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Involvement of p38 MAPK, JNK, p42/p44 ERK and NF-κB in IL-1β-induced chemokine release in human airway smooth muscle cells

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Summary Asthma is an inflammatory disease, in which eotaxin, MCP-1 and MCP-3 play a crucial role. These chemokines have been shown to be expressed and produced by IL-1β-stimulated human airway smooth muscle cells (HASMC) in culture. In the present study we were interested to unravel the IL-1β-induced signal transduction leading to chemokine production. Using Western blot, we observed an activation of p38 MAPK, JNK kinase and p42/p44 ERK when HASMC were stimulated with IL-1β. We also observed a significant decrease in the expression and the release of eotaxin, MCP-1 and MCP-3 in the presence of SB203580, an inhibitor of p38 MAPK ($71\pm6\%$, $P<0.05$, $n=8$ and $39\pm10\%$ $P<0.01$, $n=10$ respectively), curcumin, an inhibitor of JNK kinase ($83\pm4.9\%$ and $88\pm3.4\%$ respectively, $P<0.01$, $n=4$). U0126, an inhibitor of p42/p44 ERK, also produced a significant decrease in chemokine production ($46.3\pm9\%$, $P<0.01$ $n=10$ and $67.8\pm12\%$, $P<0.01$, $n=12$)· Pyrrolydine dithiocarbamate, an inhibitor of NF-κB was also able to reduce the eotaxin, MCP-1 and MCP-3 expression and production ($50\pm13\%$, $P<0.05$, $n=10$ and $23\pm7\%$, $P<0.05$, $n=12$). We conclude that p38 MAPK, JNK kinase, ERK and NF-κB are involved in the IL-1β-induced eotaxin, MCP-1, and MCP-3 expression and release in HASMC.

Introduction

Asthma is a chronic inflammatory disease characterised by variable airflow obstruction, bronchial hyperresponsiveness and airway inflammation. Mast cells, lymphocytes and eosinophils are the most important inflammatory cells found in the airways of untreated asthmatics.[1,2] It is now widely recognised that eosinophils, can cause bronchial mucosal damage in asthmatic airways, which may contribute to the symptoms of asthma.[3] Eosinophils are thought to be attracted by a special type of cytokines, called chemokines. Eotaxin, MCP-1, and MCP-3 are all members of the C–C chemokine subgroup. Eotaxin is known to attract eosinophils, basophils and dendritic cells,[4,5] whereas MCP-1 and MCP-3 also attract other types of inflammatory cells, such as monocytes and lymphocytes. These three chemokines can be detected in bronchial tissue and in bronchoalveolar lavage fluid from asthmatic subjects,[6–8] therefore they might be important in the pathogenesis of asthma.

IL-1β is a potent proinflammatory cytokine that has a central role in inflammatory reactions such as in asthma. IL-1β has been detected in the bronchoalveolar lavage fluid from asthmatics but not in normal subjects and its concentration correlates

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with the severity of asthma.[9,10] We have previously demonstrated that human airway smooth muscle cells (HASMC) in culture, when stimulated with IL-1β, can express and release MCP-1, MCP-2 and MCP-3 mRNA and protein, respectively.[11]

In the present study, we are interested in the signalling pathways involved in the IL-1β-induced chemokine expression and production. So far, three major subgroups of MAP kinases, involved in IL-1β-signalling, have been identified: p38 mitogen activated kinase (p38 MAPK), c-Jun amino-terminal kinases (JNK kinase), and p42/p44 extracellular signal-regulated kinases (p42/p44 ERK).[12] Several transcription factors are also activated by IL-1β in different cell types. NF-κB seems to be the most important up to now. Binding sites of this transcription factor are present in the mouse MCP-1 promoter region.[13] Despite numerous reports regarding the importance of IL-1β in the cytokine network, very little is known about the molecular mechanisms governing eotaxin, MCP-1 and MCP-3 release in HASMC in particular.

As a consequence, the present study is aimed at unravelling the signal transduction cascade involved in IL-1β-induced chemokine release in HASMC.

This is the first study to investigate whether the 4 most important constituents of IL-1β-induced signal transduction are involved in the eotaxin, MCP-1 and MCP-3 expression and production in HASMC.

