ERJ Express. Published on May 30, 2007 as doi: 10.1183/09031936.00008707

MORAXELLA CATARRHALIS INDUCES ERK- AND NF-κB-DEPENDENT COX-2 AND PGE_2 IN LUNG EPITHELIUM

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Running title: *M. catarrhalis* induces COX-2 and PGE_2

ABSTRACT:

Moraxella catarrhalis is a major cause of infectious exacerbations of chronic obstructive lung disease (COPD). Cyclooxygenase (COX) derived prostaglandins like prostaglandin E2 (PGE_2) are considered as important regulators of lung function. Herein we tested the hypothesis that *M. catarrhalis* induced COX-2-dependent PGE_2 production in pulmonary epithelial cells.

In this study we demonstrated that *M. catarrhalis* specifically induced COX-2 and subsequent PGE_2 release in pulmonary epithelial cells. Furthermore, the prostanoid receptors EP2 and EP4 were also up regulated in these cells. The *M. catarrhalis*-specific ubiquitous cell surface protein A1 (UspA1) was important for the induction of COX-2 and PGE_2. Moreover, *M. catarrhalis*-induced COX-2 and PGE_2 expression was dependent on ERK1/2 driven activation of NF-κB, but not on the activation of p38 MAPK.

In conclusion our data suggests that UspA1 of *M. catarrhalis*, ERK1/2- and NF-κB controlled COX-2 expression and subsequent PGE_2 release by lung epithelial cells. *M. catarrhalis*-induced PGE_2 expression might counteract lung inflammation promoting colonization of the respiratory tract in COPD-patients.

Key words: Cyclooxygenase 2, ERK1/2, *Moraxella catarrhalis*, NF-κB, UspA1, Prostaglandine E_2.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide. Disease progression is characterized by frequent acute exacerbations caused by bacterial or viral infection [6, 16, 24]. Although many studies support the causative role of *Haemophilus influenzae* and *Streptococcus pneumoniae* in the pathogenesis of COPD [16, 25], *Moraxella catarrhalis* was widely ignored for decades because this pathogen was considered to be an irrelevant saprophyte of the upper respiratory tract [15]. However increasing evidence underlines the importance of *M. catarrhalis* for acute exacerbations and disease progression of COPD [15].

The outer membrane protein UspA1 of *M. catarrhalis*, an antigenically conserved high molecular weight adhesin, is expressed by a majority of *Moraxella* isolates from COPD-patients [2]. This virulence factor has been implicated in the targeting of epithelial cell-associated fibronectin and laminin as well as the human CEA-related cell adhesion molecule CEACAM1 [9, 20, 30]. However, little is known about *M. catarrhalis*-bronchial epithelium interaction.

Lipid metabolites of arachidonic acid, including prostaglandins and leukotrienes, have emerged as potent endogenous mediators and modulators of innate immunity in the lung [31, 33]. There is growing evidence that increased concentration of PGE_2 in the lung of patient is a key event in the pathogenesis of COPD [14]. Increased PGE_2 in the lung has been shown to stimulate the secretion of surfactant by alveolar type II cells and wound closure in airway epithelium [33]. It has also been reported that PGE_2 down regulates the production of important inflammatory cytokines such as IL-8, IL-12, MCP-1 and GM-CSF, which are essential for leukocyte migration [33]. In particular prostaglandin E_2 (PGE_2) produced at sites of infection was shown to modulate immune and inflammatory responses [31, 33] and is liberated by lung epithelial cells [17, 18]. The activity of PGE_2 is mediated by four receptors, termed E prostanoid receptors (EP1–EP4) [31, 33]. Although the exact roles of each receptor type are not definitively established, it is

plausible that cAMP accumulation, promoted by the EP2 and EP4 receptors, is associated with inhibition of effector cell functions. However, the EP1 and EP3 receptors are known to increase intracellular calcium and to promote cellular activation [31, 33]. The existence of four subtypes of receptors and the possible expression of multiple receptors in a single cell can explain the multiplicity of biological responses elicited by PGE_2 and how these responses might be diverse in different cells and tissues [31, 33]. It is also probable that during inflammation the repertoire of receptors expressed changes, leading to a wide array of effects.

PGE_2 is a product of the cyclooxygenase (COX)/ prostaglandin H synthase-pathway, which includes two distinct isoforms of COX. The constitutively expressed COX-1 and the (generally) inducible COX-2 [3]. The regulation of the *cox2* promoter is subjected to a tight regulatory network involving nuclear factor-κB (NF-κB) which can be activated by complex kinase pathways centered around p38 and ERK1/2 mitogen-activated protein kinase (MAPK) [3, 17, 18].

The MAPK family is involved in multiple cell functions, including inflammation, proliferation, and apoptosis [3, 17, 18]. Five distinguishable MAPK subfamilies have been identified in mammalian systems; the best described of these are the ERK1/2 (p42/p44), p38, and c-Jun N-terminal kinase pathways [11].

Activation of pro-inflammatory signaling pathways in lung epithelial cells by bacterial infection, including the p38-, ERK1/2-MAPK and, NF-κB pathways are suggested to contribute significantly to disease process in COPD and pneumonia [22, 23, 26]. Although *M. catarrhalis* efficiently infected and stimulated lung epithelial cells [21, 26-28], mechanisms of *M. catarrhalis*-induced activation of COX-2 and PGE_2 release in lung epithelial cells are widely unknown.

In this study we tested the hypothesis that *M. catarrhalis* induces COX-2 expression and subsequent PGE_2 synthesis by stimulation of MAPK pathways and NF-κB in lung epithelial

cells. We report here, that *M. catarrhalis* induced COX-2 expression and a subsequent PGE_2 release. In addition the prostanoid receptors EP2 and EP4 were also up regulated in *M. catarrhalis*-infected cells. The *M. catarrhalis* outer membrane protein UspA1 was found to be important for COX-2 expression and PGE_2 release in bronchial epithelial cells. Furthermore, we found that PGE_2 release and COX-2 expression was depended on the activation of ERK1/2 MAP kinase driven activation of NF-κB but not on the activation of p38 MAPK. Therefore, *M. catarrhalis* induced COX-2 dependent PGE_2 liberation by lung epithelial cells may contribute significantly to the pathogenesis of COPD.

MATERIAL AND METHODS

Bacterial strains

M. catarrhalis wild-type strain O35E (Serotype A) and the isogenic *UspA*1 deficient mutant of O35E (O35E.1) were kindly provided by Eric Hansen (University of Texas Southwestern Medical Center, Dallas, Texas, USA). Antimicrobial supplementation for the *M. catarrhalis* mutant O35E.1 involved kanamycin (15 µg/ml). *M. catarrhalis* strain was grown overnight at 37°C on brain-heart infusion (BHI) agar (Difco Laboratories, BD Heidelberg, Germany) supplemented with 5% heated sheep blood. For infection experiments, single colonies of bacterial overnight cultures were expanded by resuspension in BHI broth and incubation at 37 °C for 2-3 h to midlog phase (A_{405} 0.4–0.6). Subsequently, bacteria were harvested by centrifugation, resuspended in cell culture medium without antibiotics and adjusted to an optical density (OD) at 405 nm of 0.3 (≈1x10^6 CFU/ml) and used for epithelial cell infection at the indicated multiplicity of infection (MOI). To confirm the viability of *M. catarrhalis* in cell culture medium, bacteria were resuspended and OD was measured over time. Data were verified by different CFU countings at different OD of *M. catarrhalis* suspensions.

Cell lines

Human bronchial epithelial cell line BEAS-2B was a kind gift of C. Harris (National Institutes of Health, Bethesda, MD) [12]. Each well was seeded with 4x10^5 BEAS-2B cells per ml and grown in Keratinocyte-SFM (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown to confluence in 75-cm^2 flasks and subsequently cultured in different well plates (both Falcon; Corning Star, Wiesbaden, Germany). Twelve hours before the experiment, cells were grown in medium

without antibiotic supplements. Human embryonic kidney cells (HEK293) were purchased from ATCC (Manassas, VA) and cultured according to supplier instructions.

