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The Role of MAPK and Nrf2 Pathways in Ketanserin-Elicited Attenuation of Cigarette Smoke–Induced IL-8 Production in Human Bronchial Epithelial Cells

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Cigarette smoking is a major risk factor in chronic obstructive pulmonary disease (COPD) with chronic airway inflammation as a key feature. Blockade of serotonin receptor 2A (5-HTR_{2A}) with ketanserin has been found to improve lung function in COPD patients. Furthermore, ketanserin has been shown to possess anti-inflammatory properties *in vivo*. In this study, we investigated the antioxidative and anti-inflammatory properties of ketanserin and its underlying mechanism of action on cigarette smoke–induced interleukin (IL)-8 release *in vitro*. Primary normal human bronchial epithelial cells and human bronchial epithelial cell line (BEAS-2B) were treated with or without ketanserin prior to exposure to cigarette smoke medium (CSM). Exposure to CSM caused elevation of both mRNA and release of IL-8 with increased phosphorylation of p38 and extracellular signal-regulated kinases 1 and 2 (ERK1/2). Consistently, CSM-induced IL-8 release was blocked by SB203580, U0126, or MEK1 small interfering RNA (siRNA) but not SP600125. On the other hand, CSM caused a dose-dependent decrease in the ratio of reduced glutathione to oxidized glutathione (rGSH/GSSG) together with an increased translocation of Nrf2 to the nucleus demonstrated by Western blot analysis. Knock down of Nrf2 by siRNA completely blocked CSM-induced IL-8 release. Ketanserin suppressed CSM-induced IL-8 release by inhibiting p38, ERK1/2 MAPK, and Nrf2 signaling pathways and partially inhibited CSM-induced reduction of rGSH/GSSG ratio. Our data demonstrated the novel antioxidative and anti-inflammatory role of ketanserin via the Nrf2 signaling pathway in CSM-exposed human bronchial epithelial cells. This may open up new perspectives in the development of novel therapeutic targets in the treatment of cigarette smoke–related COPD.

Key Words: **chronic obstructive pulmonary disease; cigarette smoke; human bronchial epithelial cells; interleukin-8; serotonin.**

Chronic obstructive pulmonary disease (COPD) is characterized by airway inflammation and progressive tissue destruction leading to airflow obstruction. Cigarette smoking is the major etiologic cause of COPD. Exposure to cigarette smoke results in airway inflammation, in which different cell types are involved, such as neutrophils, macrophages, lymphocytes, and, in particular, airway epithelial cells. As the first site of contacting cigarette smoke, airway epithelial cells play an important role in monitoring the airway inflammation process. Exposure to cigarette smoke has been reported to elevate the release of interleukin (IL)-8 in airway epithelial cells, and this is positively correlated to the airway dysfunction (Kodama *et al.*, 2009). IL-8 is a chemoattractant that stimulates the recruitment of neutrophils that provokes further inflammatory responses (Kunkel *et al.*, 1991).

Activation of 5-hydroxytryptamine (5-HT, serotonin) receptor (5-HTR) subtypes with 5-HT or selective 5-HTR agonists has been reported to increase the release of proinflammatory cytokines, IL-6, and IL-8 in the human bronchial epithelial cell line BEAS-2B and alveolar epithelial cells type II (AEC-II) (Bayer *et al.*, 2007). Ketanserin, a selective 5-HTR_{2A} antagonist has been reported to improve lung function in two observational clinical studies of COPD patients (Cazzola *et al.*, 1987, 1990) and to possess anti-inflammatory properties *in vivo* (Hong *et al.*, 2006). However, little is known regarding the mechanisms of how ketanserin suppresses inflammation in the airway. Activation of 5-HTR_{2A} stimulates mitogen-activated protein kinase (MAPK) signaling cascade (Greene *et al.*, 2000; Knauer *et al.*, 2009), in which MAPK, including p38 and extracellular signal-regulated kinases 1 and 2 (ERK1/2), have recently been shown to be involved in cigarette smoke–induced IL-8 release in cultured human epithelium (Moretto *et al.*, 2009). In particular, p38 activation has been found in COPD patients, and this is inversely correlated to forced expiratory volume in 1 s (FEV1) and the ratio FEV1/forced vital capacity (Renda *et al.*, 2008). A p38α-selective inhibitor, SD-282, has been reported to suppress cigarette smoke–induced lung inflammation *in vivo* (Medicherla *et al.*, 2008). Moreover, ERK1/2 has been suggested to be involved in cigarette smoke–induced inflammation via modulating the

binding activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) in human alveolar basal epithelial cell line A549 (Zhao *et al*., 2007).

Oxidative stress caused by the abundance of reactive oxygen species (ROS) in cigarette smoke also induces the elevation of proinflammatory cytokines, such as IL-8 in the airway (Crapo, 2003). In oxidant-induced lung injury or inflammation, the glutathione (GSH) system plays an important role in antioxidant defense mechanisms. Reduced state of GSH (rGSH) is a nonprotein thiol found ubiquitously in cells. When oxidative burden, glutathione peroxidases (GPx) and peroxiredoxin 6 (Prdx 6) catalyze the reduction of hydrogen peroxide by GSH to oxidized glutathione (GSSG) and water. Accumulation of GSSG is potentially cytotoxic, therefore, cells normally maintain a high activity of glutathione reductase (GR) to restore the proper ratio of rGSH/GSSG (Forman *et al*., 2009). Exposure to cigarette smoke reduces the levels of total GSH in A549 (van der Toorn *et al*., 2007). Depletion of intracellular GSH and/or elevation of ROS may result in the activation and translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) to the nucleus (Rahman, 2005b). Nrf2 is a transcriptional factor which is essential for antioxidant-related element–induced transcription of phase II detoxifying genes, including the catalytic heavy subunit of glutamylcysteine ligase (GCLC), a rate-limiting enzyme for the synthesis of GSH, and proinflammatory cytokines (Zhang *et al*., 2005). It is unclear whether ketanserin blocks cigarette smoke–induced IL-8 via an antioxidative pathway.

