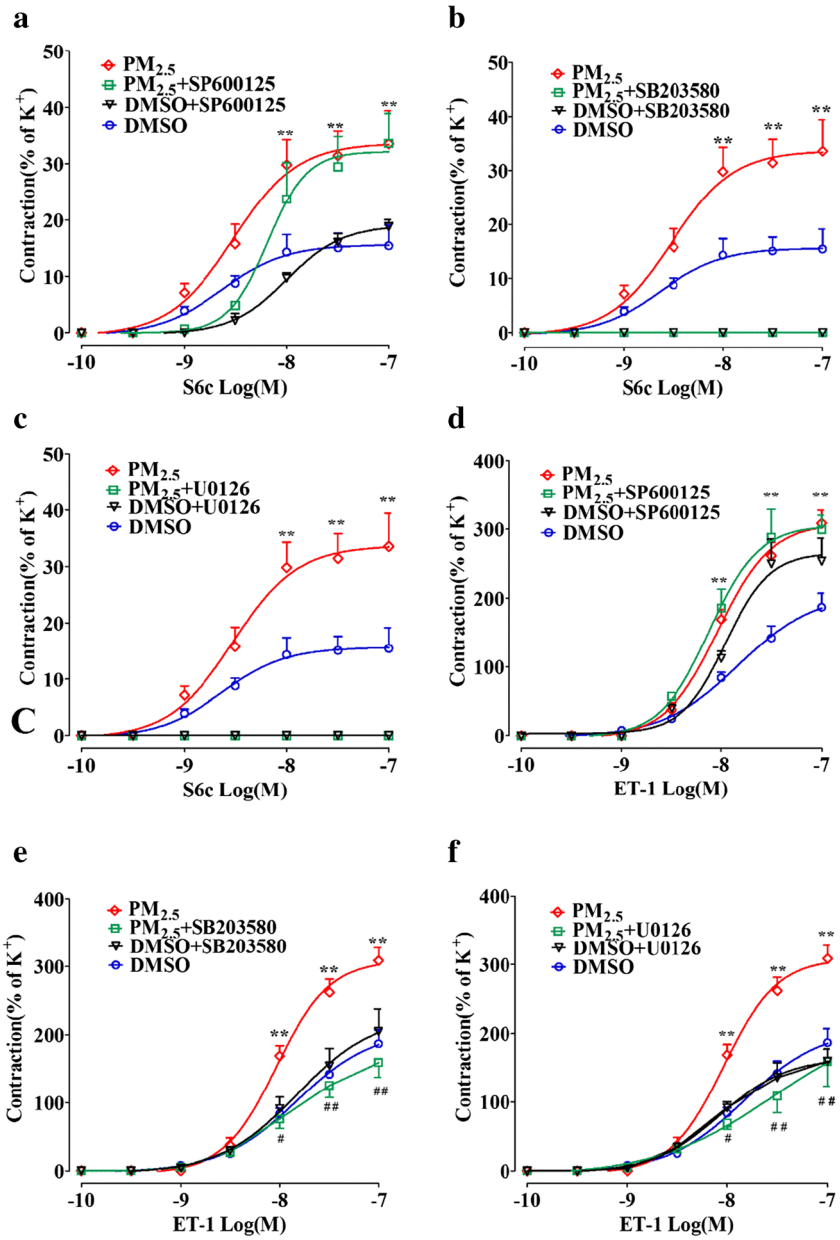

Fig. 3 The effects of MAPK pathway inhibitors on the $PM_{2.5}$-enhanced contraction mediated by ET_B and ET_A receptors in rat mesenteric artery. The isolated rat mesenteric artery segments were cultured with 1.0 μg/ml $PM_{2.5}$ for 16 h in the presence of JNK inhibitor SP600125 (**a**, **d**), p38 inhibitor SB203580 (**b**, **e**) or ERK1/2 inhibitor U0126 (**c**, **f**). Values are presented as mean ± SE, $n = 6 \sim 8$. $^{*}P < 0.05$ vs. DMSO group, $^{\#}P < 0.05$ vs. $PM_{2.5}$ group

suppressed the $PM_{2.5}$ induced ET_B and ET_A receptor up-regulation, supporting that p38 and MEK/ERK1/2 pathway is involved in ET_B and ET_A receptors up-regulation.