Materials

Keratinocyte-SFM culture Medium was purchased by Gibco BRL Life Technologies (Paisley, UK). Fetal calf serum, trypsin-EDTA solution, CA-650, and antibiotics were obtained from Life Technologies (Karlsruhe, Germany). Protease inhibitors, Triton X-100, 4-dichloroisocumarin, and Tween20 were purchased from Sigma Chemical Co. (Munich, Germany). The MAP kinase inhibitors U0126 and SB202190, indomethacin, SC-560, and NS-398 were purchased from Calbiochem (Merck, Bad Soden, Germany). TNF-α and IL-lβ were obtained from R&D Systems (Wiesbaden, Germany), and IKK-NEMO binding domain (NBD) from Biomol (Plymouth). All other chemicals used were of analytical grade and obtained from commercial sources.

PGE_2 ELISA

Confluent BEAS-2B cells were infected with *M. catarrhalis* or stimulated with TNF-α and IL-1β as indicated. After incubation, supernatants were collected and processed for PGE_2-quantification by immunoassay, according to manufacturer instructions (R&D Systems, Wiesbaden, Germany) [17, 18].

Immunoblot analysis

For determination of COX-1-, COX-2-, EP1-, EP2-, EP3-, EP4-expression and p38 MAPK and ERK1/2 phosphorylation, BEAS-2B cells were infected or incubated with TNF-α and IL-1β as indicated, washed twice, and harvested. Cells were lysed in buffer containing Triton X-100, subjected to SDS-PAGE and blotted on Hybond-ECL membrane (Amersham Biosciences,

Freiburg, Germany). Immunodetection of target proteins was carried out with specific antibodies: COX-2 (Santa Cruz Biotechnologies, Santa Cruz, CA), COX-1 (Upstate Biotechnology, Lake Placid, N.Y), phospho-specific p38 MAPK antibodies [17, 18] (Cell Signaling, Frankfurt, Germany), phospho-specific ERK1/2 [17, 18] and EP1-4 antibodies (Santa Cruz Biotechnologies), respectively. In all experiments, actin (Santa Cruz Biotechnologies), p38 or p42 (Santa Cruz Biotechnologies) were detected simultaneously to confirm equal protein loading [17-19]. Detection was performed by visualization of IRDye800- or Cy5.5-labeled secondary antibodies (Odyssey infrared imaging system; LI-COR Inc., Lincoln, Nebr.).

RT-PCR

For analysis of COX-2 and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) gene expression in BEAS-2B cells, total RNA was isolated with the RNeasy Mini kit (Qiagen, Hilden, Germany) and reversely transcribed using AMV (Avian Myeloblastosis Virus) reverse transcriptase (Promega, Heidelberg, Germany) [17, 18]. Generated cDNA was amplified by PCR using intron-spanning specific primers for COX-2 (forward: 5'-TGCTGTGGAGCTGTATCC-3', Reverse: 5'-GACTCCTTTCTCCGCAAC-3'), COX-1 (forward, 5′-TGTTCGGTGTCCAGTTCCAATA-3′, reverse, 5′-ACCTTGAAGGAGTCAGGCATGAG-3′), EP1 (forward: 5'-CACGTGGTGCTTCATCGCCCTGGGTC-3', Reverse: 5'-CACCACCATGATACCGACAAG-3'), EP2 (Forward: 5'-TCCAATGACTCCCAGTCTGAGGA-3', reverse: 5'-TCAAAGGTCAGCCTGTTTAC-3'), EP3 (forward: 5'-CTGGTATGCGAGCCACATGAA -3', Reverse: 5'-TGAAGCCAGGCGAACAGCTAT-3'), EP4 (Forward: 5'-TCTGACCTCGGTGTCCAAAAATCG-3', Reverse: 5'-TGGGTACTGCAGCCGCGAGCTA-3'), and GAPDH. All primers were purchased from TIB MOLBIOL, Berlin, Germany. After 35 amplification cycles, PCR products were analyzed on 1.5 % agarose gels, stained with ethidium

bromide and subsequently visualized. To confirm use of equal amounts of RNA in each experiment, all samples were checked for GAPDH mRNA expression [17, 18].

Electrophoretic mobility shift assay (EMSA)

After stimulation of BEAS-2B cells, nuclear protein was isolated and analyzed by EMSA as described previously [18, 23, 26]. IRDye800-labeled consensus NF-κB oligonucleotides were purchased from Metabion, Planegg-Martinsried, Germany. Briefly, EMSA binding reactions were performed by incubating 2 μg of nuclear extract with the annealed oligos according to the manufacturer's instructions. The reaction mixture was subjected to electrophoresis on a native gel PAGE and analyzed by Odyssey infrared imaging system (LI-COR Inc.).

Plasmids and transient transfection procedures

HEK-293 cells were cultured in 12-well plates with DMEM supplemented with 10% FCS. Subconfluent cells were cotransfected by using the calcium phosphate precipitation method according to the manufacturer's instructions (Clonetech, Palo Alto, CA) with 0.2 μg of NF-κB-dependent luciferase reporter [23], 0.2 μg of respiratory syncytial virus -galactosidase plasmid, 0.1 μg of human Toll-like receptor 2 (hTLR2; generously provided by Tularik, San Francisco, CA) [29] expression vectors or control vector, respectively. We used a luciferase reporter-gene assay (Promega, Mannheim, Germany) to measure luciferase activity, and results were normalized for transfection efficiency, with values obtained by respiratory syncytial virus-galactosidase as described previously [23].

Statistical analysis

Data are shown as means ± S.E.M. of at least three independent experiments. A one-way ANOVA was used for data of Fig. 1, 3C, 4, 5B, 6C, 7C, 8. Main effects were then compared by a Newman-Keul`s post-test. Statistical significance was accepted at a $p < 0.01$ indicated by asterisks or H-key.

RESULTS:

***M. catarrhalis* induces COX-2-dependent release of PGE_2 in human bronchial epithelial cells.**

To study the effect of *M. catarrhalis* on lung epithelium, human bronchial epithelial cells BEAS-2B cells, were infected with *M. catarrhalis* strain O35E (MOI 0.1 and 1) or exposed to TNF-α (50 ng/ml) and IL-1β (10 ng/ml) for 4, 8 and 16 h and PGE_2 release was analyzed by ELISA (Fig. 1). *M. catarrhalis* infection of lung epithelial cells time- and concentration-dependently induced the release of PGE_2. PGE_2 activities are mediated through its binding to the prostanoid receptors EP1, 2, 3, 4. Furthermore we noticed a time-dependent induction of EP2 and EP4 in infected cells (Fig. 2). The expression pattern of EP1 and EP3 did not change in these cells (Fig. 2).

PGE_2 release is dependent on the expression of COX-1 and/or COX-2. COX-2 expression may be increased after pro-inflammatory stimulation of cells. Therefore, we analyzed the expression of both iso-enzymes in *M. catarrhalis*-infected lung epithelium. As shown in Fig. 3A, *M. catarrhalis* (MOI 1) induced the transcription of COX-2 mRNA after 1 h of infection. Moreover, we noted a time (1-8 h)-dependent increase in the expression of COX-2 protein, but not of COX-1 protein (Fig. 3B, C) in *M. catarrhalis*-infected BEAS-2B cells.

To test the role of COX-1 and COX-2 in *Moraxella*-induced PGE_2 synthesis in lung epithelium, cells were infected in the absence or presence of the non-selective COX inhibitor indomethacin (1 μM), the selective COX-2 blocker NS-398 (1 μM), or the selective COX-1 inhibitor SC-560 (1 μM). 30 min prior to infection cells were pre-incubated with these drugs.

