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Periostin mediates cigarette smoke extract-induced proliferation and migration in pulmonary arterial smooth muscle cells

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ABSTRACT

Cigarette smoking is an important risk factor for pulmonary arterial hypertension (PAH). Pulmonary arterial smooth muscle cells (PASMCs) play a critical role in the pathogenesis of PAH-associated arterial remodeling. This study was done to explore the expression and biological roles of periostin in PASMCs following exposure to cigarette smoke extract (CSE). PASMCs were exposed to different concentrations of CSE and tested for gene expression and reactive oxygen species (ROS) production. PASMCs were incubated with recombinant periostin protein or transfected with small interfering RNA targeting periostin before CSE exposure and then examined for cell proliferation and migration. Compared to control cells, exposure to CSE led to a significant upregulation of periostin. Pretreatment with 5 mM *N*-acetyl-L-cysteine (an inhibitor of ROS formation) or 10 μM U0126 (an inhibitor of ERK1/2) significantly prevented the induction of periostin in CSE-treated PASMCs. The addition of recombinant periostin protein significantly enhanced the proliferation and migration of PASMCs. In contrast, knockdown of endogenous periostin counteracted the proliferation and migration of PASMCs induced by CSE treatment. In conclusion, CSE induces the expression of periostin in PASMCs via promotion of ROS and activation of ERK1/2. Periostin mediates the effects of CSE on PASMC proliferation and migration. These findings warrant further exploration of the roles of periostin in cigarette smoking-associated pulmonary arterial remodeling.

1. Introduction

Pulmonary arterial hypertension (PAH) is a chronic and progressive disease manifested as elevation of pulmonary arterial pressure and pulmonary vascular resistance, eventually leading to right heart failure and even death [1]. Pulmonary arterial remodeling is a hallmark feature of PAH [2]. Increased proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs) plays a central role in the pathogenesis of PAH-associated artery remodeling [3]. Understanding the mechanisms for regulation of PASMC behaviors is of importance in developing effective therapies for PAH.

Cigarette smoking is an important risk factor for PAH in patients with chronic obstructive pulmonary disease [4]. Cigarette smoke (CS) contains enormous amounts of free radicals and reactive oxygen species (ROS). At cellular level, CS exposure can induce the proliferation of PASMCs [5,6], consequently contributing to pulmonary arterial remodeling. At the molecular level, CS-derived ROS is capable of activating numerous signaling pathways, in particular those mediated by mitogen-activated protein kinases (MAPKs) [7,8]. MAPKs, comprising ERK, JNK, and p38 MAPK, are involved in the regulation of the behaviors of PASMCs [9,10]. A recent study has demonstrated that FHL1 is required for cigarette smoke extract (CSE)-induced proliferation in PASMCs [11]. However, the mechanisms underlying CS-mediated alteration of PASMC biology are not completely understood.

Periostin, originally identified as osteoblast-specific factor 2 [12], is a secreted extracellular matrix protein implicated in many respiratory disorders [13]. It has been documented that periostin can facilitate pulmonary fibrosis and serve as an indicator of disease progression in patients with idiopathic pulmonary fibrosis [14]. Periostin shows the ability to induce eosinophil infiltration in allergic lung and esophageal responses [15]. This protein can also affect lung tumorigenesis [16]. Li et al. [17] reported that hypoxia-responsive growth factors fibroblast growth factor-1 and angiotensin II can upregulate periostin expression in rat PASMCs via

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MAPK signaling. These studies suggest a possibility that periostin may has the ability to regulate the behaviors of PASMCs, thereby contributing to pulmonary vascular remodeling.

To this end, in the present study, we examined the expression of periostin in CSE-exposed PASMCs and explored its biological roles in CSE-induced PASMC proliferation and migration.

2. Materials and methods

2.1. Cell culture

Human PASMCs were purchased from Cambrex (Walkersville, MD, USA) and maintained in Sm-GM2 media (Cambrex) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA). PASMCs at passages 4–9 were used in this study.

2.2. Preparation of CSE

CSE were prepared as previously described [18], using commercially available filter cigarettes (tar 13 mg, nicotine 1 mg in each cigarette; White Shark brand, Hunan Tobacco Company, Changsha, China). In brief, the smoke of 6 cigarettes was generated by a respiratory pump and bubbled into a flask containing 30 mL of Sm-GM2 medium. The CSE solution was sterilized by filtration through a 0.22-μm membrane and regarded as 100% CSE.