Material and methods

Culture of HASMC

HASMC were grown from explants of human bronchial smooth muscle, as previously described.[11] Airway tissue was obtained from patients undergoing surgery for lung carcinoma in accordance with procedures approved by the local ethical committee. None of the patients had characteristics of asthma. Bronchial smooth muscle tissue was isolated by dissection. Small explants (2×2 mm) were prepared and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine calf serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.25 μg/ml). DMEM was added, just to cover the explants. The medium was changed every day until the cells started to grow, then the medium was changed every 3 days. When the cells reached confluency, the explants were removed and 24 h later the cells were harvested with trypsin/EDTA and plated into a 75 cm^2 flask. After subculturing the cells twice, the cultures were characterised immunohistochemically, using anti-human smooth muscle actin antibody. Primary cell cultures used for the experiments showed $>95\%$ of cells staining for smooth muscle actin. After reaching confluency the cells were washed and incubated with serum-free DMEM, for 24 h before stimulation. All experiments were carried out between passages 3 and 6. The cells used for these experiments were obtained from 5–6 different donors.

Experimental protocol

HASMC were stimulated with Il-1β (10 ng/ml) alone or in combination with several inhibitors for 4 h (RNA) or 24 h (protein). The mRNA expression and protein release were compared with those measured in HASMC stimulated with IL-1β alone. In some experiments HASMC were stimulated with IL-1β in the presence of 10 μM SB203580, an inhibitor of p38 MAP kinase, or 1 μM curcumin, an inhibitor of JNK kinase, or 10 μM U0126, an inhibitor of p42/p44 ERK, or 10 μM pyrrolydine dithiocarbamate (PDTC), an inhibitor of NF-κB. HASMC stimulated with IL-1β alone were used as controls and the results were expressed as a percentage of the values measured in cells stimulated with IL-1β alone.

Control cells were stimulated in the presence of an equivalent amount of vehicle.

Measurement of secreted eotaxin and MCP-1 protein by use of enzyme-linked immunosorbent assay (ELISA)

MCP-1 and eotaxin proteins were measured in supernatant of cultured HASMC. This was done with a commercially available sandwich ELISA kit, as specified by the manufacturer. These ELISA kits are highly specific, and there was no significant interference between any of the cytokines being investigated. The sensitivity of these assays is high, with a lower limit of detection of 5.0 pg/ml for both MCP-1 and eotaxin. The level of MCP-3 protein was too low to be measured with ELISA.

Northern blot analysis

Total RNA was isolated by phenol/chloroform extraction and isopropanol precipitation.[14] The RNA samples were subjected to a 1% agarose/formaldehyde gel, containing 20 mM morpholinosulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA (pH 7.0), blotted onto a nylon membrane. Hybridisation was performed with a 227 bp

fragment specific to the human eotaxin complementary DNA (cDNA), a 170 bp fragment specific to the human MCP-1 cDNA, a 700 bp fragment specific to human MCP-3 cDNA, and a 1200 bp cDNA fragment specific to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. They were labelled by a random primer labelling kit using [α-^{32}P]dCTP (3000 Ci/mmol). After prehybridisation for 4 h at 42°C in a buffer containing 50% formamide, 4 × standard sodium citrate (SSC), 50 mM Tris-HCl (pH 7.5), 5 × Denhardt's solution, 0.1% sodium dodecyl sulphate (SDS), 5 mM EDTA and 250 μg/ml denaturated salmon sperm DNA, the blots were hybridised overnight at 42°C with the labelled probes (1–2 × 10^6 cpm/ml). Following hybridisation, the blots were washed to a high stringency of 0.1 × SSC, 0.1% SDS at 55°C before exposure to X-OMAT-S film. After the adequate exposure time, the autoradiographs were developed and analysed by a laser densitometer. The RNA levels were expressed as the ratio of chemokine mRNA to GAPDH mRNA.

Immunoblot analysis of p38 MAP kinase

These were performed as previously described.[15]

