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Modulation by cAMP of IL-1β-induced eotaxin and MCP-1 expression and release in human airway smooth muscle cells

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ABSTRACT: Inflammatory cells, such as eosinophils, seem to be key players in the inflammatory process of asthma. These cells are attracted by chemokines, for example eotaxin and monocyte chemotactic protein (MCP-1).

In this study, the authors investigated whether eotaxin and MCP-1 expression and release in human airway smooth muscle cells could be modulated by an increase in intracellular cyclic adenosine monophosphate (cAMP) concentration. The possible involvement of cAMP-dependent protein kinase A (PKA) was also studied.

Forskolin, a direct stimulator of adenylyl cyclase, decreased the interleukin (IL)-1β-induced eotaxin and MCP-1 release by 73±8 and 65±6%, respectively. 8Bromo-cAMP, a cAMP analogue, similarly decreased the chemokine production by 58±9 and 63±8% for eotaxin and MCP-1, respectively. Prostaglandin E2, known as an activator of the prostanoid receptors EP_2 and EP_4, which are positively coupled to adenylyl cyclase, also decreased the IL-1β-induced eotaxin and MCP-1 production by 57±17 and 53±4%, respectively. H-89, an inhibitor of PKA, was able to inhibit the decrease in eotaxin and MCP-1 protein release induced by forskolin. Using Western-blot analysis, no effect of cAMP was found on the IL-1β-induced p38 mitogen-activated protein kinase, extracellular signal-related kinase or cJun *N*-terminal kinase activation.

This study shows that an increase in intracellular cyclic adenosine monophosphate concentration may decrease the interleukin-1β-induced eotaxin and monocyte chemotactic protein-1 expression and production. This can be inhibited by addition of H-89, an inhibitor of cyclic adenosine monophosphate-dependent protein kinase. No decrease was observed in interleukin-1β-induced p38 mitogen-activated protein kinase, extracellular signal-related kinase or cJun *N*-terminal kinase activation. These findings may be important for the further development of new anti-inflammatory drugs.
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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 that are found in the airways of untreated asthmatics [1, 2]. It is now widely recognised that eosinophils and their toxic derived proteins can cause bronchial mucosal damage in asthmatic airways, which may contribute to the symptoms of asthma [3].

Eosinophils are thought to be attracted by chemokines, such as eotaxin (which mainly attracts eosinophils) and monocyte chemotactic protein (MCP)-1 (which also attracts other types of inflammatory cells). The current authors have previously demonstrated that human airway smooth muscle cells (HASMC) in culture, when stimulated with interleukin (IL)-1β, can express and release MCP-1, -2 and -3 [4]. IL-1β is a potent pro-inflammatory cytokine that has a central role in inflammatory reactions as seen in asthma. These cytokines can be detected in bronchial tissue and in bronchoalveolar lavage fluid from asthmatic subjects [5–7].

The formation of cyclic adenosine monophosphate (cAMP) is performed by adenylyl cyclase (AC). Forskolin, a direct stimulator of AC increases intracellular cAMP concentration ($[cAMP]_i$). 8Bromo (8Br)-cAMP is a known analogue of cAMP and prostaglandin (PG)E_2 acts *via* the prostanoid receptors EP_2 and EP_4, which are positively coupled *via* a stimulatory G-protein to AC. Changes in $[cAMP]_i$ are able to modulate the expression of several genes in various cell types, such as Il-1β-induced IL-6 release in human lung fibroblasts [8], IL-1α-induced IL-6 and IL-8 gene expression and release in human mesangial cells [9], and tumour necrosis factor (TNF)-α-induced MCP-1 release in mesangial cells [10]. It is classically believed that cAMP acts through activation of the cAMP-dependent protein kinase A (PKA) [11], but there are some reports demonstrating that cAMP also induces some PKA-independent effects [12].

In a previous study, the present authors have already shown that p38 mitogen-activated protein kinase (MAPK), extracellular signal-related kinase (ERK) and cJun *N*-terminal kinase (JNK) are involved in the IL-1β-induced eotaxin and MCP-1 expression and production in HASMC [13].