Taking these findings together, we hypothesized that ketanserin might suppress cigarette smoke–induced oxidative stress and hence inflammation in human airway epithelial cells. The present study aimed at elucidating the signaling mechanisms involved in cigarette smoke–induced IL-8 release from human bronchial epithelial cells and evaluating the protective mechanisms of ketanserin in cigarette smoke–induced inflammation.

MATERIALS AND METHODS

Reagents. Cell culture reagents were from Gibco (Carlsbad, CA) unless otherwise stated. Ketanserin, 5-HT, actinomycin D, and cycloheximide were purchased from Sigma-Aldrich Corp (St Louis, MO). SB203580 and SP600125 were from Calbiochem, Inc. (La Jolla, CA), and U0126 was from Promega (Madison, WI). Phosphorylated and total form ERK1/2 antibodies, Nrf2 and lamin A/C antibodies, small interfering RNA (siRNA) transfection reagent, control siRNA-A, MEK1, and Nrf2 siRNA were from Santa Cruz Biotechnology (Lake Placid, NY); phosphorylated and total form p38 and JNK antibodies were from Cell Signaling Technology (Beverly, MA). Primers were synthesized according to published sequences from Invitrogen (Carlsbad, CA). Sources of other materials are noted accordingly in the text.

Cell culture and treatments. The primary normal human bronchial epithelial (NHBE) cells were a gift from Dr Michael CW Chan, Department of Microbiology, The University of Hong Kong. Undifferentiated NHBE cells (passage 2–4) were cultured with bronchial epithelial basal medium (BEBM) supplemented with growth factors and hormones (Lonza Walkersville, Inc., Walkersville, MD). Cells were maintained in a humidified incubator at 37°C with 95% (v/v) air and 5% (v/v) CO_2. Medium was replaced by fresh treatment medium (BEBM without growth factors or hormones) 24 h before treatment. The human bronchial epithelial cell line BEAS-2B (American Type Culture Collection, Rockville, MD) was cultured as described previously (Pan *et al*., 2006). Briefly, passages 42-50 BEAS-2B cells were cultured in keratinocyte serum-free medium (K-SFM; Gibco) supplemented with 5 ng/ml epidermal growth factor (EGF) and 50 mg/ml bovine pituitary extract. Medium was replaced by fresh treatment medium (K-SFM without EGF or bovine pituitary extract) 24 h before treatment. Cells were pretreated with or without various inhibitors as indicated for 1 h before exposure to cigarette smoke medium (CSM). After treatment, medium was collected for the measurement of IL-8 release; cells were reserved for cell viability test, total mRNA or protein extraction in independent experiments.

RNA interference. Knock down of MEK1 and Nrf2 was achieved by transfecting BEAS-2B cells with siRNA of MEK1 and Nrf2 from Santa Cruz Biotechnology according to the manufacturer's instructions.

Preparation of aqueous phase CSM. The preparation of CSM was based on a previous study (Culpitt *et al*., 2003). Briefly, smoke from two cigarettes (11 mg Tar, 0.8 mg nicotine; Camel; filter, R.J. Reynolds, Winston-Salem, NC) with the mouthpiece filter removed (by cutting the wrapping paper circumferentially at the point where the glass-fiber filter meets the tobacco leaves) was bubbled through 20-ml treatment medium and filtered through a 0.22-μm filter. The filtered CSM was regarded as 100%. This was diluted with treatment medium, and the absorbance of diluted samples was measured by spectrophotometer at 320 nm. A standard curve was generated accordingly in order to standardize the concentration of CSM from each independent experiment. CSM was then diluted to the desired percentage with treatment medium.

Cell viability assay. Cell viability was assessed by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) assay from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. Results are expressed in term of percentage of control.

Reverse transcription PCR. The IL-8 mRNA level was quantified using semiquantitative reverse transcription PCR. Total RNA was extracted from cells using TRI reagent (Molecular Research Center Inc., Cincinnati, OH). cDNA was generated by reverse transcription as follows: total RNA (1 μg) was added to a mixture of 5× RT buffer (USB Corp., Cleveland, OH), dNTP (20mM), Oligo (dT) (1 μg, Invitrogen), RNaseOUT Recombinant Ribonuclease Inhibitor (40 U, Invitrogen), and M-MLV reverse transcriptase (400 U, USB Corp.), topped up to 40 μl with DEPC water. The reaction mixture was incubated at 37°C for 30 min and then at 75°C for 10 min. cDNA was stored at 4°C until further analysis. Gene transcript level was assessed by PCR. The guanine nucleotide-binding protein β-polypeptide 2-like 1 (GNB2L1) mRNA level served as an internal control. For quantitative analysis, band intensity was measured with the online software ImageJ (NIH, Bethesda, MD). Results are expressed as the ratio of IL-8 to GNB2L1. The primer sequences and product sizes are listed in Table 1.

