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The tyrosine phosphatase, SHP-1, is involved in bronchial mucin production during oxidative stress

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ABSTRACT

Mucus hypersecretion is a clinically important manifestation of chronic inflammatory airway diseases, such as asthma and Chronic obstructive pulmonary disease (COPD). Mucin production in airway epithelia is increased under conditions of oxidative stress. Src homology 2 domain-containing protein tyrosine phosphatase (SHP)-1 suppression is related to the development of airway inflammation and increased ROS levels. In this study, we investigated the role of SHP-1 in mucin secretion triggered by oxidative stress. Human lung mucoepidermoid H292 carcinoma cells were transfected with specific siRNA to eliminate SHP-1 gene expression. Cultured cells were treated with hydrogen peroxide (H_2O_2), and Mucin 5AC(MUC5AC) gene expression and mucin production were determined. Activation of p38 mitogen activated protein kinase (MAPK) in association with MUC5AC production was evaluated. *N*-acetylcysteine (NAC) was employed to determine whether antioxidants could block MUC5AC production. To establish the precise role of p38, mucin expression was observed after pre-treatment of SHP-1-depleted H292 cells with the p38 chemical blocker. We investigated the *in vivo* effects of oxidative stress on airway mucus production in SHP-1-deficient heterozygous (mev/+) mice. *MUC5AC* expression was enhanced in SHP-1 knockdown H292 cells exposed to H_2O_2, compared to that in control cells. The ratio between phosphorylated and total p38 was significantly increased in SHP-1-deficient cells under oxidative stress. Pre-treatment with NAC suppressed both MUC5AC production and p38 activation. Blockage of p38 MAPK led to suppression of MUC5AC mRNA expression. Notably, mucin production was enhanced in the airway epithelia of mev/+ mice exposed to oxidative stress. Our results clearly indicate that SHP-1 plays an important role in airway mucin production through regulating oxidative stress.

Introduction

Mucus production is a normal defensive mechanism that plays an important role in protecting airway epithelium from various harmful particles and microbes in contact with the respiratory tract and maintaining the normal function of epithelium [1]. However, mucus hypersecretion and airway goblet cell hyperplasia observed in chronic airway inflammatory disorders [1–3], can lead to agonizing respiratory symptoms, even life-threatening airway obstruction. Thus, appropriate control of airway mucin generation is crucial in the management of chronic inflammatory airway diseases.

While various stimuli related to airway inflammatory conditions can cause mucus hypersecretion, significant evidence shows that oxidative stress promotes mucin gene and protein expression, even in the absence of airway inflammation [1,4,5]. The respiratory tract is one of the organs most frequently exposed to oxidative stress, which may be generated either endogenously via mitochondrial respiration and activation of inflammatory cells or exogenously from air pollutants and cigarette smoking [6,7]. Oxidative stress is closely associated with the disease pathogenic mechanisms of several chronic airway inflammatory diseases [6,7]. However, its precise mechanism of action in mucus hyper-production is currently unclear.

The src homology 2-containing protein tyrosine phosphatase (SHP-1), a negative regulator in intracellular signaling, is expressed in hematopoietic, neuronal and epithelial cells [8]. SHP-1 knockout homozygous mice (mev/mev) spontaneously develop a Th2-dominant immune response and display significantly enhanced airway

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mucus secretion with no antigen challenge [9]. Moreover, increased intracellular ROS leads to inhibition of the enzymatic activities of protein tyrosine phosphatases (PTPs), such as SHP-1 [10]. Suppression of the SHP-1 function, in turn, promotes a further increase in the intracellular ROS level by eliciting amplified and prolonged activation of endogenous ROS [11].

These studies collectively suggest that SHP-1 may be involved in mucin gene expression induced under conditions of oxidative stress. Elucidation of the underlying mechanisms should thus provide novel information on the regulatory pathway of mucus hypersecretion. In the present study, we examined the role of SHP-1 in mucin hypersecretion promoted by oxidative stress using a human bronchial epithelial cell line, H292. Furthermore, the *in vivo* effect of SHP-1 on mucus hypersecretion was evaluated with SHP-1-deficient heterozygous (mev/+) mice.

Materials and methods

Cell culture and animals. The human mucoepidermoid bronchiolar carcinoma cell line, NCI-H292, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U penicillin and 100 μg streptomycin per milliliter at 37 °C in a 5% CO_2 incubator. Experiments were performed after overnight serum starvation.

We employed 6–8 week old heterozygous female motheaten mice (mev/+) as SHP-1-deficient models and wild-type controls. All mice, purchased from the Jackson Laboratory (Bar Harbor, ME, USA), were from a C57BL/6 background. Mice were maintained under specific pathogen-free conditions. Animal experiments were approved by the institution.

SHP-1 knockdown in H292 cells with specific siRNA. SHP-1 siRNA was purchased from Santa Cruz Biotechnology Inc. (CA, USA sc-29478). The sequences were as follows: CUGGUGGAGCAUUUCAAG ATT (Duplex 1 Sense Strand), CGCAGUACAAG UUCAUCUATT (Duplex 2 Sense Strand), CAACCCUUCUCCUCUUGUATT (Duplex 3 Sense Strand), mRNA Accession Number NM_002831). H292 cells were seeded in a 6-well plate (5–7 × 10^5 cells/well) 1 day before transfection. At 70% confluence, the medium was altered to serum/antibiotic-free medium. Cells were maintained in this medium for 24 h prior to transfection experiments. SHP-1 siRNA (0.5, 1, 1.5 μg) and scrambled siRNA as a control was gently mixed with 250 μL of Opti-MEM-reduced serum medium. Lipofectamine 2000 (5 μL, Invitrogen Inc., Carlsbad, CA, USA) was diluted with Opti-MEM (250 μL) for 5 min at room temperature. Each siRNA/Lipofectamine 2000-diluted solution was mixed for 30 min at room temperature. The mixture was added to cells, and the medium was changed to antibiotic-free growth medium.

