

Cigarette smoke stimulates the production of chemokines in mast cells

Esmail Mortaz,^{*,†,1} Frank A. Redegeld,^{*} Hadi Sarir,^{*} Khalil Karimi,[‡] Danielle Raats,^{*} Frans P. Nijkamp,^{*} and Gert Folkerts^{*}

^{*}Division of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Sciences, Utrecht University, Utrecht, The Netherlands; [†]Department of Basic Science, Section of Biochemistry, Faculty of Veterinary Medicine, Urmia University, Iran; and [‡]Department of Pathology and Molecular Medicine, Centre for Gene Therapeutics, McMaster University, Ontario, Canada

Abstract: Chronic obstructive pulmonary disease is a major health problem and will become the third largest cause of death in the world by 2020. It is currently believed that an exaggerated inflammatory response to inhaled irritants, in particular, cigarette smoke (CS), causes the progressive airflow limitation, in which macrophages and neutrophils are attracted by chemokines, leading to oxidative stress, emphysema, small airways fibrosis, and mucus hypersecretion. Smoking is also associated with an increase in mast cell numbers in bronchial mucosa. This study was conducted to determine the direct effects of CS on mast cell function, using murine bone marrow-derived mast cells (BMMC) as an *in vitro* model. BMMC were cultured from BALB/cBy mice for 3 weeks. Cells were treated with CS medium (CSM) for 30 min or 16 h. The effects of CSM on mast cell degranulation and chemokine production were measured. Moreover, we investigated the effect of CSM on I κ B- α degradation and p38, Erk1/2, p65, and CREB expression by Western blotting. We found that CSM stimulated the release of chemokines in a noncytotoxic manner but did not induce mast cell degranulation. CSM induced phosphorylation of Erk1/2, p38, and CREB and increased translocation of p65 without degradation of I κ B- α NF- κ B in mast cells. The induction of chemokine production by CSM in mast cells could promote and prolong the inflammatory process. Our observations suggest that mast cells may contribute to the pathogenesis of emphysema through a direct effect of CS on the production of proinflammatory chemokines. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: bone marrow-derived mast cells · COPD · thioredoxin

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major and increasing global health problem. Although there have been major advances in the understanding and management of

asthma, there are no current therapies that reduce the progression of lung function in COPD [1], which is complex with inflammatory and structural cells, all of which have the capacity to release multiple inflammatory mediators [2]. Cigarette smoke (CS) has been considered a major player in the pathogenesis of COPD [2]. Exposure to CS activates an inflammatory cascade in the airways, resulting in the production of a number of potent cytokines and chemokines with accompanying damage to the lung epithelium, increased permeability, and recruitment of macrophages and neutrophils [3].

Mast cells normally reside close to epithelia, blood vessels, nerves, smooth muscle cells, and mucus-producing glands [4]. Mast cells in these locations will be exposed to inhaled, environmental challenges. As mast cell activation results in the coordinated release of proinflammatory mediators into the surrounding tissue, activation of this cell type following exposure to environmental challenges may result in chronic inflammatory pathology [5].

In the lungs and skin of smokers, mast cells increase in absolute numbers, and smoking may be associated with activation of mast cells [6, 7]. Earlier studies demonstrated elevated histamine and tryptase levels in bronchoalveolar lavage fluid of smokers [7]. Furthermore, recently, it has been reported that exposure of RBL-2H3 with tobacco-derived materials induces overproduction of proteinases [8] but attenuates degranulation via the release of NO [9].

Mast cell-derived TNF- α or MIP-2 (mouse analogue of human IL-8) induces the influx of neutrophils into sites infection. Thus, mast cell-derived cytokines, chemokines, and proteases are important mediators in the inflammatory process.

Extracts from CS bubbled through water have been used extensively to show that CS can directly activate cells to promote proinflammatory effects. These studies have been performed predominantly in macrophages or monocytes. In this study, we investigated whether CS medium (CSM) had imme-

¹ Correspondence: Division of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Sciences, Utrecht University, P.O. Box 80082, 3508 TB, Utrecht, The Netherlands. E-mail: e.mortaz@uu.nl
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diate and long-term effects on mast cells. In particular, the effect on chemokine production was studied. Here, we show that CSM promotes expression of chemokines MIP-1 α and MIP-2 in mast cells. The role of mast cells in COPD has yet to be fully elucidated, and so, the study presents an interesting, alternative pathway by which smoke can affect the airways.