Therefore, in the present study the authors investigated whether the IL-1β-induced chemokine release in HASMC could be modulated by agents that increase $[cAMP]_i$ and whether this modulation was PKA dependent. The authors also wanted to investigate whether this effect of cAMP was due to a modulation of p38 MAPK, ERK or JNK.

Materials and methods

Culture of human airway smooth muscle cells

HASMC were grown from explants of human bronchial smooth muscle, as previously described [4]. Airway tissue was obtained from patients undergoing surgery for lung carcinoma, who had never had any chemotherapeutic treatment before, 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 modified Eagle medium (DMEM) (Gibco Life Technologies, Merelbeke, Belgium) supplemented with 10% foetal bovine calf serum, L-glutamine (2 mM), penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$) and amphotericin B (1.25 $\mu g \cdot mL^{-1}$). 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 using trypsin/ ethylenediamine tetraacetic acid (EDTA) and plated into a 75 cm^2 flask. After subculturing the cells twice, the cultures were characterised immunohistochemically, using antihuman 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 passage three and six. Cells were cultured from four different patients.

Experimental protocol

HASMC were pretreated with several $[cAMP]_i$-increasing agents and 30 min later IL-1β (10 $ng \cdot mL^{-1}$) was added for another 4 h (ribonucleic acid (RNA)) or 24 h (protein). The mRNA expression and protein release were compared with those measured in HASMC stimulated with IL-1β alone at the same time intervals.

HASMC were stimulated with 10 μM forskolin (Merck, Leuven, Belgium), a direct stimulator of AC, 1 mM of 8Br-cAMP (Merck), an analogue of cAMP, or 1 μM PGE_2 (Sigma-Aldrich, Bornem, Belgium), an activator of the prostanoid receptors EP_2 and EP_4. In the second part of the study, in different experiments, HASMC were stimulated with IL-1β, forskolin (10 μM) and different concentrations of H-89 (0.1, 0.3 and 3 μM), a specific PKA inhibitor (Calbiochem, Nottingham, UK).

In the third part of the study, HASMC were stimulated with IL-1β (10 $ng \cdot mL^{-1}$) and forskolin (10 μM) for several time points (5–120 min) to investigate the effect of an increase in $[cAMP]_i$ on p38 MAPK, ERK or JNK activation.

HASMC stimulated with IL-1β alone were used as controls and values measured were expressed as a percentage of the values measured in these control cells.

Northern-blot analysis

Total RNA was isolated by guandinium thiocyanated-phenol-chloroform extraction and isopropanol precipitation [14]. The RNA samples were subjected to a 1% agarose/ formaldehyde gel, containing 20 mM morpholinosulphonic acid, 5 mM sodium acetate and 1 mM EDTA (pH 7.0), and blotted onto a nylon membrane. Hybridisation was performed with a 227 base pair (bp) fragment specific to the human eotaxin complementary (c) deoxyribonucleic acid (DNA), a 170 bp fragment specific to the human MCP-1 cDNA and a 1,200 bp cDNA fragment specific to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontec, Heidelberg, Germany). cDNA was labelled by a random primer labelling kit using [α-^{32}P]dCTP (3,000 $Ci \cdot mM^{-1}$) (MBI Fermentas, St Leon-Rot, Germany). 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 $\mu g \cdot mL^{-1}$ denatured salmon sperm DNA, the blots were hybridised overnight at 42°C with the labelled probes (1–2×10^6 $cpm \cdot mL^{-1}$). 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 messenger (m)RNA to GAPDH mRNA.

Measurement of total protein

Total protein was measured by the Bradford method [15] using a commercially available assay (Bio-RAD laboratories GmbH, Munich, Germany).

Measurement of secreted eotaxin and MCP-1 by use of ELISA

Eotaxin and MCP-1 were measured in the supernatant of cultured HASMC. This was carried out with a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Europe Ltd, Abingdon, UK), 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 lower limit of detection was 5.0 $pg \cdot mL^{-1}$ for both MCP-1 and eotaxin.