Extraction of cytosolic proteins: following treatment, the cells were rinsed 2 times with cold PBS and scraped into lysis buffer (25 mM HEPES, 0.3 M NaCl, 1.5 mM $MgCl_2$ 20 mM β-glycerolphosphate, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 nM sodium orthovanadate), vortex-mixed and incubated on ice for 30 min. The threonine- and tyrosine-phoshorylation of p38-MAPK, JNK kinase and p42/p44 ERK were analysed by Western blot analysis, using specific polyclonal antibodies. Protein samples (100 μg, in sample buffer: 62.5 mM Tris, 10% glycerol, 2% SDS and 10 mM β-mercaptoethanol) were separated by SDS-PAGE electrophoresis on 14% acrylamide gels and then transferred to a PVDF-hybond membrane. In order to block non-specific protein binding, the membrane was incubated at room temperature for 1 h in TBST blocking buffer (0.1% Tween 20 in Tris-buffered saline containing 5% w/v skim powdered milk). The membrane was then incubated overnight at 4°C with either Phospho-p38 MAP kinase polyclonal antibodies, JNK kinase or p42/p44 ERK polyclonal antibodies in TBST without Tween 20. The membrane was washed 2 times for 5 min with TBST and then incubated with a 1:10 000 dilution of HRP-conjugated anti-rabbit secondary antibody in TBST. The membrane was washed 5 times for 15 min each and protein detection was carried out using enhanced chemiluminescence (ECL) plus detection reagent and exposed against ECL-Hyperfilm.

Materials and reagents

Cell culture medium (DMEM), fetal bovine serum, and culture reagents were purchased from Gibco Life Technologies (Merelbeke, Belgium). The anti-human smooth muscle actin antibody was obtained from Dako (Glostrup, Denmark)

Recombinant human IL -1β and ELISA kits for eotaxin, and MCP-1, were obtained from R&D Systems Europe Ltd. (Abingdon, UK). SB203580 was purchased from Merck (Leuven, Belgium).

The 227 bp fragment specific to the human eotaxin complementary DNA (cDNA) was a kind gift from A. Yokoyama (Ehime, Japan), a 170 bp fragment specific to the human MCP-1 cDNA, and a 700 bp fragment specific to human MCP-3 cDNA, was a kind gift from G. Opdenakker (Rega institute, Leuven, Belgium). The 1200 bp cDNA fragment specific to rat GAPDH cDNA was purchased from Clontech (Heidelberg, Germany).

Hybridisation was performed with a random primer labelling kit from MBI Fermentas (St Leon-Rot, Germany) using [α-^{32}P]dCTP obtained from Amersham (little Chalfont, UK). The autoradiographs were analysed by a laser densitometer obtained from Kodak (Rochester, USA).

Phospho-p38 MAPK, JNK kinase polyclonal antibodies were purchased from New England Biolabs, (Beverly, USA). PVDF-hybond membrane and ECL-Hyperfilm were obtained from Amersham (little Chalfont, UK).

All the other used reagents were obtained from Sigma-Aldrich (Bornem, Belgium).

Statistics

All data are presented as means ± SEM. Statistical analysis was performed using the Mann–Whitney *U*-test [Prism 3.0 (GraphPAD) software was used].

Results

Effect of IL-1β on expression and release of eotaxin, MCP-1 and MCP-3 in HASMC in vitro

Neither mRNA expression nor the protein release of these chemokines was measurable in unstimulated HASMC. However, after 24 h of stimulation with IL-1β, there was an increase in eotaxin and MCP-1 release (11 ± 2 and 107 ± 9 ng/ml, respectively). The level of MCP-3 protein was too low to be

measured with ELISA (data not shown). Eotaxin, MCP-1, and MCP-3 mRNA expression in HASMC was clearly present after stimulation with IL-1β (data not shown).

MAP kinase activation

Immunoblot analysis showed that amounts of phosphorylated threonine and tyrosine of p38 MAP kinase in cells stimulated with IL-1β 10ng/ml were increased at 5 min. Peak phosphorylation levels occurred at 30 min and remained significantly elevated for 1 h after which time the levels returned to near base line (Fig. 1A).

The amounts of phosphorylated threonine and tyrosine of JNK kinase in IL-1β-stimulated cells were significantly increased at 10 min. Peak phosphotyrosine levels occurred at 30 min and after 1 h we observed a complete loss of activation (Fig. 1B).

IL-1β also induced phosphorylation of tyrosine of p42/p44 ERK with a peak activation at 15 min and a decrease thereafter (Fig. 1C).

Effects of SB203580, curcumin, U0126 and PDTC on IL-1β-induced release and expression of eotaxin, MCP-1, and MCP-3 in HASMC in vitro

Stimulation of the HASMC with SB203580 (10 μM), a p38 MAPK inhibitor in the presence of IL-1β (10 ng/ml) for 24 h resulted in a significant reduction of the eotaxin ($71 \pm 6\%$, $P<0.05$, $n=8$) and MCP-1 ($39 \pm 10\%$, $P<0.01$, $n=10$) protein release, compared to IL-1β-stimulated HASMC alone (Fig. 2). After 4 h of IL-1β stimulation in the presence of SB203580 (10 μM), the expression of eotaxin, MCP-1, and MCP-3 mRNA were decreased by $34 \pm 10\%$, $24 \pm 7\%$, and $44 \pm 4\%$, respectively, compared with IL-1β alone ($n=4$) (Fig. 3).