MATERIALS AND METHODS

Reagents

Recombinant mouse IL-3 and stem cell factor (SCF) were purchased from Peprotech (Sanvertech, Heerhugowaard, The Netherlands). LPS (*Escherichia coli* 055:B5) was purchased from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands). RPMI 1640, Tyrode's buffer, FCS, and nonessential amino acids were purchased from Gibco-BRL Life Technologies (Gibco-BRL Invitrogen Corp., Carlsbad, CA, USA). Penicillin, streptomycin, L-glutamine, sodium pyruvate, and 2-ME were obtained from Sigma-Aldrich. The MIP-1 α and MIP-2 ELISA kits were from R&D Systems (Minneapolis, MN, USA; ITK Diagnostic BV, Uithoorn, The Netherlands). N-Acetylcysteine (NAC), SB203580, and curcumin were obtained from Sigma-Aldrich. Rabbit polyclonal antibody against I κ B- α , mouse mAb specific for phosphorylated (p)-Erk1/2, and mouse mAb against p38 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA; Tebu-bio, Heerhugowaard, The Netherlands), New England Biolabs (Beverly, MA, USA), and Stressgen (Sanbio, Uden, The Netherlands), respectively. The precision protein standards were purchased from Bio-Rad Laboratories (Veenendaal, The Netherlands). HRP-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG were purchased from Dako Diagnostics (Heverlee, Belgium).

Mouse bone marrow (BM) cultures

BM-derived mast cells (BMMC) were generated from BM of male BALB/cBy mice as described before [10]. Briefly, mice were sacrificed, and intact femurs were removed. Sterile, endotoxin-free medium was flushed repeatedly through the bone shaft using a needle and syringe. The suspension of BM cells was centrifuged at 320 *g* for 10 min and cultured at a concentration of 0.5×10^6 nucleated cells/ml in RPMI 1640 with 10% FCS (Sigma-Aldrich, Axell, The Netherlands), 100 units/ml penicillin, 100 μ g/ml streptomycin (Life Technology, Breda, The Netherlands), 10 μ g/ml gentamycin, 2 mM L-glutamine, and 0.1 mM nonessential amino acids (referred to as enriched medium) and a combination of IL-3 (5 ng/ml) and SCF (50 ng/ml) for 3 weeks at 37°C in a humidified atmosphere with 5% CO₂. Nonadherent cells were transferred to fresh medium at least once a week. After 3–4 weeks when a mast cell purity of >95% was achieved, as assessed by toluidine blue staining, the cells were used for the experiments.

Production of CSM

CSM was produced following the method described before [11]. Briefly, a smoking machine (Teague Enterprises, Davis, CA, USA) was used to direct main and side-stream smoke from one cigarette through a 5-ml culture medium (RPMI without phenol red). Hereafter, absorbance was measured spectrophotometrically, and the media were standardized to a standard curve of CSM concentration against absorbance at 320 nm. The pH of the resultant extract was titrated to pH 7.4 and diluted with medium. This solution is considered to be 100% CSE. Solutions ranging from 0.75% to 1.5% were used in the present study following preliminary experiments, which indicated that these were nontoxic concentrations (viability $\geq$ 96%).

Nontoxic concentrations of CSM were detected performing different toxicological assays (lactate dehydrogenase) and FACS analysis (Annexin-V and 7-amino-actinomycin staining).

Activation of BMMC

In all experiments, unless specified otherwise, cells were cultured at a density of 1×10^6 cells/ml in 10 mL tubes containing 2 ml complete medium at 37°C in a humidified atmosphere with 5% CO₂. BMMC were incubated with various concentrations of CSM for 2 h. After washing twice with culture medium, cells

were suspended at 2×10^6 /ml in medium and stimulated with LPS (100 ng/ml) for various time-points. Subsequent incubation times were chosen for optimal effects, i.e., 30 min for I κ B- α expression and 16 h for MIP-1 α and MIP-2 release. mRNA levels were measured at 2–4 h after incubation, and incubations were stopped by centrifugation at 1200 *g* for 5 min at 4°C.