$PM_{2.5}$ is a component of air pollution and can usually deposit in the tracheobronchial and alveolar regions and even penetrated through the lung tissue to reach the capillary blood vessel and subsequently other target organs (Schwarze et al. 2006). So, exposure to $PM_{2.5}$ has been linked with cardiovascular morbidity and mortality. Hypertension is an important risk factor for cardiovascular diseases. The link of $PM_{2.5}$ and hypertension has been investigated in a few epidemiological studies. Schwartz et al. estimated that exposure to air pollution could chronically raise blood pressure, thereby increasing hypertension (Schwartz et al. 2012). Lin′s finding suggested that $PM_{2.5}$ might be associate with increased risk of hypertension and increased blood pressure and is responsible for significant hypertension burden in adults in China (Lin et al. 2017). However, the link between $PM_{2.5}$ and hypertension is clearly established but the mechanisms is not clear.

ET-1 is the strong vascular constrictor and plays an important role in vascular contraction. ET-1 induces vascular contraction through ET_A and ET_B receptors. Previous reports have demonstrated that ET receptor-mediated contraction is markedly increased in arteries from hypertensive patients and spontaneously hypertensive rats (Li et al. 2007). Previous studies showed that exposure to $PM_{2.5}$ increased ET-1 levels in plasma (Cherng et al. 2009; Lund et al. 2007; McDonald et al. 2007).

Table 3 E_{max} and pEC_{50} values of contractile responses mediated by ET receptors in rat mesenteric artery segments cultured with $PM_{2.5}$ in the presence of inhibitors

Groups	*n*	Contraction by K^+ (mN)	Contraction by ET_B		Contraction by ET_A	
			E_{max} (% of K^+)	pEC_{50}	E_{max} (% of K^+)	pEC_{50}
$PM_{2.5}$	6	4.05 ± 1.05	35.0 ± 4.6*	8.47 ± 0.16	344.4 ± 30.1	7.87 ± 0.074
$PM_{2.5}$ + SP600125	6	4.46 ± 0.28	37.7 ± 5.7*	7.98 ± 0.17	318.1 ± 21.9	8.05 ± 0.050
$PM_{2.5}$ + SB203580	8	4.85 ± 0.54	–#	–	219.9 ± 33.2#	7.89 ± 0.066
$PM_{2.5}$ + U0126	6	4.47 ± 1.42	–#	–	146.5 ± 43.1#	7.091 ± 0.98
DMSO	6	4.59 ± 1.10	16.8 ± 3.1	8.60 ± 0.13	263.4 ± 43.9	7.90 ± 0.072
DMSO + SP600125	6	4.01 ± 0.64	20.5 ± 1.4	7.90 ± 0.-095	263.4 ± 41.5	7.79 ± 0.067
DMSO + SB203580	7	4.50 ± 0.40	–	–	252.3 ± 37.3	7.85 ± 0.089
DMSO + U0126	6	5.20 ± 0.75	–	–	166.9 ± 18.4*	8.06 ± 0.068

The rat mesenteric artery segments were isolated and cultured with DMSO or 1.0 μg/ml of $PM_{2.5}$ in the presence of MAPK inhibitors. K^+-induced responses were represented as absolute values of contraction (mN). ET_B and ET_A receptors-mediated contractions were recorded and expressed as percentage of K^+-induced contraction. The E_{max} values represent maximal contraction; the pEC_{50} values represent the negative logarithm of the concentration that produces 50% of E_{max}; values are presented as mean ± SE. *n* denotes the number of rats. * $P < 0.05$ vs. DMSO group. # $P < 0.05$ vs. $PM_{2.5}$ group