Inhibition of COX-2 but not COX-1 in *M. catarrhalis*-infected BEAS-2B cells blocked PGE_2 release. The nonselective cyclooxygenase inhibitor indomethacin also strongly reduced PGE_2

secretion (Fig. 4). Thus, *M. catarrhalis* induced COX-2 dependent release of PGE_2 secretion by cultured lung epithelial cells. The concentration of indomethacin, NS-398, and SC-560 used in this study did not alter bacteria growth within the time frame tested (data not shown). The inhibitors did not reduce epithelial cell numbers or induce morphological signs of cytotoxicity (data not shown).

The COX-2-dependent release of PGE_2 is induced by the *M. catarrhalis*-specific ubiquitous cell surface protein 1 (UspA1).

By infecting bronchial epithelial cells with *M. catarrhalis* strain O35E or its UspA1-deficient mutant strain O35E.1, we analyzed the impact of this bacterial virulence factor in more detail. The UspA1 deficient mutant O35E.1 induced COX-2 expression (4 h p.i.) and PGE_2 release (16 h p.i.) to a significantly lower extent (Fig. 5) than the wild type strain O35E, respectively. Our data suggested that UspA1 plays an important role in the *M. catarrhalis* induced COX-2 expression and subsequent PGE_2 release in airway epithelial cells.

Inhibition of ERK1/2 and p38 MAPK blocked *M. catarrhalis*-induced expression of COX-2 and PGE_2 release in human bronchial epithelial cells.

Because MAP kinases are considered as important regulators of proinflammatory gene expression, we analyzed MAP kinase activation and its impact on *M. catarrhalis*-related COX-2 expression and PGE_2 release. Infection of BEAS-2B cells with *M. catarrhalis* was associated with the activation of the MAPK p38, ERK1/2 as demonstrated by immunoblot analysis of phosphorylated ERK1/2 and p38 (Fig. 6A). The degree of MAP kinase phosphorylation observed was comparable to that seen following TNF-α/IL-1β exposure. The ERK1/2 inhibitor U0126, significantly inhibited COX-2 activation and PGE_2 release by *M. catarrhalis* in BEAS-2B cells

whereas the p38 MAPK inhibitor SB202190 had no effect on either target (Fig. 6B, C). Similar results were obtained with SB203580, another specific p38 inhibitor (data not shown).

M. catarrhalis-induced COX-2 expression and PGE_2 release depended on NF-κB activation in bronchial epithelial cells.

Expression of COX-2 and subsequent PGE_2 release in cells is considered to be regulated by NF-κB, which is released of its cytosolic sequestration by phosphorylation of its inhibitor IκBα by IKKβ and subsequent proteolytic degradation [3]. To assess NF-κB activation, *M. catarrhalis*-infected BEAS-2B cells were examined for different time periods by electrophoretic mobility shift assay (EMSA). As shown in Fig. 7A, *Moraxella* induced NF-κB activation within 60 min. In the next step, the role of IκB kinase, the central kinase complex of the canonical NF-κB pathway was analyzed. The IκB kinase complex was blocked by pre-incubation of BEAS-2B cells with the cell permeable peptide inhibitor IKK-NBD [13]. IKK-NBD strongly reduced COX-2 protein expression (Fig. 7 B) and release of PGE_2 (Fig. 7 C) in *Moraxella*-infected cells. Overall, our data demonstrates that activation of the NF-κB signaling pathway by *M. catarrhalis* was necessary for the expression of COX-2 and PGE_2-release in lung epithelium. IKK-NBD did not alter bacteria growth within the concentration and time frame tested (data not shown).

M. catarrhalis activated NF-κB via ERK1/2 but not via p38 MAPK.

Our data suggest that activation of ERK1/2 and NF-κB but not p38 MAPK-dependent signaling contributed to *M. catarrhalis*-related expression of COX-2 and subsequent PGE_2 release in BEAS-2B cells. Thus, we hypothesized that ERK1/2 activity is necessary for NF-κB-dependent gene transcription in *Moraxella*-infected cells. We found, that ERK1/2 pathway inhibitor U0126 (10 μM) but not p38-inhibitor SB202190 (10 μM) blocked *M. catarrhalis*-induced NF-κB

activation as shown by NF-κB luciferase reporter assay (Fig. 8). This data indicates that ERK1/2 controlled COX-2 expression and PGE_2 secretion via NF-κB in *M. catarrhalis*-infected bronchial epithelial cells.

DISCUSSION:

An increasing number of epidemiologic studies demonstrating an association between *M. catarrhalis* and respiratory morbidity in the course of COPD [15, 16, 25] prompted us to a detailed analysis of the *M. catarrhalis*-bronchial epithelium interaction.

In this study we demonstrated that infection of *M. catarrhalis* induces ERK1/2-dependent NF-κB activation and subsequent COX-2 expression and PGE_2 release in cultured bronchial epithelial cells. We previously demonstrated that *M. catarrhalis* significantly contribute to the activation of lung tissue cells [21, 26, 27]. In the study presented we found, that *M. catarrhalis*-infection resulted in increased expression of COX-2 in BEAS-2B cells. Increased COX-2 protein expression was followed by PGE_2 liberation, which is known to be the major cyclooxygenase product released by pulmonary epithelial cells [17, 18]. The secretion of several cytokines involved in the cellular inflammatory and reparative processes are known to be modulated by PGE_2. Interestingly, PGE_2 has been shown to increase the secretion of G-CSF in human airway smooth muscle cell [4]. Additionally, the ability of PGE_2 to down regulate the production of important cytokines, such as IL-8, monocyte chemotactic protein-1 (MCP-1) and granulocyte–macrophage-colony stimulating factor (GM-CSF) which are significantly involved in the recruitment of inflammatory cells has also been reported [4, 33]. Montuschi *et al.* demonstrated that exhaled PGE_2 was increased in patients with stable COPD and suggested this to be a mechanism counteracting lung inflammation in COPD [14]. Here we report, that COX-2 expression and PGE_2 release was dependent on UspA1 of *M. catarrhalis*. UspA1, an important adhesin, mediating the adherence of *M. catarrhalis* to human respiratory epithelial cells, has been described as being present on the surface of most *M. catarrhalis* disease isolates examined to date [2, 28, 30, 32]. UspA1 is known to adhere to the epithelial cell-associated laminin and fibronectin [30]. In addition, UspA1 targets the human CEA-related cell adhesion molecule (CEACAM1) a member of the carcinoembryonic antigen family and the immunglobulin

superfamily [8]. Recently we demonstrated that adhesion of *M. catarrhalis* wildtype strain O35E to BEAS-2B cells did not differ compared to the UspA1 deficient mutant [28]. Thus, our findings suggest, that the UspA1-dependent interaction to epithelial cells is essential for COX-2 expression and PGE_2 release. Taken into account that *M. catarrhalis* colonizes the lower respiratory tract of up to 32% of adults with COPD [15], it is likely that the UspA1-dependent induction of PGE_2 release might promote the ability of *M. catarrhalis* to colonize the bronchial epithelium in COPD-patients. A significant UspA1-independent induction of COX-2 expression and PGE_2 release in *M. catarrhalis* infected lung epithelial cells could also be observed. These results suggest that other receptors like TLR2 and TLR4 may partly mediates COX-2 [10] dependent or *Moraxella* related signalling as published elsewhere [26, 27].

The activity of PGE_2 is mediated by four receptors, termed E prostanoid receptors (EP1–EP4) [33]. In our study we demonstrated that infection of bronchial epithelial cells with *M. catarrhalis* increased the transcription and expression of the prostanoid receptors EP2 and EP4.