Mast cell degranulation assay

To assay degranulation, BMMC were sensitized with anti-DNP IgE at 37°C for 1 h or left as control. Then cells were washed twice with modified Tyrode's buffer supplemented with 0.1% BSA and 10 mM HEPES, pH 7.2, and resuspended in this supplemented buffer at a density of 0.6×10^6 cells/ml. Cells were aliquoted in 96-well plates (3×10^4 cells per well) and incubated with an indicated concentration of DNP-human serum albumin or CSM for 30 min. Release of β -hexosaminidase was determined as described before [10].

Chemokine assays

MIP-1 α and MIP-2 protein concentrations in supernatants of cells were quantified using ELISA kits, according to the manufacturer's instructions.

Preparation of nuclear extracts

Cells were washed twice with PBS and allowed to equilibrate for 5 min in ice-cold cytoplasmic extraction reagent (Pierce, Rockford, IL, USA) containing protease inhibitors [MiniTM protease inhibitors (Roche Diagnostics BV, Almere, The Netherlands)]. Cells were lysed on ice for 5 min in the mentioned reagent. Following centrifugation at 3500 *g* for 5 min, the supernatants (cytoplasmic extracts) were collected and frozen at –70°C. The pellets were suspended in nuclear extraction buffer containing protease inhibitors. After vigorous mixing and incubating for 10 min on ice, the solution was clarified by centrifugation at 14,000 *g* for 5 min, and the supernatant (nuclear extract) was collected and stored at –70°C. Protein concentrations were determined by using a bicinchoninic acid (BCA) protein assay kit (Pierce). Cytoplasmic or nuclear fractions (25–50 μ g) were subjected to SDS/PAGE [10% (w/v) gel] for detection of I κ B- α , p-Erk1/2 and total Erk, p-p38 and total p38, p65, and CREB expression.

Western blotting

After activation, BMMC were washed once with PBS and lysed in lysis buffer containing 1% Triton X-100 or Nonidet P-40, NaCl, Tris, and MiniTM protease inhibitor. The protein concentration was determined by BCA protein assay (Pierce). The cytoplasmic or nuclear fractions were subjected to SDS-PAGE [10% (w/v)] gel. The separated proteins were electroblotted on polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories), which were then washed once with Tris/HCl, pH 7.4, containing 159 mM NaCl and 1% Tween 20 (TBS-T), and then blocked in superblocking buffer (Pierce) for 1 h. After washing with TBS-T, the membranes were probed with antibodies in cytoplasmic and in nuclear fractions at a dilution of 1:3000 in TBS-T. After three washes with TBS-T, membranes were treated for 1 h with HRP-conjugated, indicated antibodies diluted to 1:20,000 in TBS-T. After three washes with TBS-T, immunoreactive protein bands were revealed with an ECL Western blot analysis system or ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Films were scanned and analyzed on a GS7-10 calibrated imaging densitometer equipped with Quantity One, Version 4.0.3, software (Bio-Rad Laboratories).

Thioredoxin (Trx)/NF- κ B and I κ B- α /phosphotyrosine (PY) coprecipitation

Cells were lysed in ice (10 min) in 20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, and 15 μ g/ml aprotinin and centrifuged at 18,000 *g* for 15 min. Immunoprecipitations of clarified extracts were performed with protein G sepharose (Amersham Pharmacia Biotech), 2.5 μ l Trx-specific (Stressgen), or I κ B- α (Santa Cruz Biotechnology) antibodies. The purified precipitates were dissolved in sample buffer, separated on SDS-PAGE gel, and blotted on PVDF membranes. Western analysis was performed with NF- κ B (p65)-specific or PY (61000; BD Biosciences, San Jose, CA, USA)-specific antibodies. In parallel, Western analysis of whole extract proteins was performed with Trx- or NF- κ B (p65)-specific and I κ B- α antibodies.