After addition of curcumin (an inhibitor of JNK kinase), we observed a significant decrease in eotaxin and MCP-1 production (with a decrease of $83 \pm 4.9\%$ and $88 \pm 3.4\%$ with curcumin 1 μM, for eotaxin and MCP-1, respectively)($P<0.01$, $n=4$) (Fig. 2). U0126, an inhibitor of p42/p44 ERK, produced a significant decrease of eotaxin and MCP-1 production ($57.3 \pm 9\%$ and $57.8 \pm 12\%$ $P<0.01$ decrease for eotaxin and MCP-1, respectively for 10μM U0126, $n=10$ and 12 respectively)(Fig. 2).

Treatment of the HASMC with PDTC (10 μM), an NF-κB inhibitor, in the presence of IL-1β, resulted in a significant decrease of chemokine protein release

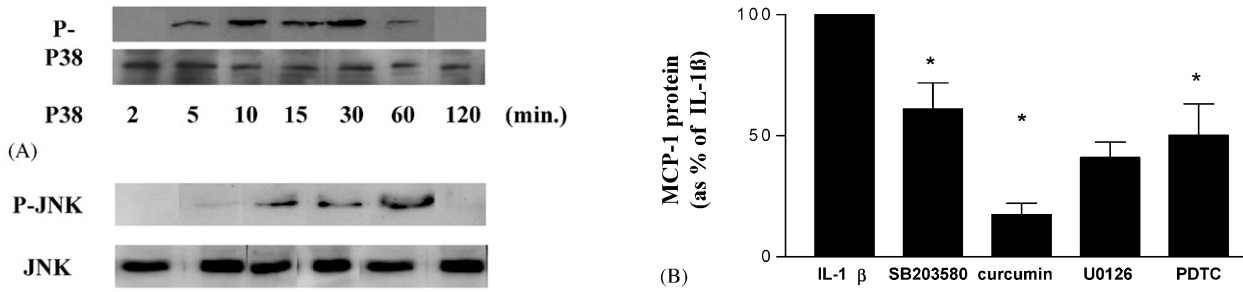

Figure 1 HASMC are stimulated with IL-1β for the time points indicated. Proteins in cell lysates were separated by SDS-PAGE prior to immunoblot detection of phosphorylated p38 MAP kinase (panel A), JNK (panel B), and ERK kinase (panel C).

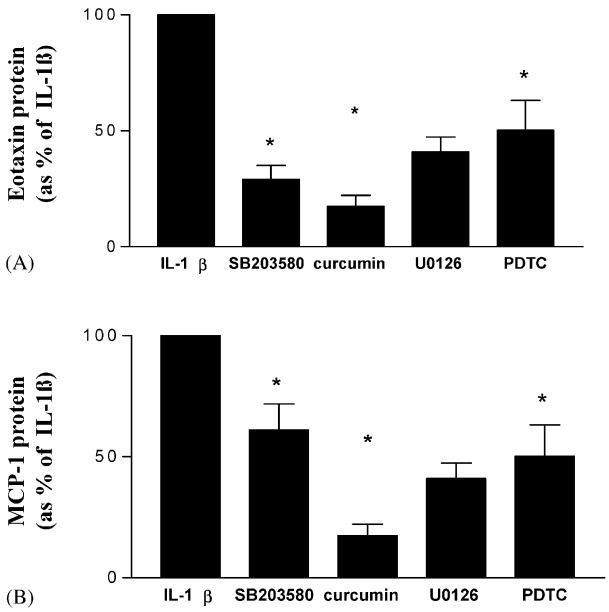

Figure 2 HASMC were treated with IL-1β (10 ng/ml) for 24 h, in the presence or absence of SB203580 (10 μM), an inhibitor of P38 MAP kinase activity, or U0126 (10 μM), a selective inhibitor of ERK kinase, or curcumin (1 μM), an inhibitor of JNK kinase or PDTC (10 μM), an inhibitor of NF-κB. Cells were pretreated for 30 min with these inhibitors. The effects of these inhibitors are shown, on eotaxin (panel A) and MCP-1 (panel B) release from cells stimulated with IL-1β. Data are expressed as a percentage of the control response (IL-1β alone) and are represented as mean $\pm$ SEM of duplicate values from independent experiments using cells cultured from five different donors, cell passages 3–6. *$P<0.05$, **$P<0.01$ compared with stimulated levels in the absence of inhibitor.