2.3. Cell treatment

PASMCs were exposed to 2% or 10% CSE for 48 or 72 h and tested for gene expression and cell proliferation and migration. In some experiments, cells were pretreated with 5 mM *N*-acetyl-L-cysteine (NAC), 10 μM U0126, or 10 μM SP600125 for 1 h before CSE exposure or incubated with recombinant periostin protein (50 ng/mL) or bovine serum albumin (BSA; 50 ng/mL) for 48 h or 72 h. NAC, U0126, SP600125, and BSA were purchased from Sigma (St. Louis, MO, USA) and periostin protein was purchased from R&D Systems (Minneapolis, MN, USA).

2.4. Transfection of small interfering RNA (siRNA)

A synthetic siRNA targeting human periostin and scrambled siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Cells were grown to 80% confluence and transfected with periostin siRNA or control siRNA (50 nM) using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were exposed to 10% CSE and tested for cell migration and proliferation. Transfection efficiency (>85%) was determined in parallel using a fluorescent-labeled siRNA (Invitrogen).

2.5. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from cells using Trizol (Invitrogen) and treated with DNase I (Qiagen, Hilden, Germany). cDNA was synthesized from 0.5 μg of RNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen). The following PCR primers were used: human periostin, forward 5′-AGGCAAACAGCTCAGAGTCTTC-3′, reverse 5′-TGCAGCTTCAAGTAGGCTGAGGA-3′; human β-actin, forward 5′-GGGRCAGAAGGATTCCTATG-3′, reverse 5′-GGTCTCAAACATGATCTGGG-3′. PCR amplifications were detected with SYBR Green I (Stratagene, La Jolla, CA, USA). Relative periostin mRNA levels were calculated after normalization to β-actin mRNA levels using the comparative cycle threshold (ΔΔCt) method [19].

2.6. Western blot analysis

The following primary antibodies were used in this study: anti-periostin (Abcam, Cambridge, MA, USA), anti-ERK1/2, anti-JNK1/2, anti-p38, anti-phospho-ERK1/2, anti-phospho-JNK1/2, anti-phospho-p38, and anti-β-actin (Cell Signaling Technology, Beverly, MA, USA).

Cells were lysed in radioimmune precipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) supplemented with a protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). Protein concentration was measured using the BCA Protein Assay kit (Beyotime Institute of Biotechnology). Protein samples (60 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were incubated at 4 °C overnight with the primary antibodies (diluted at 1:1000), followed by incubation for 1 h with horseradish peroxidase conjugated-secondary antibodies (Santa Cruz Biotechnology). The blots were visualized using an Enhanced Chemiluminescence Detection Kit from Amersham Biosciences (Piscataway, NJ, USA). Protein expression was quantified via densitometry with Quantity One program (Bio-Rad, Hercules, CA, USA).

2.7. ROS measurement

Intracellular ROS generation was measured using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA), which can be oxidized by peroxide to produce the fluorescent product 2′,7′-dichlorofluorescein (DCF). In brief, cells were incubated with 10 μM DCHF-DA (Sigma) for 30 min at 37 °C. After washing, cells were analyzed by flow cytometry. The fluorescence of DCF was measured at an excitation wavelength of 485 nm.

2.8. Cell proliferation assay

Cells were seeded at 5×10^3 cells per well in 96-well cell culture plates. After treatment with CSE or recombinant periostin protein for 48 or 72 h, cells were collected and tested for the proliferation by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. In brief, cells were incubated with 0.5 mg/mL MTT (Sigma) at 37 °C for 4 h. Precipitates were dissolved in dimethyl sulfoxide at 37 °C for 5 min. Absorbance of the samples at 570 nm was determined using a microplate reader (Bio-Rad).

2.9. BrdU incorporation assay

For 5-bromo-2′-deoxyuridine (BrdU) labeling, cells were incubated for 12 h with 10 μM BrdU (Beyotime Institute of Biotechnology) before fixation with 4% paraformaldehyde. Cells were incubated with an anti-BrdU antibody (Sigma) overnight at 4 °C, followed by incubation with FITC-labeled anti-mouse IgG (Santa Cruz Biotechnology). Nuclei were counterstained with 2 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma). At least 600 cells per sample were examined under a fluorescence microscope, and the percentage of BrdU positive staining was calculated.

2.10. Wound-healing assay

Cells were cultured to confluence, and the cell monolayer was scratched manually with a pipette tip to produce a “wounded” zone. The detached cells were removed. Cells were exposed to 10% CSE or incubated with recombinant periostin protein for 48 h. Cell