Semiquantitative RT-PCR

RNA from untreated and treated cells was isolated using the TRIzol reagent (Invitrogen), and RT was performed in a 20- μ l reaction with 1 μ g total RNA, 50 mM Tris-HCl (pH 8), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 500 μ M each deoxynucleotide triphosphates, 500 ng random hexamers, and SuperScript III RT (Invitrogen) at 42°C for 1 h. The cDNA was diluted tenfold, and then 1 μ l of the dilution was used in a 12.5- μ l PCR containing 66 mM Tris-HCl (pH 9), 16 mM (NH₄)₂SO₄, 140 μ g/ml BSA, 0.4 μ M each primer, 200 μ M each deoxynucleotide triphosphate, 2 mM MgCl₂, 4% glycerol, 4% DMSO, and 1 U Platinum *Taq* DNA polymerase (Invitrogen). Different dilutions of cDNA were amplified using specific primers for MIP-1 α , forward 5'-ATGAAGGTCTC-CACCACTG-3' and reverse 5'-GCATTCACTTCCAGGTCA-3' [12]; MIP-2, forward 5'-AGTTTGCCTTGACCCTGAAGCC-3' and reverse 5'-TGGGTGG-GATGTAGCTAGTTC-3' [13]. mRNA levels were assessed using RT-PCR standardized with the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) as forward 5'-AGTCCACGCTCGTGATTAGC-GATGA-3' and reverse 5'-TGGCCTGTATCCAACACTTCGAGAGGT-3', as described before [14]. PCR products were separated on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide (Bio-Rad Laboratories) and visualized using a Gel Doc 1000 system (Bio-Rad Laboratories).

Statistical analysis

Experimental results are expressed as mean $\pm$ SEM. Results were tested statistically by an unpaired two-tailed Student's *t*-test or one-way ANOVA, followed by Newman-Keuls test for comparing all pairs of groups. Analyses were performed by using GraphPad Prism (Version 2.01). Results were considered statistically significant when *P* < 0.05.

RESULTS

CSM does not induce degranulation of mast cells

We determined the effect of CSM on degranulation of mast cells. Activation with IgE/antigen caused rapid degranulation of mast cells (**Fig. 1**), but treatment of mast cells with CSM (1.5%) did not induce degranulation (**Fig. 1**).

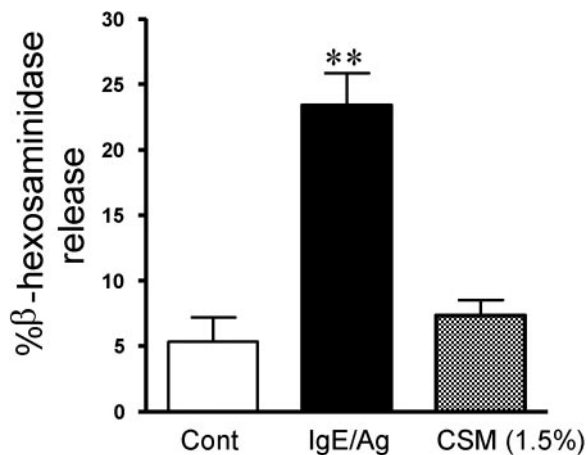


Fig. 1. CSM does not induce degranulation of mast cells. BMMC were sensitized with IgE and then activated with antigen or were exposed to CSM for 30 min. Degranulation was assessed by release of β -hexosaminidase. The percentage of degranulation was calculated as: $(a-b)/(t-b) \times 100$, where *a* is the amount of β -hexosaminidase released from stimulated cells, *b* is that of β -hexosaminidase released from unstimulated cells, and *t* is the total cellular content of β -hexosaminidase. Data are mean $\pm$ SEM of quadruplicate samples. **, *P* < 0.01, compared with nonactivated control cells (Cont).