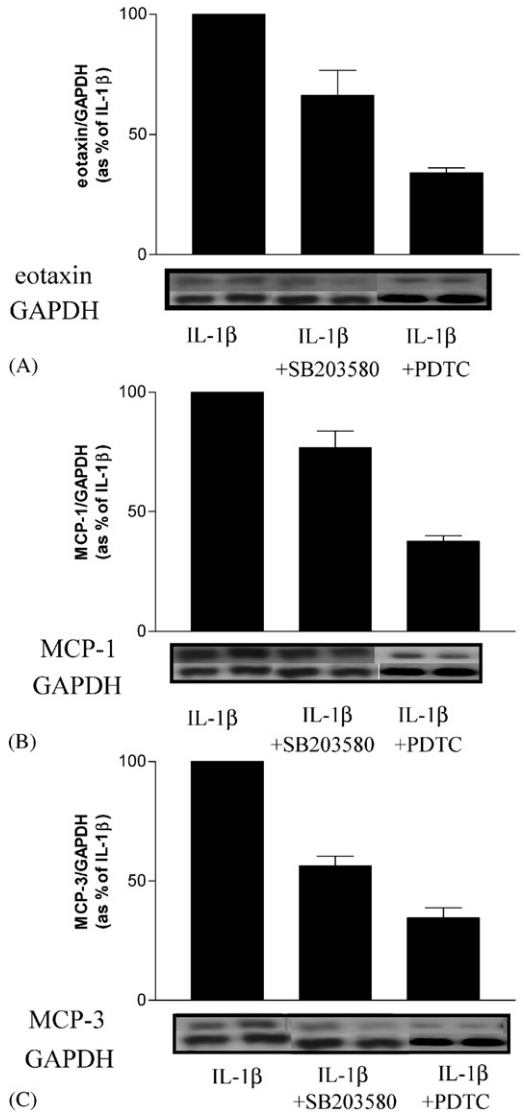

Figure 3 HASMC were incubated with IL-1β (10 ng/ml) in the presence or absence of SB203580 (10 μM), an inhibitor of p38 MAP kinase activity, or PDTC (10 μM), an inhibitor of NF-κB. Panel A shows the effects of SB 203580 and PDTC, on eotaxin (panel A), MCP-1 (panel B) and MCP-3 (panel C) expression from cells stimulated with IL-1β (10 ng/ml) for 4 h. Cells were pretreated for 30 min with pharmacological inhibitors. Data in graphs are derived from densitometric quantification of autoradiographs using a laser densitometer and software obtained from Kodak (Rochester, USA). Data are expressed as a percentage of the control response to IL-1β alone and are represented as mean $\pm$ SEM of duplicate values from independent experiments using cells cultured from five different donors, cell passages 3–6.

for eotaxin (50 $\pm$ 13%, $P<0.05$, $n=10$) and MCP-1 (50 $\pm$ 7%, $P<0.05$, $n=12$) (Fig. 2). PDTC in combination with IL-1β also induced a decrease in eotaxin, MCP-1, and MCP-3 mRNA expression by 66 $\pm$ 2%, 62 $\pm$ 2%, and 67 $\pm$ 8% respectively ($n=4$) (Fig. 3).

Discussion

In the present study we have again confirmed that HASMC in culture are able to express the chemokines eotaxin, MCP-1 and MCP-3, when stimulated with the proinflammatory cytokine IL-1β. Likewise, we have also demonstrated an increase in eotaxin, and MCP-1 protein release. Using Western blotting, we observed an activation of p38 MAPK, JNK kinase and p42/p44 ERK when HASMC were stimulated with IL-1β. The IL-1β-induced eotaxin, MCP-1 and MCP-3 release and expression were significantly decreased by SB203580, an inhibitor of p38 MAPK, curcumin, an inhibitor of JNK kinase, U0126 an inhibitor of p42/p44 ERK and PDTC, an inhibitor of NF-κB. These findings imply an important role for p38 MAPK, JNK kinase, p42/p44 ERK and NF-κB in the IL-1β-induced chemokine expression and release.