Activation of EP2 and EP4 increases intracellular cAMP concentrations which is associated with inhibition of effector cell functions [31, 33]. Human tracheobronchial epithelial cells express all four EP subtypes, but only activation of EP2 or EP4, mediates respiratory mucin MUC5AC expression [7]. Respiratory mucins protect the airway epithelium against exogenous insults. In chronic airway diseases such as COPD, mucin hyperproduction contributes to airway obstruction, accelerated decline of lung function, morbidity and mortality [1]. Their hyperproduction is evoked by a variety of pro-inflammatory stimuli as a part of the inflammatory response in airways in COPD [7]. Therefore *M. catarrhalis*-induced expression of EP2 and 4 may also be important for the pathogenesis of this diseases.

In contrast to the constitutively expressed COX-1, a complex signaling network regulates the expression of inducible COX-2 [3]. In *Moraxella*-infected lung epithelial cells we demonstrated the activation of ERK1/2 and p38 MAPK. These kinases were considered as important regulators

of COX-2 and other pro-inflammatory signaling pathways [17, 18, 22, 26]. Interestingly, we found that inhibition of ERK1/2 but not of p38 MAPK reduced *Moraxella*-related expression of COX-2 and PGE_2 liberation. We recently reported that *S. pneumoniae*-induced-COX-2 expression was dominantly mediated by p38 MAPK and JNK but not by ERK1/2 [18]. The results of the study presented suggest a possible pathogen-specific regulation of COX-2 expression in lung tissue.

NF-κB mediates multiple aspects of host response to bacterial infection [17, 18, 22, 23, 26] and activation of the transcription factor NF-κB is considered to contribute significantly to COX-2 expression and PGE_2 liberation [17, 18]. In resting cells, IκB molecules sequester NF-κB in the cytosol. After cell activation, the signaling cascade containing IKK-complex results in degradation of IκBα, thus allowing NF-κB transfer into the nucleus [13]. Concurred with this findings *M. catarrhalis*-infected cells showed an increased NF-κB activation. Moreover, the highly specific cell permeable inhibitor of IKK, IKK-NBD [13] abolished *Moraxella*-related COX-2 protein expression and subsequent PGE_2 release. Recently, Di Stefano *et al*. observed a marked increase in the expression of p65 protein, the major subunit of NF-κB, in bronchial biopsies of COPD patients [5]. This finding was significantly correlated with the degree of airflow limitation and with increasing severity of the disease [5]. Overall, our results emphazise a crucial involvement of NF-κB in *Moraxella*-caused COX-2 and PGE_2 induction.

Because both ERK1/2 and NF-κB pathways seem to be essentially involved in COX-2 and PGE_2 expression in *Moraxella*-infected lung epithelium, we analyzed the impact of ERK1/2 on NF-κB activation in more detail.

Since BEAS-2B cells could only be poorly transfected, we made use of TLR2-overexpressing HEK-293 epithelial cells as a model that has been applied successfully in earlier studies investigating *Moraxella*-related cell activation [23, 26]. A chemical inhibitor of ERK1/2, but not

an inhibitor of p38 MAPK, blocked *Moraxella*-driven NF-κB-dependent reporter-gene expression in HEK-293 epithelial cells. Thus, the data confirmed an important role of ERK1/2 for *Moraxella*-caused COX-2 and PGE_2 induction.

In conclusion, our data suggests, that *M. catarrhalis* contribute to COX-2 dependent PGE_2 release of bronchial epithelium. Moreover, this required a ERK1/2 dependent activation of NF-κB as well as an increased expression of the E prostanoid receptors EP2 and EP4.

M. catarrhalis-induced PGE_2 expression might counteract lung inflammation promoting colonization of the respiratory tract in COPD-patients and may thus play an important role in the pathogenesis of this disease. Additional studies are required to follow up on this in an *in vivo* model.

ACKNOWLEDGEMENTS

We greatly appreciate the excellent technical assistance of Frauke Schreiber, Sylwia Schapke and Jacqueline Hellwig. This work was supported in part by grants from the Rahel-Hirsch Stipendium of the Charité-Universitätsmedizin Berlin, Germany, to Hortense Slevogt, and the Bundesministerium für Bildung und Forschung to Stefan Hippenstiel (CAPNETZ C15) and Norbert Suttorp (CAPNETZ C4) and the Deutsche Gesellschaft für Pneumologie (Stefan Hippenstiel, Janine Zahlten, and Bastian Opitz).

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FIGURE LEGEND

Fig. 1: *M. catarrhalis*-increased PGE_2 expression in bronchial epithelial cells is time-and concentration dependent.

BEAS-2B cells were infected for 4, 8 and 16 h with *M. catarrhalis (M.c.)* (MOI 0.1, 1) or stimulated with TNF-α (50 ng/ml) plus IL-1β (10 ng/ml) and PGE_2 release was measured by ELISA. The data represent the mean +/- S.E.M. of 4 values. *, $p<0.05$ vs. infected control.

Fig. 1

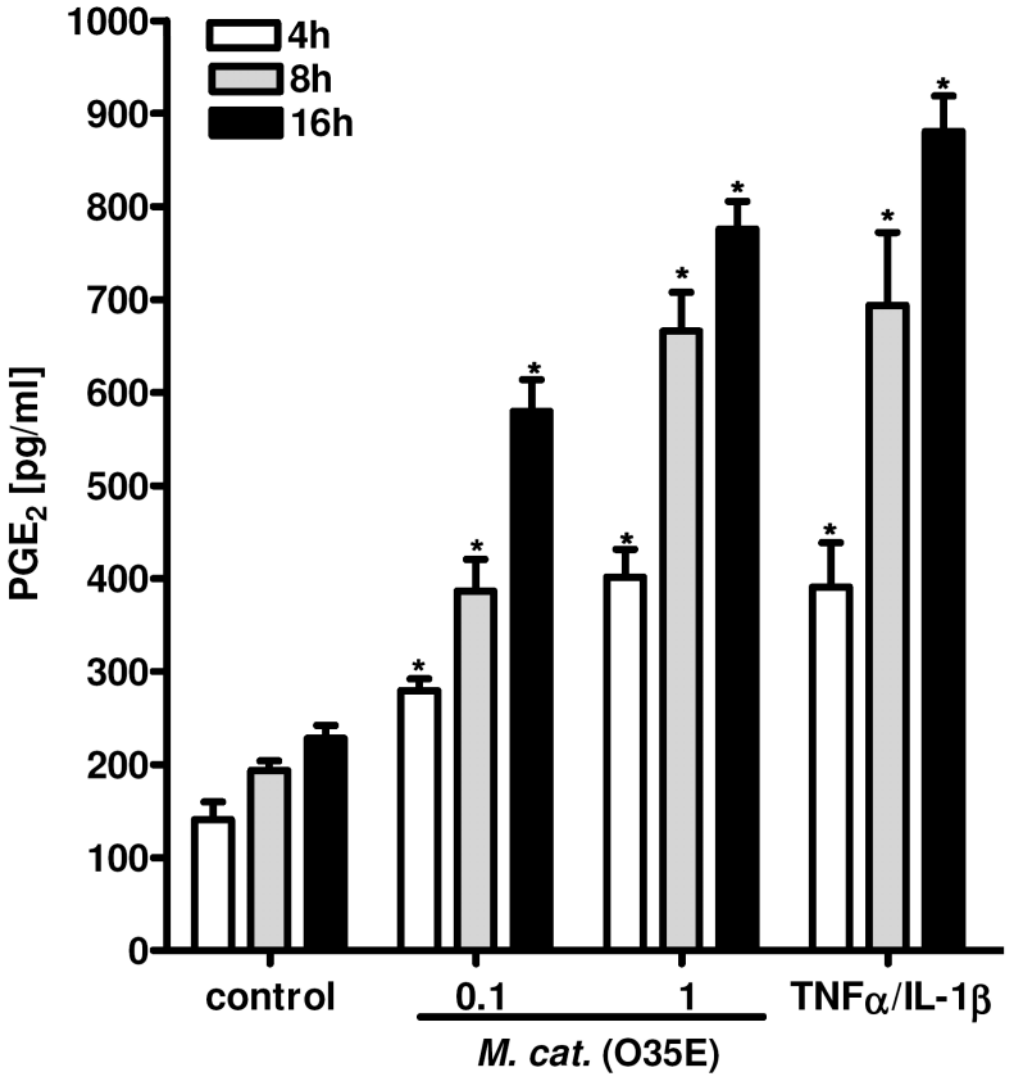
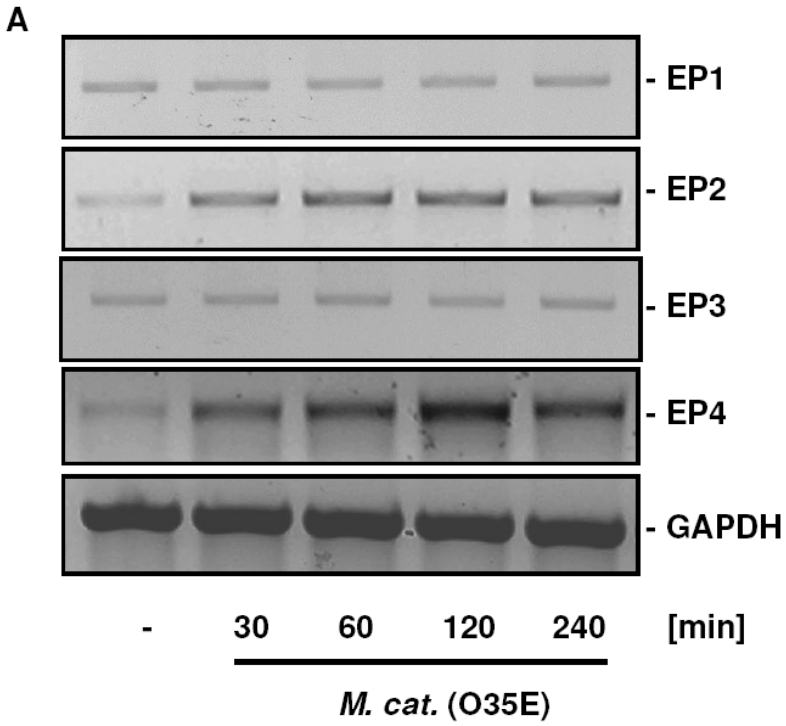