CSM induces the production of chemokines MIP-1 α and MIP-2 in mast cells

To explore whether CSM induces mediator production by mast cells, BMMC were exposed for 4 h and 16 h to different concentrations of CSM (0.35–6%). The production of MIP-1 α and MIP-2 was determined at protein and RNA level. As shown in **Figure 2, A and B**, stimulation of cells with LPS induces the release of MIP-1 α and MIP-2. Moreover, incubation of mast cells with CSM dose-dependently induced production of MIP-1 α and MIP-2 (**Fig. 2, A and B**) with an optimum at 1.5% CSM. The decrease observed at higher CSM concentration is most likely a result of the cytotoxic effects of these concentrations (see Materials and Methods). The CSM-increased expression of MIP-1 α and MIP-2 in mast cells was confirmed at mRNA level (**Fig. 2C**).

Inhibition of CSM-induced chemokine production by pharmacologic inhibitors

To investigate the involvement of reactive oxygen species (ROS) and oxidative stress in the production of chemokines by CSM-exposed mast cells, we examined the effect of the oxidative stress inhibitor NAC. As shown in **Figure 3**, NAC attenuated the production of MIP-1 α (**A**) and MIP-2 (**B**) induced by CSM.

Next, to gain insight in the mechanism of mast cell activation by CSM, we determined the effect of inhibition of NF- κ B and other protein kinases on the CSM-induced chemokine release. Inhibition of NF- κ B by curcumin and MG-132 resulted in a 50–60% reduction of CSM-induced chemokine production, inhibition of Erk1/2 by PD98059 in a 35% reduction, and inhibition of p38 MAPK by SB239063 in a 33% reduction (**Fig. 3, A and B**). The results of the pharmacologic inhibitors led us to explore the effect of CSM on the activation of several key proteins in the intracellular signal transduction pathway leading to chemokine production in more detail.

CSM inhibits NF- κ B activity and induces Erk1/2 MAPK, CREB phosphorylation

NF- κ B exists as an inactive form bound to the inhibitory protein I κ B- α in the cytoplasm, and degradation of I κ B- α must occur for NF- κ B to translocate to the nucleus. We explored the possibility that CSM stimulates chemokine production by activation the NF- κ B pathway. Protein levels of I κ B- α were analyzed by immunoblotting of whole cell extracts with I κ B- α -specific antibodies. Activation of cells with LPS resulted in a reduction of I κ B- α levels, but exposure to CSM did not lead to an enhanced degradation of I κ B- α (**Fig. 4A**). Alternatively, it has been demonstrated that tyrosine phosphorylation of I κ B- α can activate NF- κ B without degradation of I κ B- α [15]; however, increased tyrosine phosphorylation of I κ B- α was not observed after exposure of mast cells to CSM (**Fig. 4B**). Next, we determined the phosphorylation of MAPK members in mast cells. We found that exposure to CSM induced phosphorylation of p38 and Erk1/2 in mast cells (**Fig. 4A**).

In the nuclear fractions, we studied the effects of CSM on transcription factors p65 and CREB. The majority of p65 was