Measurement of IL-8 release. Measurement of the released IL-8 was determined by enzyme-linked immunosorbent assay (ELISA) kits from BD Biosciences-Pharmingen (San Diego, CA) according to the manufacturer's instructions. The detection range of the kit was 3.1–200 pg/ml.

Nuclear protein extraction. After treatment, cells were harvested with NE-PER nuclear and cytoplasmic extraction reagents (PIERCE, IL) together with the working concentration of protease inhibitor cocktail (Calbiochem) according to the manufacturer's instructions. Nuclear proteins were used for determining the translocation of Nrf2. Lamin A/C served as a loading control.

Western blot analysis. Protein extracts were separated on 12.5–15% SDS-PAGE gel and then transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20. Subsequently, the blocked membrane was incubated with diluted primary antibody (1:250–1:1000) at 4°C overnight. The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) for 1 h at

TABLE 1
Primer Sequences and Product Sizes

Gene of interest	Primer	Sequence, (5′ → 3′)	Tα (°C)/ cycles	Product size (bp)
IL-8	Forward	AGATGTCAGTGCATAAAGACA	56/35	200
	Reverse	TGAATTCTCAGCCCTCTTCAAAAA		
GNB2L1	Forward	GAGTGTGGCCTTCTCCTCT	50/30	224
	Reverse	GCTTGCAGTTAGCCAGGTT		

room temperature. Bands were visualized on Fuji x-ray film (Fujifilm, Tokyo, Japan) using an enhanced chemiluminescence (ECL) kit. After developing the target proteins, the membranes were stripped and reprobed for the corresponding internal control. Quantitative analysis of the chemiluminescent signal was done by an online software Image J. Results are expressed as the ratio of the protein of interest to its corresponding loading control.

Total glutathione and oxidized glutathione assays. The GSH and GSSG concentrations were measured based on a protocol established previously (Rahman *et al.*, 2006). For GSSG measurement, proteins from cell lysate were precipitated out by incubating with 5% sulfosalicylic acid. Protein-free samples were then incubated with 2-vinylpyridine for 1 h on ice. The mixture was then incubated with a master mix containing sodium phosphate buffer (143mM), EDTA (6.3mM), nicotinamide dinucleotide phosphate (NADPH, 2.39mM), glutathione reductase, and 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 10mM) (Sigma-Aldrich) in dark for 30 min at room temperature. The absorbance was measured at 405 nm. The procedure for measuring total GSH was the same as mentioned above except that no incubation with 2-vinylpyridine was done. Reduced GSH (rGSH) was calculated by subtracting the amount of GSSG from total GSH. The rGSH/GSSG ratio was calculated and corrected for total amount of protein. The results are expressed in term of fold of control.

Statistical analysis. Numerical data are expressed as mean ± SEM from at least three independent experiments. Differences in parameters between two groups were analyzed by two-tailed independent Student's *t*-test; multiple comparisons of the means of multiple treatment groups were conducted using one-way ANOVA with the Tukey *post hoc* test, using the Statistical Program for the Social Sciences (SPSS/PASW, version 18.0, Chicago, IL). A p value less than 0.05 was regarded as statistically significant.

RESULTS

Concentration-Dependent Effect of CSM on Cell Viability

To study the cytotoxic effect of CSM, BEAS-2B and NHBE cells were exposed to CSM (0–4%). The MTT assay was performed after 24 h exposure. CSM at 4% caused a significant decrease (~60%) in the viability in BEAS-2B cells but not of NHBE cells (Figs. 1A and 1B). Since 4% CSM was toxic to BEAS-2B cells, 2% CSM was used throughout the rest of the study in both BEAS-2B and NHBE cells.

Effect of CSM on IL-8

To further study the effect of CSM on the release of IL-8, BEAS-2B and NHBE cells were exposed to CSM at various concentrations for 24 h. CSM (1–2%) caused a concentration-dependent increase in IL-8 release, which then decreased to

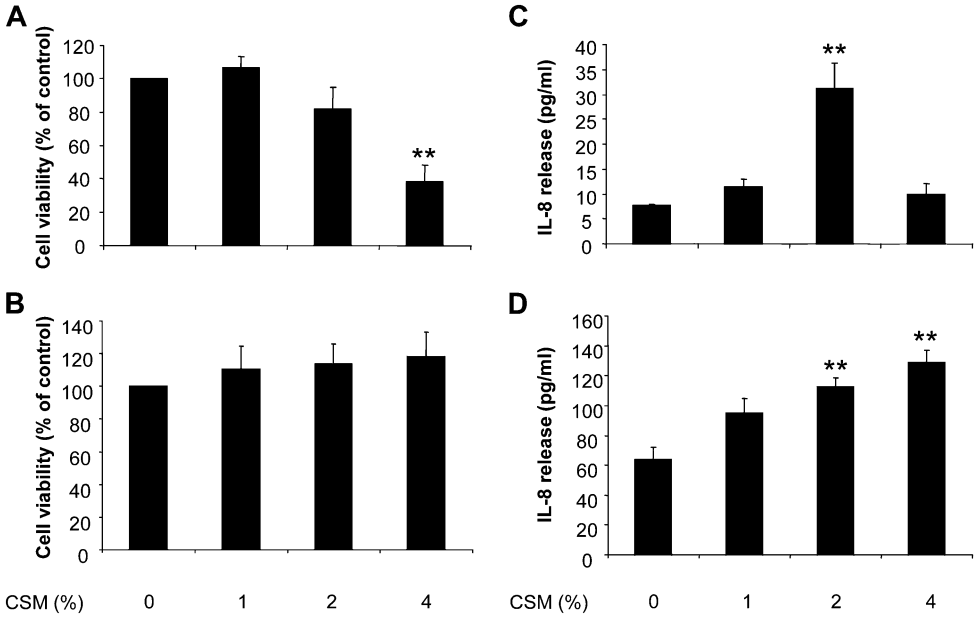

FIG. 1. Cell viability and released IL-8 levels after exposure to different concentration of CSM for 24 h in BEAS-2B cells (A, C) and NHBE cells (B, D). Values are mean ± SEM (n = 3–4). **p < 0.01 (one-way ANOVA) compared with control.