The ratio of measured protein to total protein in the same sample was calculated and the levels of protein were depicted as % of IL-1β-induced protein production.

Immunoblot analysis of p38 MAPK, JNK and ERK

After extraction of cytosolic proteins, the threonine and tyrosine phosphorylation of p38 MAPK, JNK and ERK were analysed by Western-blot analysis, using phospho-p38 MAPK, JNK and ERK polyclonal antibodies, which only react with the phosphorylated form of the MAPKs studied. The assay was performed as described previously [16]. Phospho-p38 MAPK, phospho-JNK, phospho-ERK, p38 MAPK, JNK and ERK polyclonal antibodies were diluted as recommended by the manufacturer (New England Biolabs, Beverly, MA, USA).

After the adequate exposure time, the autoradiographs were developed and analysed by a laser densitometer. The levels were expressed as the ratio of phosphorylated to nonphosphorylated MAPK.

Statistics

All data are presented as means±SEM. Statistical analysis on absolute values was performed using the Mann-Whitney U and Kruskal-Wallis test.

Results

Effect of $[cAMP]_i$-elevating agents on IL-1β-induced release and expression of eotaxin

The following results are shown in figure 1. The current authors were unable to detect any eotaxin mRNA or protein in unstimulated HASMC, however, IL-1β induced a huge protein release, as described previously [4]. The actual value of secreted eotaxin was 26±3 $ng \cdot mL^{-1}$. Forskolin (10 μM) decreased the eotaxin mRNA expression by 68±4% ($p<0.01$, n=4). The use of forskolin also significantly decreased eotaxin protein release (73±8%, $p<0.01$, n=4).

8Br-cAMP (1 mM) decreased the mRNA expression for eotaxin by 38±12% ($p<0.05$, n=4) after 4 h of stimulation (n=4) (fig. 1). 8Br-cAMP also significantly inhibited the eotaxin protein release (58±9%, $p<0.05$, n=4).

PGE_2 (1 μM) decreased the eotaxin mRNA expression by 48±11% ($p<0.05$, n=4). These results were in accordance with the results of protein release. Addition of PGE_2 induced a significant decrease in chemokine release. After stimulation for 24 h with IL-1β and PGE_2, the eotaxin release decreased by 57±17% ($p<0.05$, n=4).