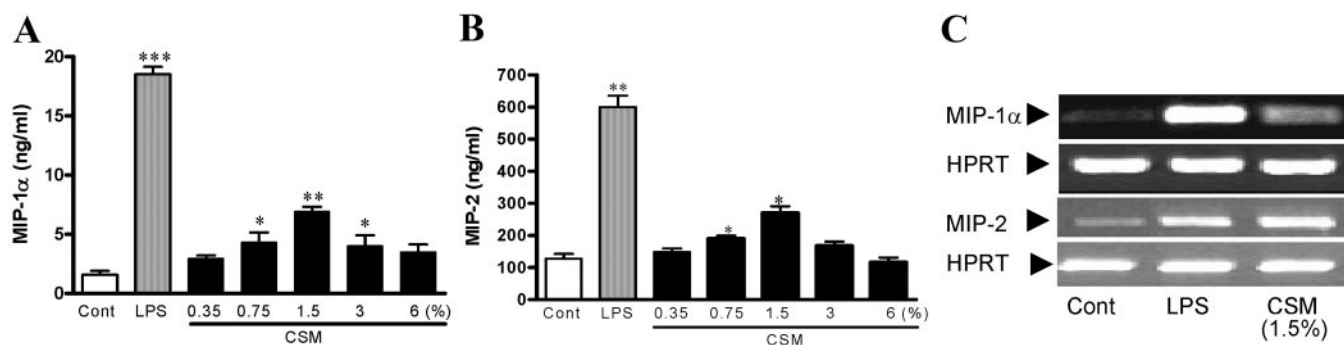


Fig. 2. CSM induces expression and production of MIP-1 α and MIP-2 in mast cells. BMMC were stimulated for 4 h or 16 h with various concentrations of CSM or activated with LPS (1 μ g/ml). Culture supernatants were tested for the indicated chemokine by ELISA after 16 h (A and B), and cells were tested for MIP-1 α , MIP-2, and HPRT mRNA levels by RT-PCR after 4 h (C). Data are representative of three independent experiments, showing the means $\pm$ SD from triplicate cultures. The asterisks represent significant differences compared with nonactivated cells (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

located in the cytoplasmic fraction of control cells at basal conditions. At 30 min of activation, an increase in the nuclear expression of p65 was seen. Exposure of cells to CSM increases nuclear translocation of p65 in mast cells. Furthermore, CSM slightly induces phosphorylation of CREB in mast cells (Fig. 4C).

CSM modulates Trx/NF- κ B formation but not serine and tyrosine phosphorylation of I κ B α in mast cells

It has been demonstrated that CSM can induce increased Trx expression in 3T3 cells. Complex formation of Trx and NF- κ B increased the nuclear transcription activity of NF- κ B without phosphorylation and degradation of I κ B- α in 3T3 cells [16]. We found that CSM did not degrade I κ B- α in mast cells; therefore, we studied the effect of CSM exposure on complex formation of Trx with NF- κ B. As shown in **Figure 5**, CSM indeed increased Trx/NF- κ B complex formation in a dose-dependent manner.

DISCUSSION

Mast cells reside at interfaces with the environment, including the mucosa of the respiratory and gastrointestinal tracts. This localization exposes mast cells to inhaled or ingested, environmental challenges. In the airway of smokers, resident immune cells will be in contact with the condensed components of CS. From these immune cells, mast cells are of particular interest as a result of their ability to promote airway remodeling and mucus hypersecretion. Therefore, in this study, we investigated the effect of CSM on mast cells.

We here demonstrate that exposure of mast cells to CSM causes the production of chemokines MIP-1 α and MIP-2. This stimulation of chemokine production was not accompanied by a general production of cytokines (e.g., TNF- α or IL-6) or degranulation of the mast cells. The chemokine production induced by CSM may be related to the induction of oxidative stress, as evidenced by an abrogation of the response in the presence of the antioxidant agent NAC.

CS may contribute to oxidant-induced damage of the cells via the oxidants and free radicals it contains or by modulation of ROS production by inflammatory cells [15]. Earlier studies show that CS induces oxidative stress by generation of ROS [17–19] and depletes intracellular glutathione [20, 21]. Moreover, CS can generate ROS by a Fenton reaction and can cause lipid peroxidation of membranes [19]. In agreement, prelimi-