almost baseline level at 4% CSM in BEAS-2B cells (Fig. 1C). In parallel, CSM at 1–4% caused a concentration-dependent increase in IL-8 release in NHBE cells (Fig. 1D).

To investigate the effect of CSM on the synthesis of IL-8, the change of IL-8 mRNA level was studied at various time points. CSM caused a significant increase in IL-8 mRNA after 6 h of exposure and continued progressively up to 24 h (Figs. 2A and 2B). To investigate whether CSM-induced IL-8 release was due to transcriptional or translational control, cells were pretreated with 10 μg/ml actinomycin D or cycloheximide for 1 h prior to exposure to CSM for 24 h. IL-8 mRNA and released IL-8 levels were measured. Drug vehicle (0.1% ethanol) showed no significant effects on IL-8 mRNA level or release. Our results demonstrated that actinomycin D, but not cycloheximide, attenuated CSM-induced IL-8 mRNA level (Figs. 2C and D). On the other hand, both actinomycin D and cycloheximide significantly suppressed CSM-induced IL-8 release (Fig. 2E).

Effects of Ketanserin on CSM-Induced IL-8 Release

To investigate the anti-inflammatory effect of ketanserin, a 5-HTR_{2A} antagonist, BEAS-2B and NHBE cells were pretreated with ketanserin (1–100nM) for 1 h prior to the exposure to CSM for 24 h. We found that ketanserin at 10nM significantly suppressed the CSM-induced IL-8 release in BEAS-2B cells (Fig. 3A), whereas ketanserin at 100nM completely blocked CSM-induced IL-8 in NHBE cells (Fig. 3B). Vehicle control (DMSO) had no such effect. The involvement of 5-HTR_{2A} in the elevation of IL-8 release was further confirmed by treating BEAS-2B cells with various concentration of TCB-2, a 5-HTR_{2A} specific agonist. TCB-2 (100μM) significantly elevated IL-8 release in BEAS-2B cells (10.8 ± 0.7 pg/ml vs. 18.9 ± 0.7 pg/ml, $p < 0.001$, one-way ANOVA).

Effects of Ketanserin on CSM-Induced Oxidative Stress in BEAS-2B Cells

To assess the antioxidative effects of ketanserin on CSM-induced oxidative stress, cells were pretreated with or without ketanserin (10nM) prior to the exposure to CSM. A concentration-dependent reduction of the rGSH/GSSG ratio was found after exposure to CSM for 15 min (Fig. 4A), indicating oxidative stress. Ketanserin but not DMSO (as vehicle control) partially attenuated CSM-induced reduction of rGSH/GSSG ratio (Fig. 4B).

Involvement of MAPK

To reveal the mechanism on how CSM induce IL-8 release, BEAS-2B cells were exposed to specific MAPK inhibitors for 1 h prior to the exposure to CSM for 24 h. BEAS-2B cells pretreated with the p38 inhibitor SB203580 (10μM) or the