$PM_{2.5}$ is a complex mixture. Therefore, it is difficult to verify which substances are responsible for vascular diseases. Cigarette smoke was regarded as an indoor $PM_{2.5}$. Ninety percent of the cigarette smoke particles diffused in air are $PM_{2.5}$ (Wilson et al. 2009). Previously we reported that DMSO-soluble cigarette smoke particles alter the expression

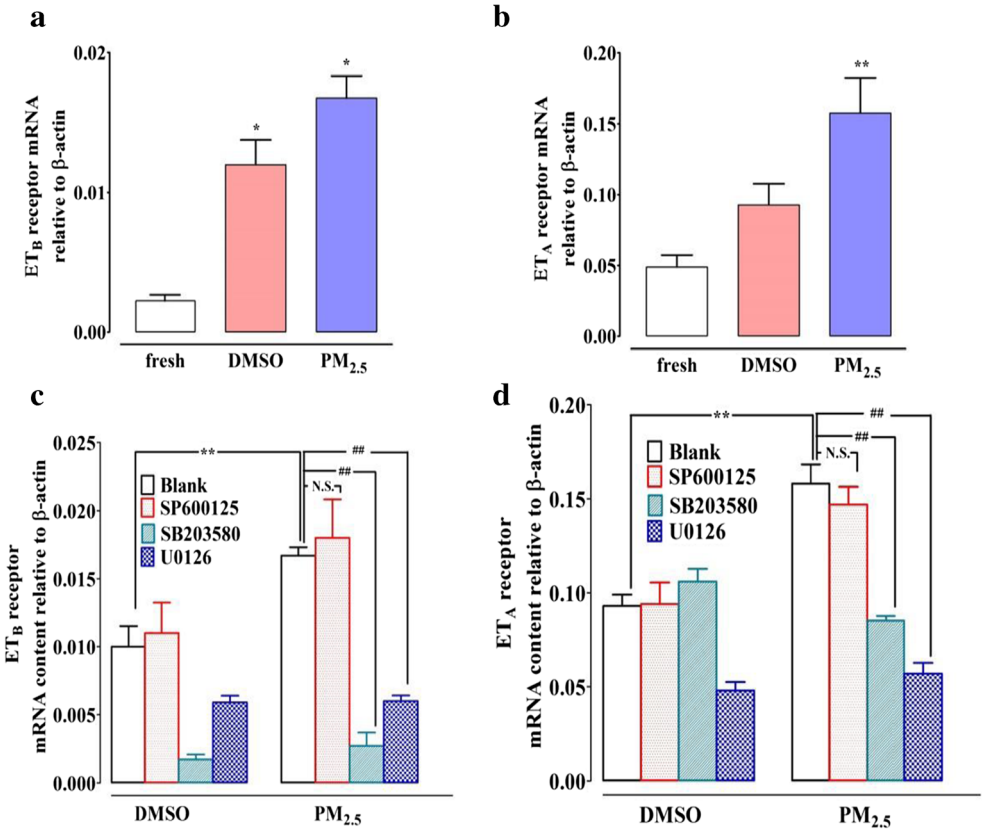

Fig. 4 The Effect of $PM_{2.5}$ and MAPK pathway inhibitors on ET receptor mRNA expressions. The isolated rat mesenteric artery segments were cultured with 1.0 μg/ml PM2.5 for 16 h in the presence of MAPK inhibitors or DMSO. The mRNA levels of ET_B (**a**, **c**) and ET_A (**b**, **d**) receptor was determined by RT-PCR. The receptor mRNA expression was relative to β-actin. Values are presented as mean ± SE, $n = 6$. * $P < 0.05$, ** $P < 0.01$ vs. DMSO group