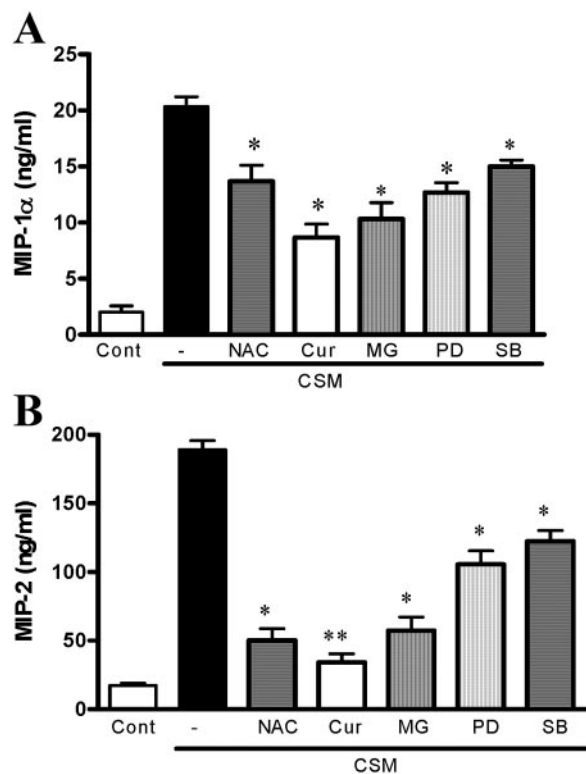


Fig. 3. Effect of pharmacologic inhibitors on production of chemokines induced by CSM in mast cells, which were pretreated with NAC (10^{-4} mol/l), curcumin (Cur; 10^{-6} mol/l), MG-132, PD98059 (10^{-3} mol/l), and SB203580 (10 μ M) for 30 min prior to treatment with CSM (1.5%) for 16 h. Culture supernatants were assayed for MIP-1 α (A) and MIP-2 (B) by ELISA. Data are the mean $\pm$ SEM of four independent experiments with triplicate dishes. The asterisks represent significant differences compared with CSM-treated cells in the absence of inhibitors (black bars; *, $P < 0.05$; **, $P < 0.01$).

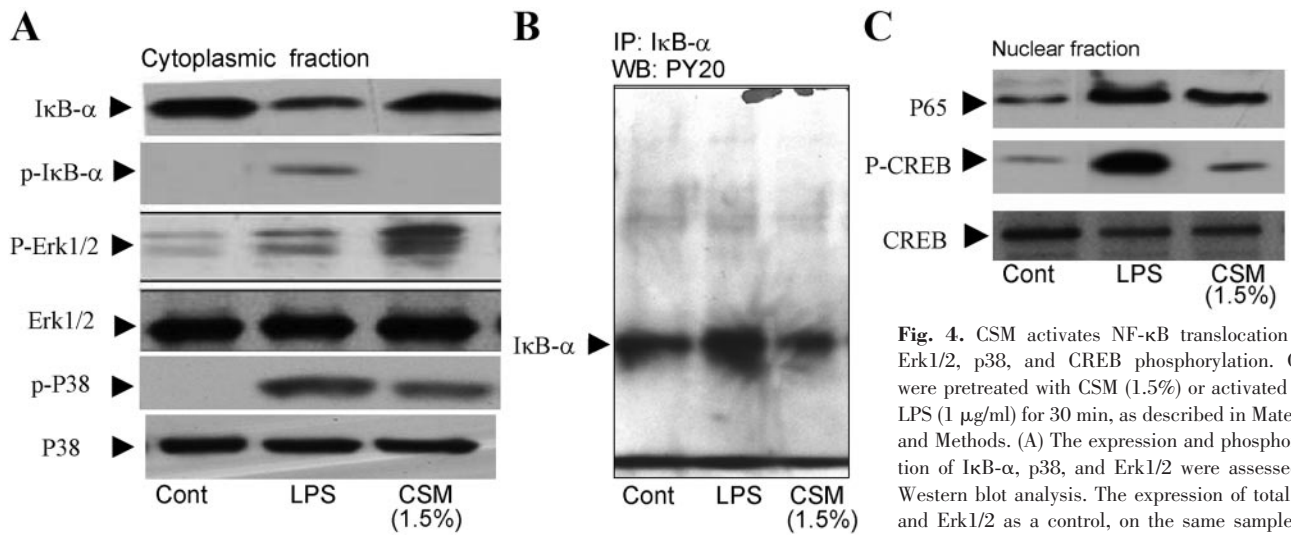


Fig. 4. CSM activates NF- κ B translocation and Erk1/2, p38, and CREB phosphorylation. Cells were pretreated with CSM (1.5%) or activated with LPS (1 μ g/ml) for 30 min, as described in Materials and Methods. (A) The expression and phosphorylation of I κ B- α , p38, and Erk1/2 were assessed by Western blot analysis. The expression of total p38 and Erk1/2 as a control, on the same samples by stripping membranes, was assessed (A). BMMC

were treated with CSM (1.5%) or LPS (1 μ g/ml) as described in Materials and Methods. Cells were lysed, and I κ B- α was immunoprecipitated (IP) from the supernatants using antibodies specific for I κ B- α . Proteins were resolved by SDS-PAGE and analyzed by Western blot (WB) with anti-PY mAb PY-20 conjugated with HRP. The data are representative of three independent experiments with similar results. (C). Nuclear fractions of samples (as described in A) were subjected to Western blot analysis by using p65, p-CREB, and CREB antibodies. Representative results of three independent experiments are shown.