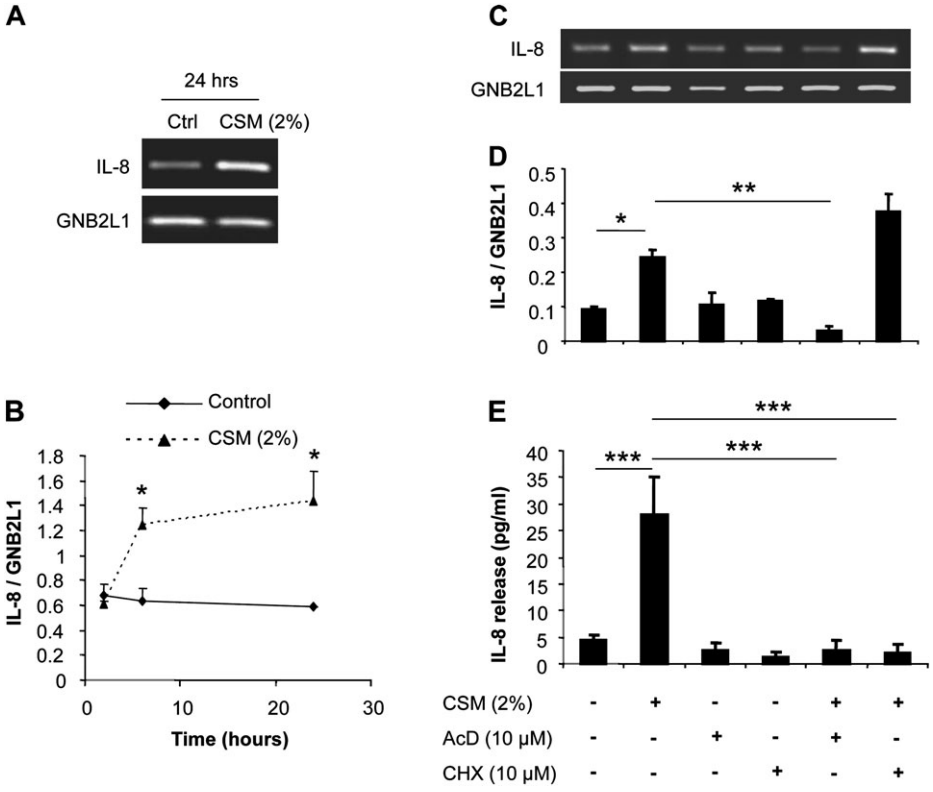

FIG. 2. Transcription and translation controls of CSM-induced IL-8 in BEAS-2B cells. (A) Increased mRNA level of IL-8 after exposure to CSM for 24 h. (B) Time-dependent increase of IL-8 mRNA after exposure to CSM. (C, D) Actinomycin D (AcD) but not cycloheximide (CHX) suppressed CSM-induced IL-8 mRNA levels. (E) Both AcD and CHX blocked CSM-induced IL-8 release. Values are mean ± SEM ($n = 3$–6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Student's *t*-test and one-way ANOVA).

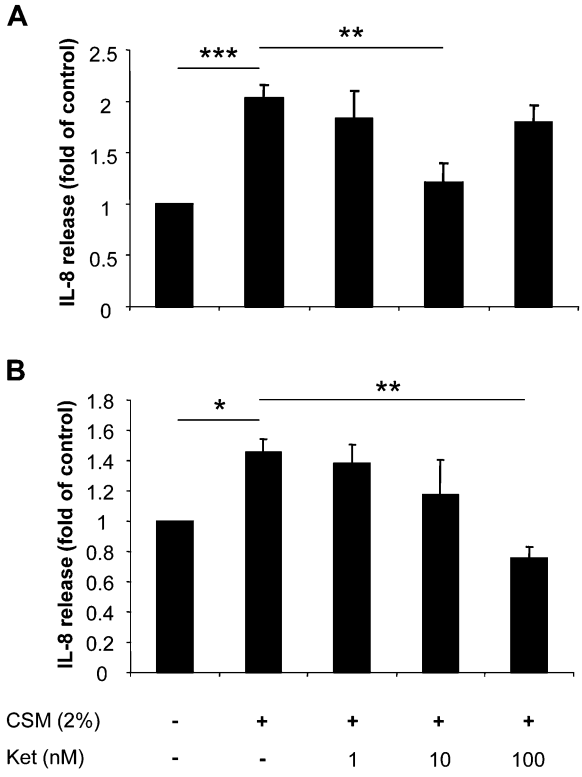

FIG. 3. Ketanserin (Ket) attenuated CSM-induced IL-8 release in BEAS-2B (A) and NHBE cells (B). Values are mean ± SEM ($n = 4$–6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (one-way ANOVA).

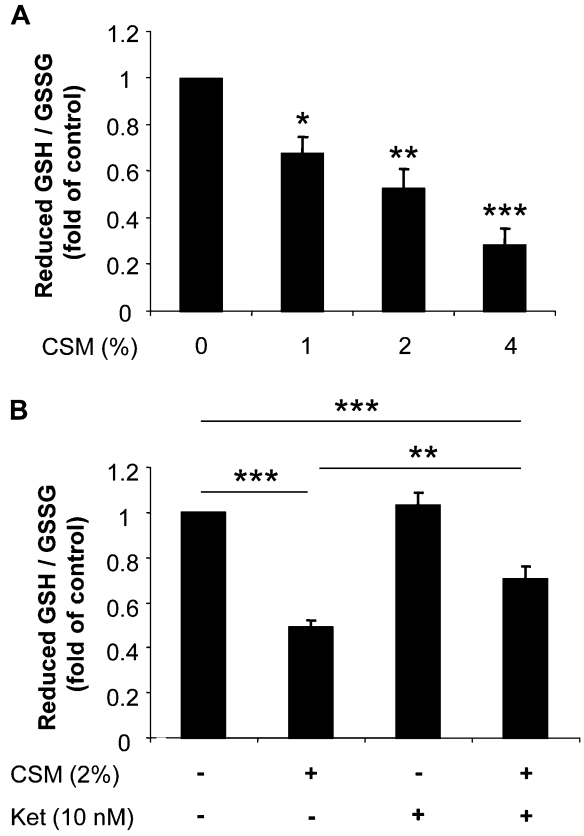

FIG. 4. Ratio of reduced to oxidized glutathione (rGSH/GSSG) after exposure to CSM for 15 min in BEAS-2B cells. (A) Dose-dependent reduction of rGSH/GSSG ratio after exposure to different concentration of CSM. (B) Ketanserin (Ket) partially restored CSM-induced reduction of rGSH/GSSG ratio. Values are mean ± SEM ($n = 3$–6). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (one-way ANOVA).

MEK inhibitor U0126 (10μM), showed significant attenuation of CSM-induced IL-8 release (Fig. 5A). Vehicle control (DMSO) had no such effect. Inhibition of JNK with SP600125 (10μM) did not suppress the CSM-induced IL-8 release. Knock down of MEK1 by siRNA significantly suppressed the CSM-induced IL-8 release (Fig. 5B).