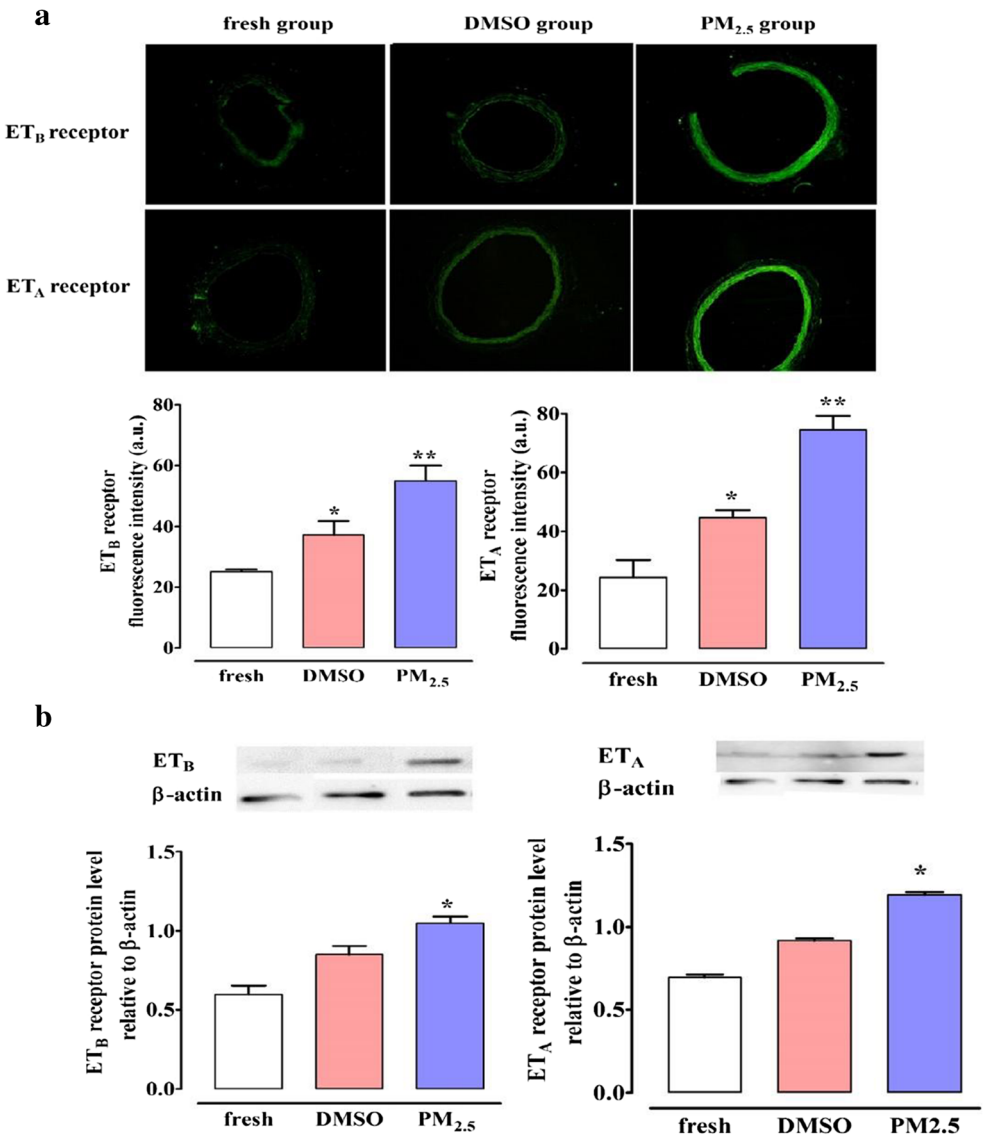

Fig. 5 The Effect of $PM_{2.5}$ on ET receptor protein expressions. The isolated rat mesenteric artery segments were cultured with 1.0 μg/ml $PM_{2.5}$ for 16 h in the presence of $PM_{2.5}$ or DMSO. The protein levels of ET receptors were determined using immunofluorescence (**a**) and Western blotting (**b**). Images were taken using a fluorescence microscope (× 100). The ET receptor protein expression levels were measured relative to β-actin. Values are presented as mean ± SE, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. DMSO group