In an attempt to identify some key elements that constitute the link between stimulation with IL-1β and the expression and production of the above mentioned chemokines, we evaluated whether p38 MAPK, JNK kinase and p42/p44 ERK, three of the most important MAP kinases, were activated by IL-1β in HASMC in vitro. Using Western blotting we observed an activation of all three elements studied. Only small numbers of studies examining the activation of these MAP kinases by IL-1β in HASMC have been published to date. Our group already demonstrated that p38 MAP kinase is activated by IL-1β in HASMC.[15] In the present study we also showed that IL-1β was able to activate JNK kinase and p42/p44 ERK in HASMC. The involvement of P38 MAP kinase and p42/44 ERK in eotaxin has meanwhile been confirmed in an article of Hallsworth et al.[16]

Since IL-1β induces a tremendous amount of different actions, the activation of these three MAP kinases not directly implies an involvement of all of them in the IL-1β-induced chemokine expression and production. Therefore, we stimulated the cells with IL-1β, in the presence of the specific inhibitors of these three MAP kinases studied.

Showing that SB203580 was able to reduce the chemokine expression and production, provides some more evidence for p38 MAPK being involved in IL-1β-induced eotaxin, MCP-1 and MCP-3 expression and production. Recently an article was published in which it is confirmed that eotaxin release is dependent on p38 MAP kinase in HASMC.[16] It has been described as well that p38 MAPK is necessary for the IL-1β-induced MCP-1 expression in human mesangial cells,[17] but this is, to our knowledge the first time that the involvement of p38 MAPK in the IL-1β-induced expression

and release of MCP-1 and MCP-3 is observed in HASMC.

We also observed a decrease of the chemokine production in the presence of curcumin, a specific inhibitor of JNK kinase,[18] which suggest the involvement of JNK kinase in the IL-1β-induced signal transduction. In rat and human mesangial cells, it has already been shown that an activation of JNK kinase is involved in the MCP-1 production.[19,20] We did not perform mRNA expression because in the former experiments mRNA and protein results were equivalent and because the protein is the most important product that finally is responsible for the effects on inflammation.

Nevertheless our paper for the first time demonstrates that JNK kinase is involved in eotaxin and MCP-1 production in HASMC.

We also tested U0126, an inhibitor, which is able to completely block the p42/p44 ERK activity as demonstrated by a complete inhibition to baseline levels of its substrate.[16] This potent blocker was able to reduce the IL-1β-induced eotaxin and MCP-1 production. We did not measure the mRNA because of the same reason mentioned above. As a consequence, our data also argue in favour of an involvement of p42/p44 ERK in the IL-1β-induced release of chemokines.

As NF-κB is also known as an important element in IL-1β-induced signal transduction, we also investigated whether NF-κB is involved in the specific process of expression and release of chemokines. We have demonstrated, in the present study, that NF-κB is involved in the IL-1β-induced release and expression of eotaxin, MCP-1, and MCP-3 in HASMC. Indeed, PDTC, an inhibitor of NF-κB,[21] induced a significant decrease in chemokine protein release, which was in accordance with our mRNA findings in HASMC. PDTC has been shown to block cytokine-induced NF-κB activation in several cell lines including HASMC.[22,23] Our observation that NF-κB is involved in the IL-1β induced chemokine release and expression is also consistent with previous data in other cell types, such as mesangial cells,[24] vascular smooth muscle[25] and A549 airway epithelial cells.[26,27]

Our results can be of a great importance in the development of future therapy for inflammatory diseases of the airways, such as asthma. It has been shown that the early stages of inflammatory cell adherence and diapedesis appear to be dependent upon p38 MAP kinase activation as suggested by the inhibition of LPS and TNF-α-induced ICAM-1 expression on pulmonary microvascular endothelial cells by SB203580.[28] It has already been suggested that the use of a p38 MAP kinase inhibitor may influence different key processes of chronic inflammation as seen in asthma.[29] Our study provides some more evidence for this hypothesis and also points to the possible role of JNK, ERK and NF-κB inhibition in the treatment of asthma.

Up to now, there is already a lot of information available about IL-1β-induced signal transduction, in different cells, however, this is the first study to investigate the involvement of the 4 most important elements in IL-1β-induced eotaxin, MCP-1 and MCP-3 expression and production in HASMC.

In conclusion we have demonstrated that in HASMC the IL-1β-induced protein release and mRNA expression of eotaxin, MCP-1, and MCP-3, in HASMC is mediated by p38 MAP kinase, JNK kinase, p42/p44 ERK and NF-κB.

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