Effect of Ketanserin on CSM-Induced MAPK Activation in BEAS-2B Cells

To further investigate the inhibitory role of ketanserin, the effects of ketanserin on the MAPK pathway were studied. Cells were pretreated with or without ketanserin (10nM) for 1 h prior to exposure to CSM for 15 min. This time point was chosen because in our pilot study using Western blot, the phosphorylation of MAPK was found to be maximal before declining at 30 min (data not shown). Ketanserin (10nM), but not the vehicle control (DMSO), attenuated CSM-induced p38 and ERK1/2 activation (Fig. 5C).

Involvement of Nrf2 Signaling Pathway

Activation of the Nrf2 signaling pathway is a self-defensive response to oxidative stress (Biswas and Rahman, 2009; Rahman, 2008). To determine the involvement of this pathway in CSM-induced IL-8 release, BEAS-2B cells were transfected with Nrf2 siRNA in order to knock down the Nrf2. In cells transfected with Nrf2 siRNA, but not the negative control siRNA-A, the CSM-induced IL-8 release was blocked (Fig. 6A).

Effect of Ketanserin on CSM-Induced Nrf2 Translocation in BEAS-2B Cells

To further determine whether ketanserin suppresses CSM-induced Nrf2 translocation to the nucleus, cells were pretreated with or without ketanserin (10nM) for 1 h prior to exposure to CSM for 15 min. Ketanserin but not vehicle control (DMSO) was found to suppress CSM-induced Nrf2 translocation to the nucleus (Fig. 6B).

Interrelation Between MAPK and Nrf2 Signaling Pathways

To determine whether CSM-induced Nrf2 translocation was due to the activation of MAPK, BEAS-2B cells were pretreated with MAPK inhibitors for 1 h prior to the exposure to CSM for 15 min. Inhibition of p38 or MEK did not suppress CSM-induced Nrf2 translocation to the nucleus (Fig. 7).

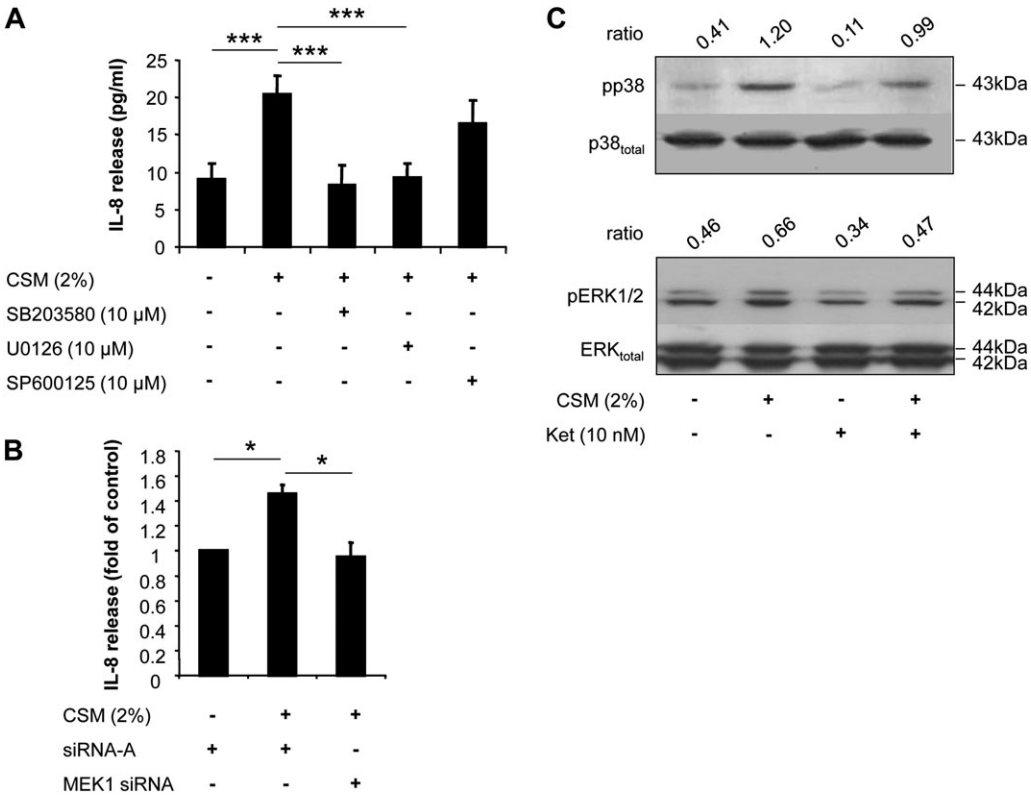

FIG. 5. Ketanserin suppressed CSM-induced IL-8 release via inhibition of MAPK in BEAS-2B cells. (A) Inhibition of p38 by SB203580 or MEK by U0126, but not JNK by SP600125, suppressed CSM-induced IL-8 release. (B) Knock down of MEK1, but not the negative control (siRNA-A), blocked CSM-induced IL-8 release. (C) Increased expression of phosphorylated p38 (pp38) and ERK1/2 (pERK1/2) occurred after exposure to CSM for 15 min, while pretreatment with ketanserin (Ket) suppressed the change. The expression of total p38 and ERK served as loading controls. The values are the ratio of target protein to its loading control from one representative blot; the patterns were consistent in four independent experiments. Values are mean ± SEM ($n = 4$–6). *$p < 0.05$, ***$p < 0.001$ (one-way ANOVA).