of ET_B receptor in coronary artery (Huang et al. 2013). Our recent researches reported that DMSO-soluble $PM_{2.5}$-induced bronchial hyper-reactivity by up-regulated smooth muscle cell muscarinic and ET receptors (Wang et al. 2016; Wang et al. 2017). So, we investigated the effect of DMSO-soluble $PM_{2.5}$ on vascular hyper-reactivity. $PM_{2.5}$ contents exist considerable regional differences. With the rapid industrialization and urbanization, coal, and motor vehicle combustion are the major sources of $PM_{2.5}$ in most Chinese cities. The major components of $PM_{2.5}$ which we used collected from Xi'an and in other studies were different, whereas the major components are similar in magnitude (Cao et al. 2012).

In the present study, we used rat mesenteric resistance artery to explore the effect of DMSO-soluble $PM_{2.5}$ on ET receptors and to reveal the underlying molecular mechanisms. The receptor up-regulation can increase the sensitivity of artery contractility. Firstly, we used a sensitive myograph to screen out an optimal concentration and culture periods of $PM_{2.5}$. The results showed that $PM_{2.5}$ significantly increased the ET_B and ET_A receptor-mediated contraction in a time- and concentration-dependent manner. $PM_{2.5}$ 1.0 μg/ml for 16 h induced maximal contractile response. In other word, 1.0 μg/ml for 16 h was the optimal work concentration and time. Interestingly, 3.0 μg/ml $PM_{2.5}$ for 16 h or 1.0 μg/ml $PM_{2.5}$ for 24 h not increased the ET_B and ET_A receptor-mediated contraction. Previous researches have demonstrated that an association exists between $PM_{2.5}$ exposure and cardiovascular diseases, and that it is dependent on particles concentration and time of exposure (Fiordelisi et al. 2017). $PM_{2.5}$ carries large amounts of toxic substances such as heavy metal ions, PAHs, and endotoxin and so on. Particle or toxic substances may destroyed vascular smooth muscle cell. So, we speculated 3.0 μg/ml $PM_{2.5}$ for 16 h or 1.0 μg/ml $PM_{2.5}$ for 24 h induced contraction which was mixture effect of