1000
900
800
700
600
500
400
300
200
100
0
PGE2 [pg/ml]
4h
8h
16h
*
control
0.1
1
TNFα/IL-1β
M. cat. (O35E)

Fig. 2: *M. catarrhalis* induced time- and concentration-dependently expression the E prostanoid receptors EP2 and EP4 in human bronchial epithelial cells.

BEAS-2B cells were incubated with *M. catarrhalis* strain O35E (MOI 1) for the indicated time. EP2 and EP4 transcription and expression were analysed by RT-PCR (A) and Western blot (B). Representative blots or gels out of three separate experiments are shown.

Fig. 2


A
- EP1
- EP2
- EP3
- EP4
- GAPDH
-
30
60
120
240
[min]
M. cat. (O35E)

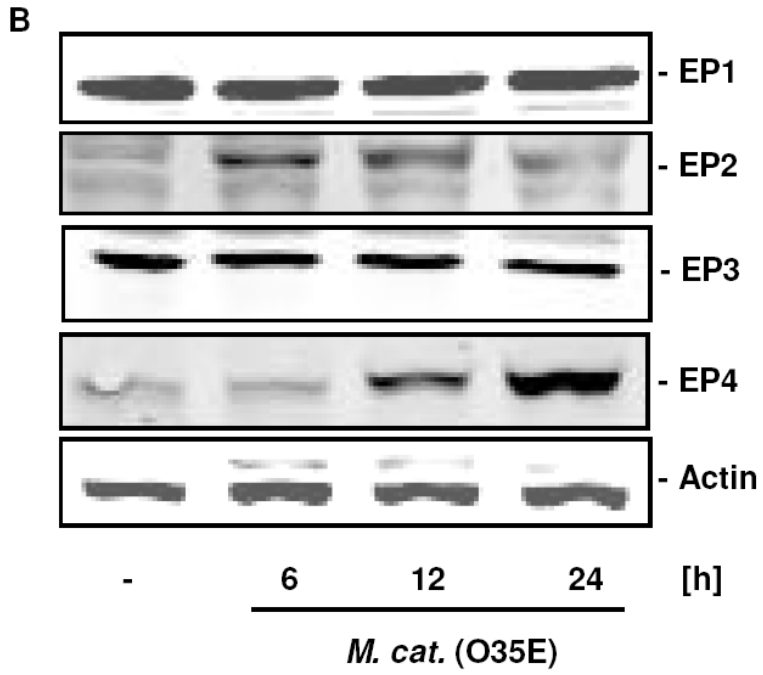

B
- EP1
- EP2
- EP3
- EP4
- Actin
-
6
12
24
[h]
M. cat. (O35E)

Fig. 3: *M. catarrhalis* induced time-dependently expression of COX-2 in human bronchial epithelial cells.

BEAS-2B cells were incubated with *M. catarrhalis* strain O35E (MOI 1) or TNF-α (50 ng/ml) plus IL-1β (10 ng/ml) for the indicated time. COX-1 and COX-2 transcription and expression were analysed by PCR (A) and Western blot (B). TNF-α (50 ng/ml) plus IL-1β (10 ng/ml) was used as a positive control by an incubation time of 2h for PCR and 4 h for Western blot.

In addition results of three responses (Western blots) have been graphically analyzed (C).

Representative blots or gels out of three separate experiments are shown. Data presented in (C) are means ± S.E.M. of three separate experiments. *, $p<0.05$ vs. unstimulated control.