DISCUSSION

Interleukin-8 (IL-8) is one of the key mediators that regulate neutrophil infiltration, leading to inflammation in the lung (Simpson *et al.*, 2009). Cigarette smoke extract has been reported to induce IL-8 release in human sinonasal epithelial cells (Mulligan *et al.*, 2009). In agreement with the literature (Witherden *et al.*, 2004), our results demonstrated a concentration-dependent effect of CSM on IL-8 release in both BEAS-2B and NHBE cells. The decrease in IL-8 release in BEAS-2B cells after exposure to 4% CSM may be explained by cell death. We further investigated the mechanisms of CSM-induced IL-8

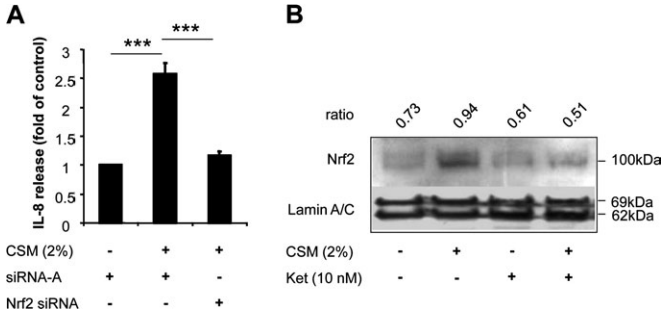

FIG. 6. Ketanserin suppressed CSM-induced IL-8 release in BEAS-2B cells via inhibition of Nrf2. (A) Knock down of Nrf2, but not the negative control (siRNA-A), blocked CSM-induced IL-8 release. (B) Increased translocation of Nrf2 to the nucleus after exposure to CSM for 15 min, while pretreatment with ketanserin (Ket) suppressed the change. Lamin A and C (A/C), the nuclear membrane structure components, served as a loading control. The values are the ratio of Nrf2 to Lamin A/C from one representative blot; the patterns were consistent in four independent experiments. Values are mean ± SEM ($n = 4$–6). ***$p < 0.001$ (one-way ANOVA).

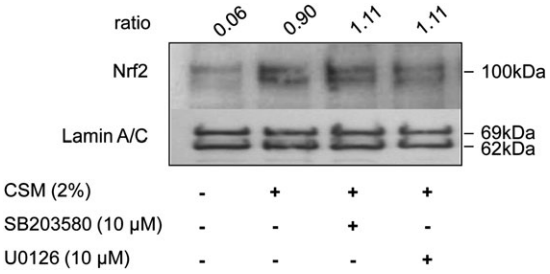

FIG. 7. Inhibition of p38 by SB203580 or MEK by U0126 did not suppress CSM-induced Nrf2 translocation to the nucleus. Lamin A/C served as a loading control. The values are the ratio of Nrf2 to Lamin A/C from one representative blot; the patterns were consistent in four independent experiments.

release in BEAS-2B cells. Actinomycin D, a transcription inhibitor, suppressed both CSM-induced IL-8 mRNA level and release. On the other hand, cycloheximide, a protein synthesis inhibitor, also suppressed CSM-induced IL-8 release. These results indicate that CSM-mediated IL-8 release is regulated at both transcriptional and translational levels.

The classical MAPK pathway including p38, ERK1/2, and JNK play an important role in regulating inflammation and oxidative stress in COPD (Rahman, 2005a). Expression of different MAPK components in response to cigarette smoke has been suggested to be cell type specific (Mercer and D'Armiento, 2006), which might explain our findings that inhibition of p38 or MEK, but not JNK, blocked CSM-induced IL-8 release. The MEK inhibitor, U0126, has been reported to inhibit not only ERK1/2, but also ERK5 (Su *et al.*, 2011). In order to further specify the role of ERK1/2, we conducted a knock down experiment using MEK1 siRNA that specifically blocked the upstream signal of ERK1/2. Our results demonstrated that blockade of MEK1 was sufficient to suppress CSM-induced IL-8, specifying the role of ERK1/2. Moretto *et al.* (2009) have reported that the α, β-unsaturated aldehydes contained in cigarette smoke elevate IL-8 release accompanied by an increased expression of active p38 and ERK1/2 in cultured normal human lung fibroblasts and small airway epithelial cells, consistent with our findings.

Although the involvement of MAPK has been widely studied in cigarette smoke–induced IL-8 production, i.e., via the NFκB pathway (Simone *et al.*, 2011), the upstream mechanisms are not fully understood. One possible mechanism is the activation of 5-HTR_{2A}. Bayer *et al.* (2007) reported the use of R-(-)-DOI-hydrochloride, a selective 5-HTR_2 agonist, to induce IL-8 release in human airway epithelial cells, indicating the involvement of 5-HTR_2 in the regulation of IL-8 in the airways. Furthermore, TCB-2, a 5-HTR_{2A}-specific agonist, significantly induced IL-8 release in our *in vitro* model, specifying the involvement of 5-HTR_{2A}. Our study provides further evidence for the involvement of 5-HTR_{2A} coupled to p38 and ERK1/2 MAPK pathways in CSM-induced IL-8 release, because the concentration of ketanserin used in suppression of CSM-induced MAPK activation and IL-8 release has been reported to selectively block 5-HTR_{2A} in airway smooth muscle *in vitro* (Zhang *et al.*, 2007). We found that the window for an effective inhibitory effect of ketanserin on CSM-induced IL-8 was narrow in BEAS-2B cells, whereas a dose-dependent inhibitory effect of ketanserin was observed in NHBE cells. We speculate that such difference may be due to the differential expression of 5-HTR subtypes between the two cell types, resulting in nonspecific bindings to other 5-HTR in BEAS-2B cells that may also contribute to the induction of IL-8, which requires further study. Although the selectivity of ketanserin at the nanomolar range has been suggested to cross-react with histamine H1 receptor (Leysen *et al.*, 1982), we found that mepyramine (10–100nM), a selective histamine H1 receptor antagonist, did not suppress CSM-induced IL-8 release (data not shown), confirming the involvement of 5-HTR_{2A} in our model.