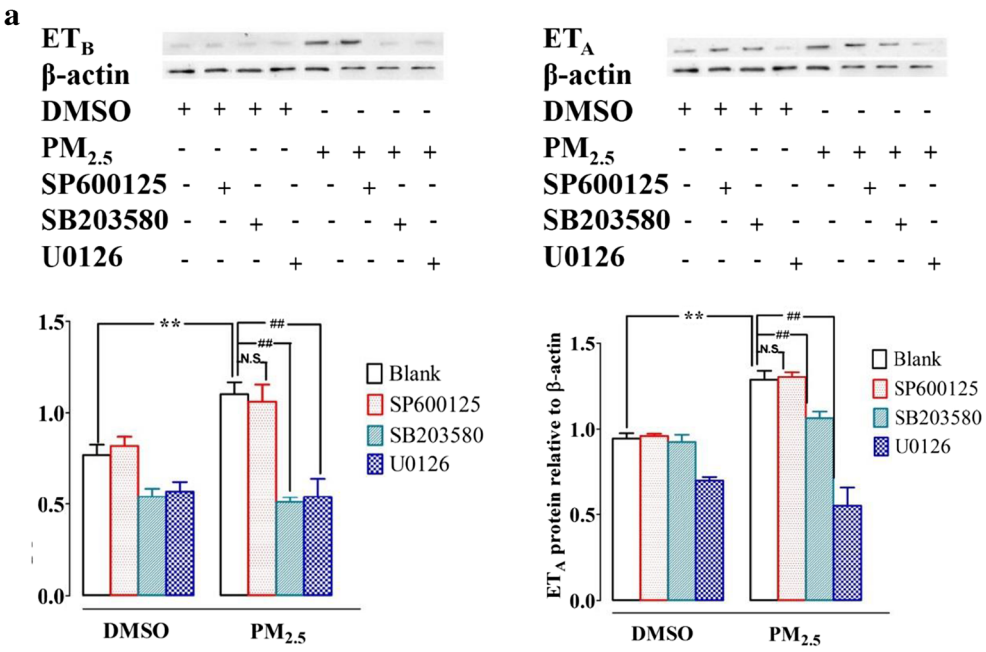

Fig. 6 The effects of MAPK pathway inhibitors on the $PM_{2.5}$-enhanced protein expression level of ET receptors in rat mesenteric artery using Western blotting. The isolated rat mesenteric artery segments were cultured with 1.0 μg/ml $PM_{2.5}$ for 16 h in the presence of MAPK pathway inhibitor or DMSO. The ET receptor protein expression levels were measured relative to β-actin. Values are presented as mean ± SE, $n = 6$. * $P < 0.05$ vs. DMSO group, $^{\#}$ $P < 0.05$ vs. $PM_{2.5}$ group