Fig. 3

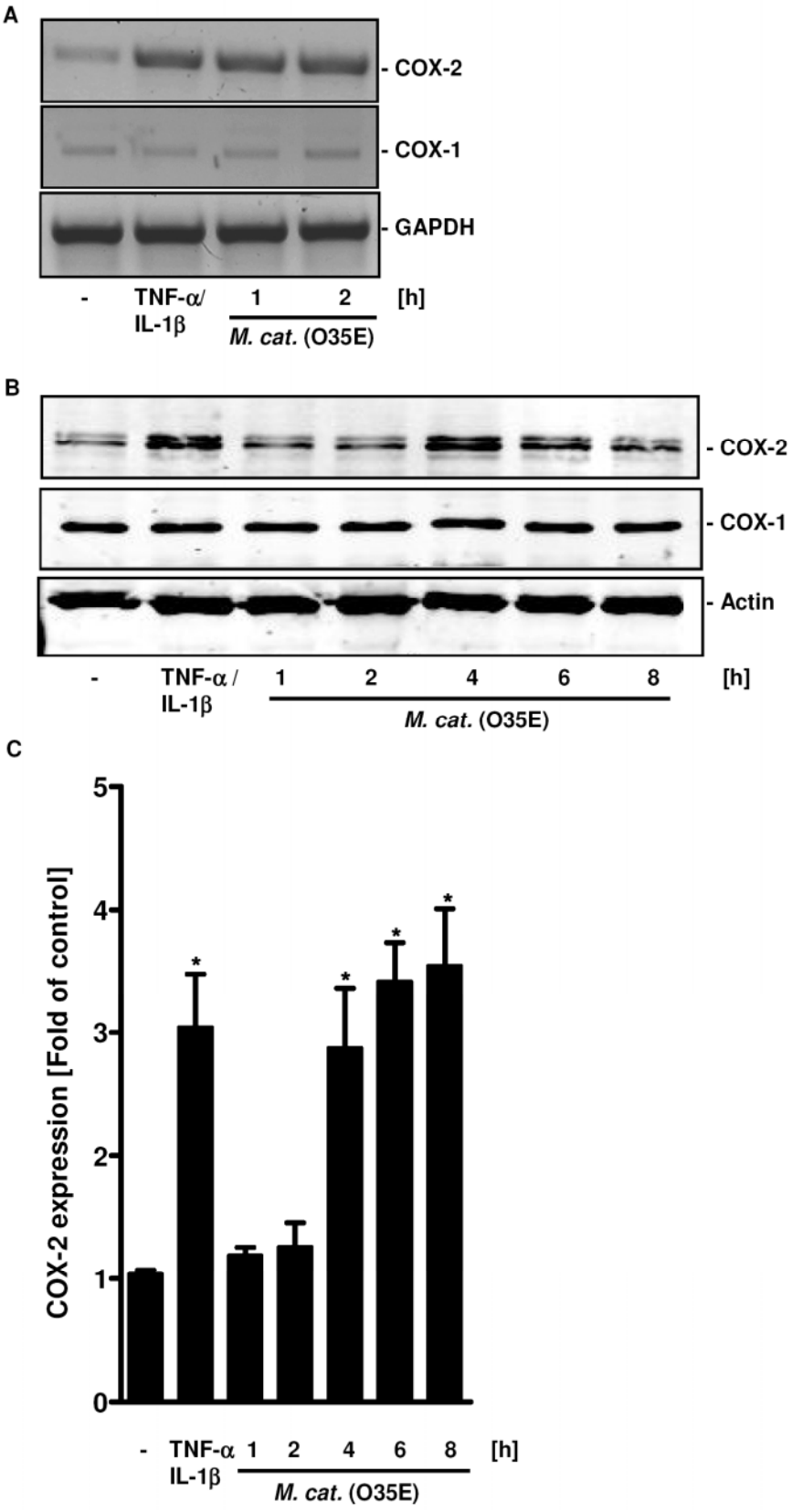

A
- COX-2
- COX-1
- GAPDH
- TNF-α/ IL-1β
1 2 [h]
M. cat. (O35E)
B
- COX-2
- COX-1
- Actin
- TNF-α / IL-1β
1 2 4 6 8 [h]
M. cat. (O35E)
C
COX-2 expression [Fold of control]
0 1 2 3 4 5
- TNF-α IL-1β
1 2 4 6 8 [h]
M. cat. (O35E)

Fig. 4: Inhibition of COX-2 but not of COX-1 abrogated *M. catarrhalis*-induced PGE_2 release in human bronchial epithelial cells.

BEAS-2B cells were pre-treated with the nonselective COX inhibitor indomethacin (INDO) (1 µM), the selective COX-1 inhibitor (SC-560; 1 µM), or with the selective COX-2 inhibitor (NS-398; 1 µM) for 30 min and then infected with *M. catarrhalis* O35E, MOI 1) for 16 h. PGE_2 release was measured by ELISA. Data presented are means ± S.E.M. of 4 separate experiments. *, $p<0.05$ vs. unstimulated control; #, $p< 0.05$ with or without inhibitors.

Fig. 4

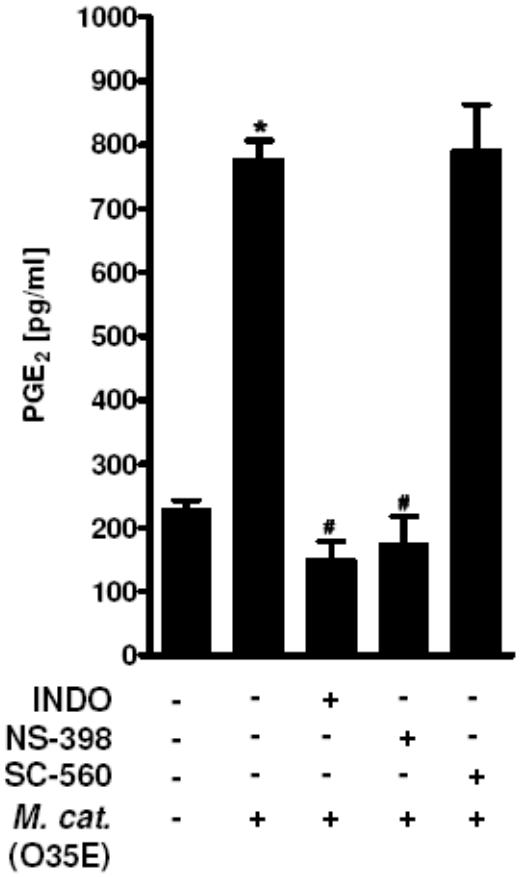


Fig. 5: The *M. catarrhalis* specific UspA1 is important for the induction of COX-2 and PGE_2 release in pulmonary epithelial cells.

BEAS-2B cells were infected with *M. catarrhalis* strain O35E (MOI 1) or with the UspA1-deficient strain O35E.1 for 4 h or 16 h. COX-2 expression (A, 4h) was analyzed by Western blot and PGE_2 (B, 16h) secretion by ELISA. Representative blots out of three separate experiments are shown. ELISA data presented are means ± S.E.M. of 4 separate experiments. *, $p<0.05$ vs. unstimulated control; #, $p< 0.05$ O35E versus O35E.1.