As well as the inhibition of CSM-induced MAPK pathways through blocking 5-HTR_{2A}, our data demonstrated a novel antioxidative property of ketanserin in CSM-induced oxidative stress. Apart from being an exogenous source of ROS, cigarette smoke induces oxidative stress in various ways, including

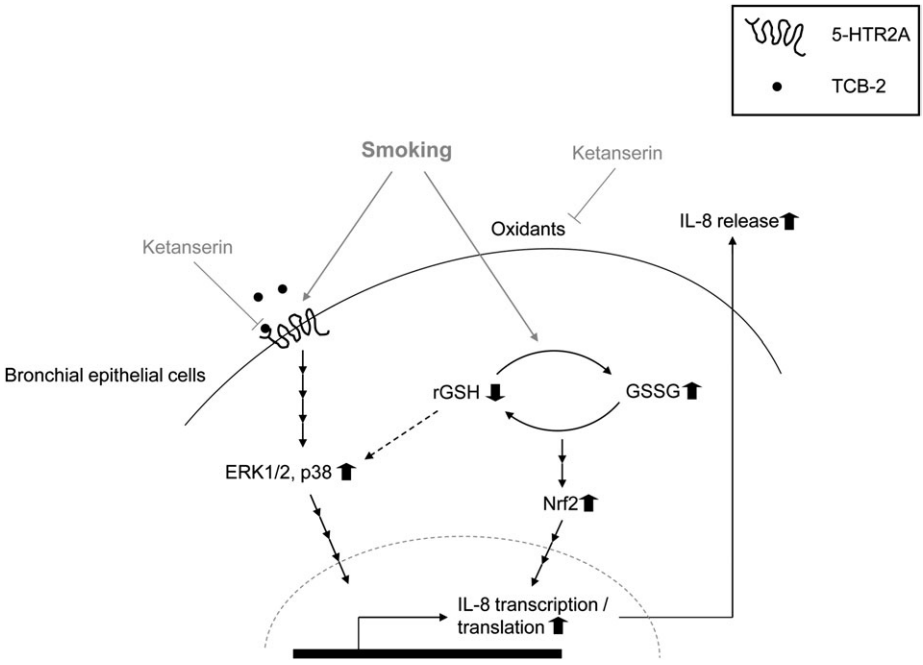

FIG. 8. Schematic diagram of the protective mechanisms of ketanserin on CSM-induced IL-8 release.

reduction of mitochondrial potential, antioxidant capacity, and enzymes activity (mitochondrial complexes I, II, III, superoxide dismutase, and glutathione peroxidase [Jia *et al*., 2007]), resulting in the increased endogenous levels of ROS. In agreement with the literature (Muller and Gebel, 1998), CSM disrupted the GSH/GSSG system in our *in vitro* model. Translocation of Nrf2 to the nucleus after treatment with CSM indicated a self-defense response in BEAS-2B cells under oxidative stress, and this phenomenon was shown to involve in the elevation of IL-8 by using the RNA interference approach. Ketanserin partially attenuated the reduction of the CSM-induced rGSH/GSSG ratio. Furthermore, ketanserin reduced the translocation of Nrf2 to the nucleus. These data suggested that ketanserin might function as an antioxidant to attenuate the IL-8 release via antioxidative pathway. In the literature, phosphorylation of Nrf2 by MAPK makes a limited contribution on Nrf2-mediated antioxidative responses (Sun *et al*., 2009). Our results demonstrated that blockade of p38 or MEK did not alter CSM-induced Nrf2 translocation in BEAS-2B cells. Although the MAPK pathway was activated by CSM exposure, MAPK activation did not seem to participate in the translocation of Nrf2, suggesting that MAPK and Nrf2 pathways might act in parallel in our *in vitro* model. We cannot, however, exclude the possibility that blockade of CSM-induced MAPK phosphorylation by ketanserin might partially involve the antioxidative mechanism (Fig. 8). N-acetylcysteine (NAC), a commonly used antioxidant, has been reported to suppressed cigarette smoke extract–induced MAPK activation and IL-8 release in small airway epithelial cells (Moretto *et al*. 2009), indicating cigarette smoke–induced IL-8 production via oxidative pathway.

In conclusion, we demonstrated that CSM-induced IL-8 release was mediated by 5-HTR_{2A}, via activation of p38 and ERK1/2 MAPK and Nrf2 signaling pathways. Our results provide evidence for a novel antioxidative and anti-inflammatory role of ketanserin in CSM-induced airway inflammation. Future study is warranted to investigate TCB-2 or oxidative stress inducers (i.e. lipopolysaccharide, LPS)-induced IL-8, and its signaling involved (i.e. the MEK3-mediated p38 MAPK pathway), in order to distinguish the antioxidative role of ketanserin from being a receptor antagonist. This study may open up new perspectives in the development of novel therapeutic targets in the treatment of cigarette smoke–related COPD.

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