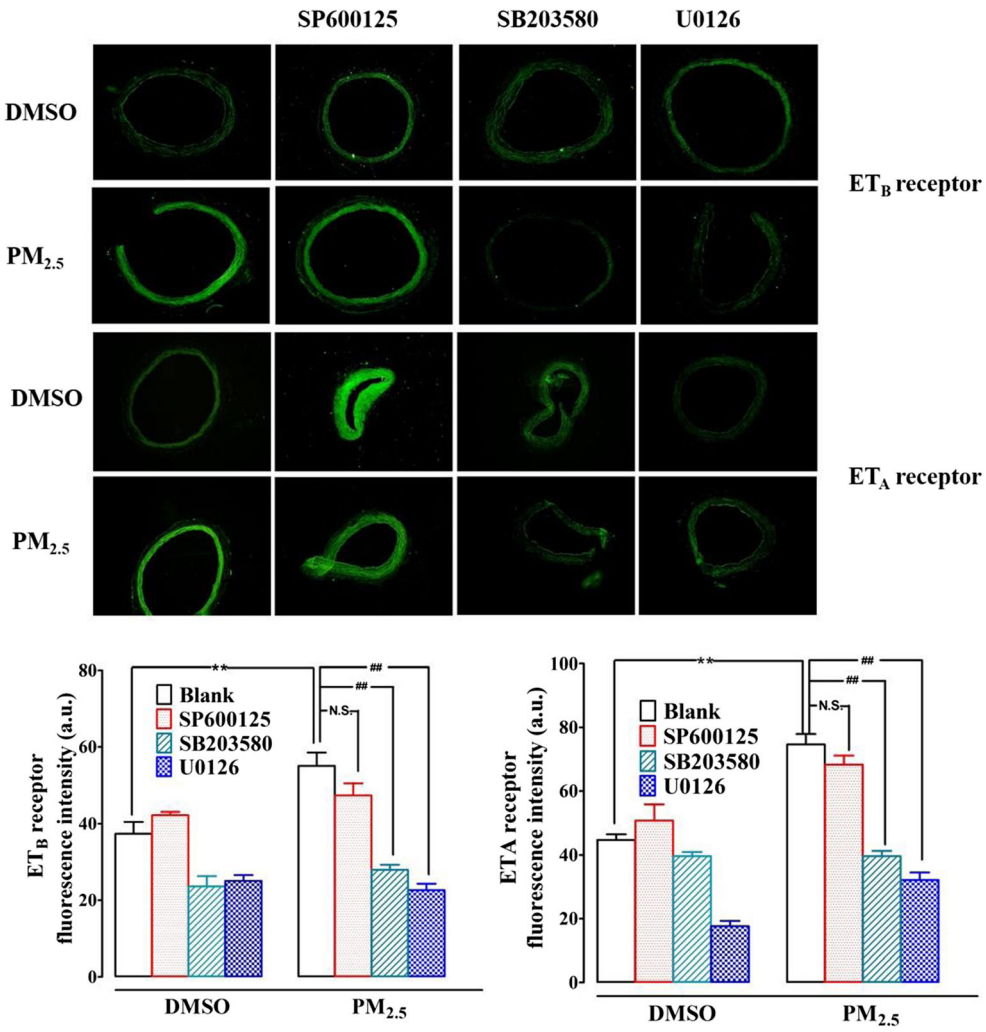

Fig. 7 The effects of MAPK pathway inhibitors on the $PM_{2.5}$-enhanced protein expression of ET receptors in rat mesenteric artery using immunofluorescence. The isolated rat mesenteric artery segments were cultured with 1.0 μg/ml $PM_{2.5}$ for 16 h in the presence of MAPK pathway inhibitor or DMSO. Images were taken using a fluorescence microscope (×100). Values are presented as mean ± SE, $n = 6$. * $P < 0.05$ vs. DMSO group, $^{\#}$ $P < 0.05$ vs. $PM_{2.5}$ group

receptors up-regulation and smooth muscle cell injured. Secondly, 1.0 μg/ml $PM_{2.5}$ for 16 h was applied to research mRNA and protein levels for the ET_B and ET_A receptors. In parallel with the functional results, the mRNA and protein expression levels of ET_B and ET_A receptors were enhanced after cultured with $PM_{2.5}$. These results suggests that ET_B and ET_A receptors are regulated and a transcription and translation mechanism is involved.

The molecular mechanisms responsible for $PM_{2.5}$ induced up-regulation of ET receptors were further investigated. MAPK pathways are serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate various cellular activities. The intracellular MAPK consists of three main signaling pathways: ERK1/2, JNK and p38 pathways. In this research, we used organ culture which is a convenient way to studying mechanisms involved in GPCRs alterations as a *vitro* model. The concentrations of MAPK inhibitors were referenced previous studies (Huang et al. 2010; Lei et al. 2011; Sandhu et al., 2010). Many studies demonstrated that MAPK signaling pathway may play a key role in mediating ET receptors up-regulation. Cigarette smoke exposure induced up-regulation of ET receptors via activation of MAPK pathways, which was an important pathogenic characteristic of cardiovascular diseases (Zhang et al. 2017). In addition, our previous studies demonstrated that MAPK activation participates in $PM_{2.5}$ induced ET receptors up-regulation in rat coronary arteries, basilar arteries and bronchi (Wang et al. 2016; Wang et al. 2017; Xiao et al. 2016; Xiao et al. 2016).

In our study, SB203580 and U0126 significantly suppressed $PM_{2.5}$-induced ET_B and ET_A receptors up-regulation by decreased the enhanced contraction and diminished the increased mRNA and protein expressions, while the SP600125 has no such effect. In addition, SB203580 only suppressed receptors-mediated contractile response and expression in $PM_{2.5}$ group, U0126 attenuated ET_B, and ET_A receptors-mediated contractile response and expression in the control group that were lower than those in $PM_{2.5}$ group. Based on these findings, we think that p38 and ERK/MEK1/2 pathway are involved in the ET_B and ET_A receptors up-regulation which induced by $PM_{2.5.}$ This finding is similar with previous studies which showed that inhibition of p38 and ERK1/2 significantly attenuates the DMSO-soluble smoking particle induced up-regulation of vascular SMC ET_B and ET_A receptors.

In summary, $PM_{2.5}$ induced an up-regulation of mesenteric artery ET_B and ET_A receptors subsequently resulting in vascular hyper-reactivity and thus contributing to the development of vascular diseases. p38 and MEK/ERK1/2 pathways are involved in ET_B and ET_A receptors up-regulation. Thus, targeting MAPK-mediated up-regulation of ET receptors might be a novel and promising therapeutic target for treating cardiovascular diseases correlating with $PM_{2.5}$.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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