At first, we determined whether the SHP-1 siRNA was effective in suppression of SHP-1 expression in H292 cells by assessing both SHP-1 mRNA and protein levels 24 and 48 h after transfection. SHP-1 mRNA expression was determined using reverse transcription-polymerase chain reaction (RT-PCR), and adjusted according to the GAPDH mRNA level. The primer sequences for SHP-1 mRNA were as follows: downstream primer, 5′-TGGCGTGGCAGGAGAACAG-3′ (forward) and upstream primer, 5′-GCAGTTGGTCACAGAGTAGGGC-3′ (reverse). The primer sequences for GAPDH were 5′-ACCACAGTCCATGCCATCAD-3′ (downstream) and 5′-TCCACCACCCTGTTGCTGTA-3′ (upstream). PCR products were resolved on 2% agarose gels, and visualized with ethidium bromide under a transilluminator. SHP-1 protein level was assessed by using Western blot with SH-PTP antibody (1:1000, Santa Cruz Biotechnology Inc.).

SHP-1 expression was significantly suppressed upon treatment with specific siRNA. Efficiency of suppression was effectively maintained till the 48 h after treatment (Fig. 1A). Accordingly, subsequent experiments were performed at 24 h after transfection with 1 μg siRNA.

Treatment of H292 cells with hydrogen peroxide, antioxidant and p38 inhibitor. SHP-1 siRNA-transfected and scrambled RNA infected control cells were treated with hydrogen peroxide (H_2O_2, Sigma, St. Louis, MO, USA) to induce oxidative stress. Following preliminary experiments to determine the optimal concentration, 1 mM H_2O_2 was used for subsequent experiments. Cells were treated with various concentrations (1, 5, and 10 mM) of the antioxidant, *N*-acetylcysteine (NAC, Sigma), 1 h before H_2O_2 stimulation. Pre-treatment with the p38 inhibitor, SB203580 (20 μM; Calbiochem, San Diego, CA, USA), was performed 30 min prior to the application of H_2O_2.

Determination of MUC5AC expression. In the present study, MUC5AC mRNA and mucin protein levels were measured at 4 and 12 h after stimulation with H_2O_2, respectively. RNA was extracted from H292 cells treated with H_2O_2, NAC and a p38 inhibitor using TRI reagent. Extracted RNA was quantified as 200–400 ng/μL on average on the ND spectrophotometer (NanoDrop Technologies, LLC, Wilmington, Delaware, USA). Following RNA extraction, 1–20 μg of RNA was used for cDNA synthesis with the RT kit (iNtRON Biotechnology Inc., Seoul, Korea). MUC5AC mRNA expression was determined using PCR, and adjusted according to the GAPDH mRNA level. The primer sequences for MUC5AC were as follows: downstream primer, 5′-TGATCATCCAGCAGCAGGGCT-3′ (forward) and upstream primer, 5′-CCGAGCTCAGAGGACATATGG-3′ (reverse).

Mucin protein production in H292 cells treated with various compounds was assessed by measuring MUC5AC protein levels in the supernatant using ELISA, as described previously [12]. Briefly, 50 μL of supernatant was incubated with bicarbonate–carbonate buffer (50 μL) at 40 °C in a 96-well plate until dry. Plates were washed three times with PBS, and blocked with 2% BSA, fraction V (Sigma), for 1 h at room temperature. Next, plates were rewashed three times with PBS, and incubated with 50 μL of monoclonal MUC5AC antibody (1:100, Abcam Ltd., Cambridge, UK, ab24070) diluted in PBS-T (PBS–0.05% Tween 20) containing 1% BSA. After 1 h, wells were washed three times with PBS-T, and 100 μL of horseradish peroxidase-conjugated anti-mouse IgG (diluted 1:2500 with PBS-T containing 1% BSA) was dispensed into each well. After 1 h, plates were washed six times with PBS-T. The color reaction was developed with substrate reagent (3,3′,5,5′-tetramethylbenzidine peroxidase solution, R&D systems, Minneapolis, MN, USA, DY999), and terminated with 2N H_2SO_4. Optical density was measured at 450 nM using a microplate reader.

SHP-1 expression and MAPK activity. Mitogen activated protein kinase (MAPK) expression was examined 15 min after H_2O_2 treatment. H292 cells treated with various compounds were disrupted in cell lysis buffer containing protease and phosphatase inhibitors. Cell lysates were centrifuged at 4 °C, 27,000*g* for 30 min, and the concentration of the proteins in the supernatant was determined using the Bradford method. Each protein sample was separated by 10% SDS–PAGE, and transferred to PVDF membrane in Tris-buffered saline (TBS) for 2 h. Blots were incubated with phospho-p44/42 (ERK) MAPK antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA) and p44/42 MAPK antibody (1:1000, Cell signaling Technology) diluted in 2.5% skimmed milk and phospho-p38 MAPK antibody (1:1500, Cell signaling Technology) or p38 MAPK antibody (1:1500, Santa Cruz Biotechnology Inc.) diluted in 5% BSA for 3 h. After washing with TBS-T (0.5% Tween 20 in TBS), blots were incubated with anti-mouse IgG or anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h. Following a wash with TBS-T, blots were visualized using chemiluminescent substrate.

Induction of oxidative stress in lungs of heterozygous mev/+ mice. Paraquat dichloride (Riedel de Haen, Seelze, Germany), which is a well known chemical agent inducing oxidative stress *in vivo,* was