nary experiments have demonstrated that CSM indeed induces production of ROS in mast cells (data not shown). The first signaling proteins to be recognized as oxidative stress-sensitive molecules are transcription factors, such as NF- κ B [22]. ROS strongly affects the activation of NF- κ B [3]. Our study indicates that the effects of CSM on the production of chemokines in mast cells can be a result of activation of NF- κ B via ROS production. We found that NF- κ B inhibitors, such as curcumin and MG-132, were able to abrogate the production of chemokines.

The MAPK pathway is an important signaling pathway leading to chemokine production. We found that CSM induces phosphorylation of the MAPK members Erk1/2 and p38 and that increased transcriptional activity for the chemokines MIP-1 α and -2 corresponded with an increased nuclear translocation of p65. The MAPKs (Erk1/2, p38, and JNK) have been reported to mediate transcription of pro-

teases and cytokines in response to a variety of stimuli. CS or other components of cigarettes indeed activate the MAPK-Erk1/2 pathway in fibroblasts, smooth muscle cells, pulmonary epithelial cells, and bronchoalveolar cells [23]. In normal human bronchial epithelial cells and small airway epithelial cells, treatment with CS extract increased phosphorylation of Erk1/2 [24]. Several reports indicate that members of the MAPK family play crucial roles in oxidant-induced signaling processes [25, 26]. Our observations suggest that the activation of MAPKs may be a cellular response to a CS-induced, altered redox state in mast cells. Normally, Erk1/2 MAPKs are activated by mitogenic and differentiation promoters, and stress-activated protein kinase/JNK and p38-MAPK are stimulated by stress inducers. Our data suggest that activation of Erk1/2 may also be under the control of the oxidation/reduction potential.

The mechanism of NF- κ B activation seems independent of degradation or tyrosine phosphorylation of I κ B- α . The activity of NF- κ B may be regulated by Trx: Several hours after exposure to CSM, enhanced complex formation between NF- κ B and Trx was measured. Recently, it has been demonstrated that the stress response in Swiss 3T3 cells after exposure to CSM was accompanied with a temporal, enhanced expression of Trx reductase transcription and an increase in Trx/NF- κ B complex formation [16].

Further research is required to explore if the enhanced Trx/NF- κ B complexation is indeed responsible for the CSM-induced chemokine production in mast cells.

Our study indicates that mast cells exposed to CS are also activated and that released chemokines could contribute to a local inflammatory response. However, the significance of this study may contribute to a new role of mast cells in pathogenesis of emphysema induced by CS, and mast cells may be interesting cellular targets in the treatment of lung emphysema.

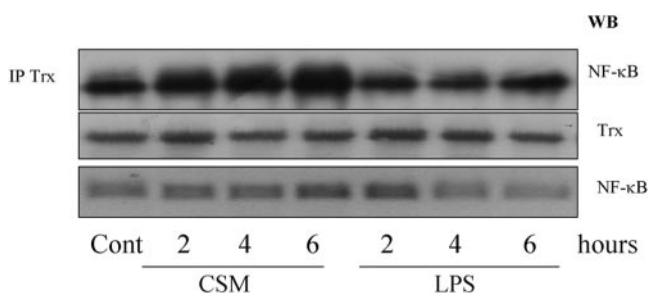


Fig. 5. CSM increases complex formation Trx and NF- κ B, kinetics of Trx/NF- κ B complex formation. Cells were treated with LPS (100 ng/ml) or CSM (1.5%) for 2, 4, and 6 h, as described in Materials and Methods. Coprecipitation of NF- κ B with Trx was detected by Western blot analysis of the immunoprecipitates using a NF- κ B (p65)-specific antibody. To evaluate possible quantitative alterations in the amounts of total Trx and NF- κ B, extracts were subjected to Western blot using Trx- or NF- κ B-specific antibodies.

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