Fig. 5

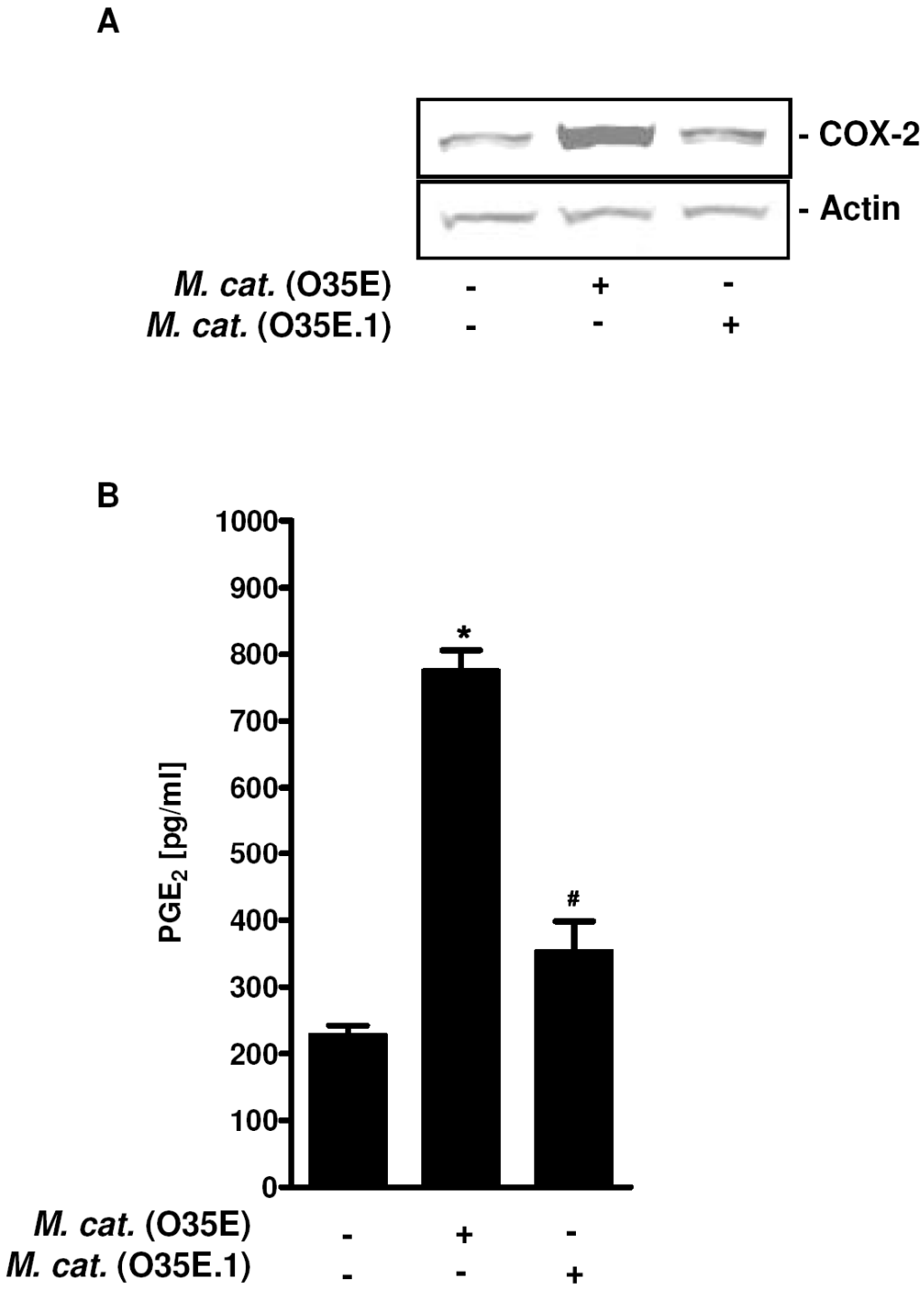
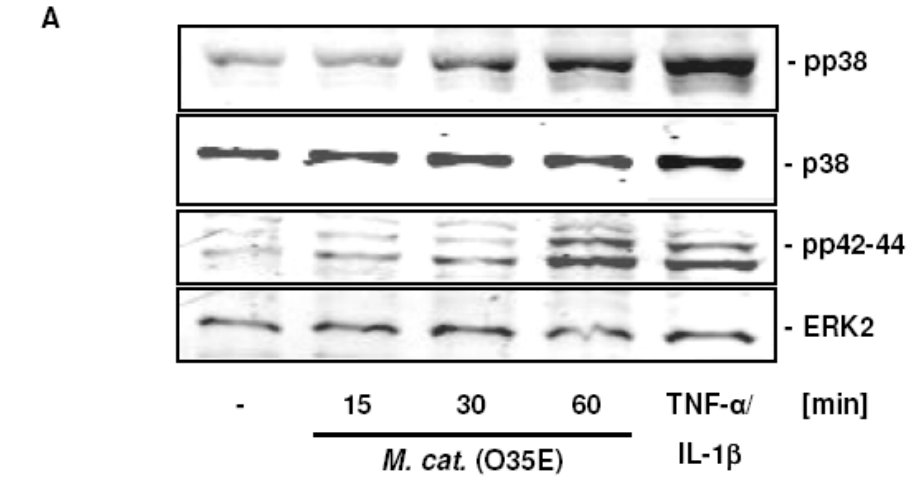
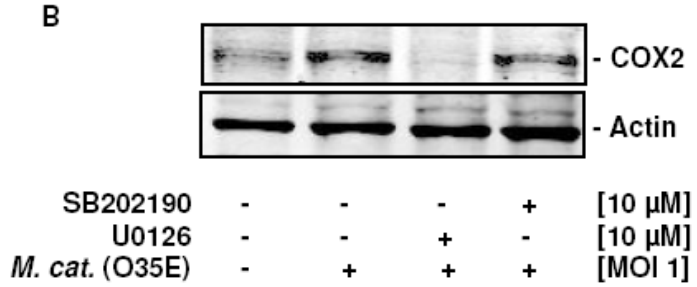

A
- COX-2
- Actin
M. cat. (O35E) - + -
M. cat. (O35E.1) - - +
B
1000
900
800
700
600
500
400
300
200
100
0
PGE2 [pg/ml]
*
#
M. cat. (O35E) - + -
M. cat. (O35E.1) - - +

Fig. 6: *M. catarrhalis* induced COX-2 expression and PGE_2 release are ERK1/2 but not p38 MAPK-dependent.

BEAS-2B cells were incubated with *M. catarrhalis* for 15, 30, 60 min, or TNF-α (50 ng/ml) plus IL-1β (10 ng/ml) for 60 min. Phosphorylated p38 and ERK1/2 MAPK were detected by Western blot (A). Expression of p38 or p42 was performed simultaneously to confirm equal protein load. Furthermore, BEAS-2B cells were preincubated with the ERK1/2 inhibitor U0126 and the p38 MAPK inhibitor SB202190 for 60 min and then infected with *M. catarrhalis* strain O35E (MOI 1) for 4 h (B) or 16 h (C). Data presented in (C) are means ± S.E.M. of 4 separate experiments. Representative blots out of three separate experiments are shown. *, $p<0.05$ vs. unstimulated control; #, $p< 0.05$ with or without inhibitors.

Fig. 6


A
- pp38
- p38
- pp42-44
- ERK2
- 15 30 60 TNF-α/ [min]
M. cat. (O35E) IL-1β


B
- COX2
- Actin
SB202190 - - - + [10 μM]
U0126 - - + - [10 μM]
M. cat. (O35E) - + + + [MOI 1]

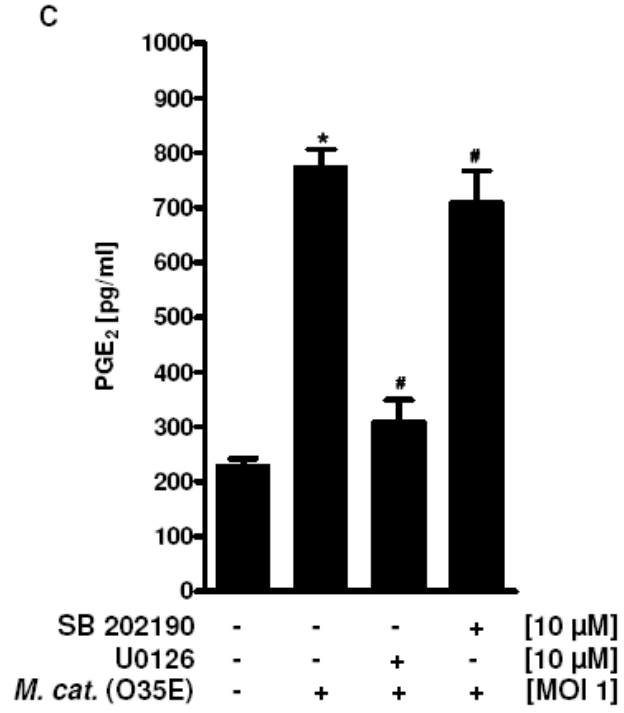

C
PGE2 [pg/ml]
1000
900
800
700
600
500
400
300
200
100
0
*
#
#
SB 202190 - - - + [10 μM]
U0126 - - + - [10 μM]
M. cat. (O35E) - + + + [MOI 1]

Fig. 7: *M. catarrhalis*-induced COX-2 expression and PGE_2 release is depended on NF-κB activation

BEAS-2B cells were infected with *M. catarrhalis* strain O35E (MOI 1) for the indicated time periods. An increased DNA binding of NF-κB in nuclear cell extracts of *Moraxella*-exposed cells was shown by EMSA (A). Furthermore BEAS-2B cells were pretreated with a specific IκB kinase inhibitor IKK-NBD (10 μM) for 60 min and infected with *M. catarrhalis* strain O35E for 4 h (B) or 16 h (C). Induction of COX-2 was assessed by Western blot (B). PGE_2 release was measured by ELISA (C). Representative blots or gels out of 3 are shown in A, B. Data presented in (C) are means ± S.E.M. of 4 separate experiments. *, $p < 0.05$ vs. unstimulated control. #, $p < 0.05$ with or without inhibitor.

Fig. 7

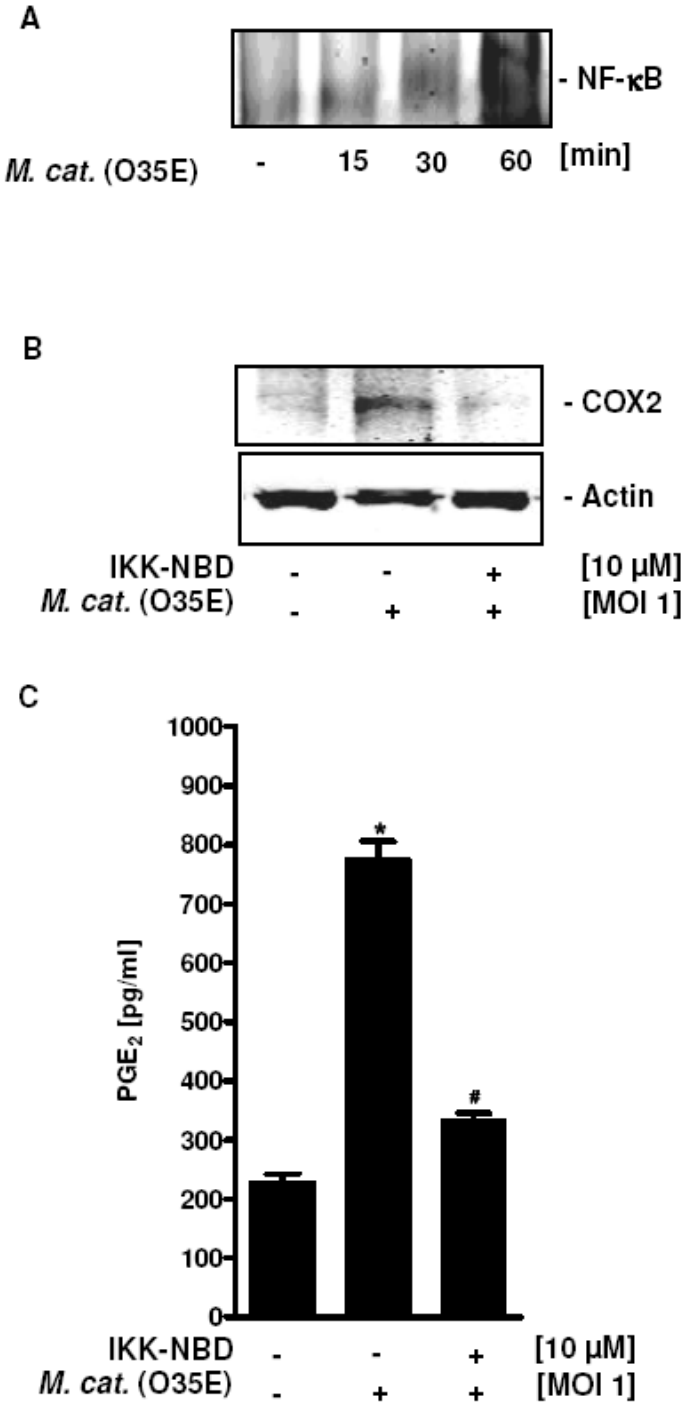
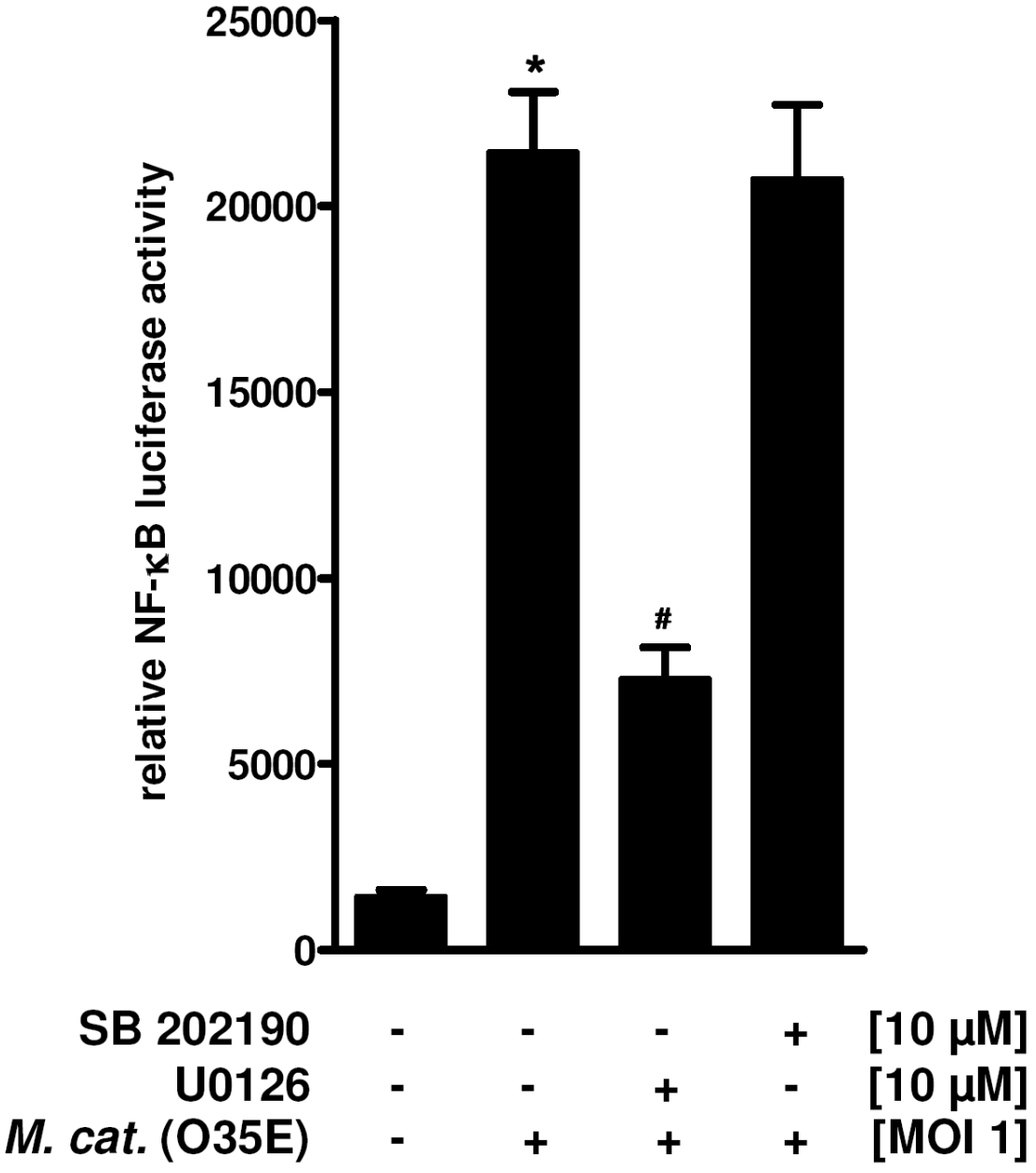

A
- NF-κB
M. cat. (O35E) - 15 30 60 [min]
B
- COX2
- Actin
IKK-NBD - - + [10 μM]
M. cat. (O35E) - + + [MOI 1]
C
PGE2 [pg/ml]
1000
900
800
700
600
500
400
300
200
100
0
*
#
IKK-NBD - - + [10 μM]
M. cat. (O35E) - + + [MOI 1]

Fig. 8: *M. catarrhalis* activated NF-κB via ERK1/2 but not via p38 MAPK.

HEK-293 cells were cotransfected with human Toll-like receptor 2 (hTLR2), an NF-κB-dependent luciferase reporter plasmid, and a β-galactosidase (β-Gal) construct. Cells pretreated with U0126 (10 μM) or SB202190 (10 μM) were infected for 6 h with *M. catarrhalis* O35E (MOI 1), and luciferase and β-Gal activities were determined and normalized. Data are means ± SE of 4 separate experiments. *P < 0.05 vs. unstimulated control. #P < 0.05 with or without inhibitor.

Fig. 8


25000
20000
15000
10000
5000
0
relative NF-κB luciferase activity
*
#
SB 202190 - - - + [10 µM]
U0126 - - + - [10 µM]
M. cat. (O35E) - + + + [MOI 1]