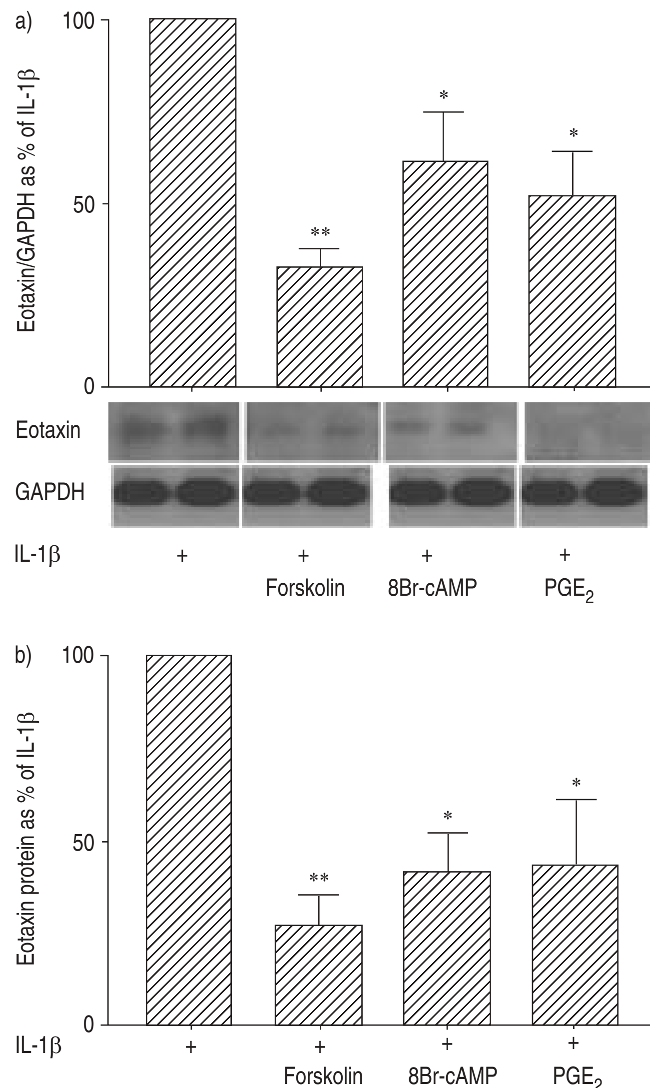

Fig. 1. – Effect of intracellular cyclic adenosine monophosphate (cAMP)-increasing agents on eotaxin expression and release. Human airway smooth muscle cells were pretreated with forskolin (10 μM), 8Bromo (8Br)-cAMP (1 mM) or prostaglandin $(PG)E_2$ (1 μM), 30 min later interleukin (IL)-1β (10 $ng \cdot mL^{-1}$) was added for 4 h. a) Eotaxin expression, presented as the ratio of eotaxin messenger ribonucleic acid (mRNA) to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data are expressed as a percentage of the control response to IL-1β alone and are represented as mean±SEM of four independent experiments, carried out in duplicate using cells cultured from four different donors, cell passages three to six. Duplicate blots from one representative experiment are also shown. b) Eotaxin release after 24 h. *: $p< 0.05$; **: $p<0.01$.

Effect of $[cAMP]_i$-elevating agents on IL-1β-induced release and expression of MCP-1

These following results are shown in figure 2. The actual value of secreted MCP-1 was 106±13 $ng \cdot mL^{-1}$; when HASMC were not stimulated, the MCP-1 production was below the detection limit (5 $pg \cdot mL^{-1}$). Forskolin (10 μM) decreased the MCP-1 mRNA expression by 57±8% ($p<0.05$, n=4) and the protein release by 65±6% ($p<0.01$, n=4).

8Br-cAMP (1 mM) decreased the MCP-1 mRNA expression by 29±9% ($p<0.05$, n=4) and the MCP-1 protein release by 63±8% ($p<0.01$, n=4).

PGE_2 (1 μM) decreased the MCP-1 mRNA expression and protein release by 51±7% ($p<0.05$, n=4) and 53±4% ($p<0.05$, n=4), respectively.

Effect of blocking cAMP-dependent protein kinase in the presence of $[cAMP]_i$-increasing agents

The IL-1β-induced eotaxin and MCP-1 production was significantly decreased (73±8 and 65±6%, respectively) by using forskolin 10 μM in HASMC. When H-89, an inhibitor of PKA activation, was added, a concentration-dependent attenuation of the effect of forskolin on the IL-1β-induced eotaxin and MCP-1 release was observed with a residual reduction of only 28±7 and 27±7% with 3 μM of H-89 ($p<0.01$, n=6) (fig. 3).

Effect of $[cAMP]_i$- increasing agents on p38 MAPK, ERK and JNK activation

In order to examine the effect of an increase in $[cAMP]_i$ on IL-1β-induced p38 MAPK activation, cells were preincubated with 10 μM forskolin for 30 min and then stimulated for 5, 15, 30, 60 or 120 min with IL-1β. Immunoblot analysis showed a peak of p38 MAPK activation between 5–15 min and was comparable to baseline after 60 min. Pre-incubation with forskolin did not significantly inhibit the phosphorylated p38 MAPK level when compared to cells treated with IL-1β alone (fig. 4a).

IL-1β (10 $ng \cdot mL^{-1}$) also induced phosphorylation of threonine and tyrosine of JNK and ERK, with a peak activation after 15–30 min. Thereafter, the activation decreased and disappeared after 60 min. There was no difference in either JNK or in ERK activation, compared to control levels after adding 10 μM forskolin (fig. 4b and c).

Discussion

In this study the current authors observed that an increase in $[cAMP]_i$ induced a decrease in eotaxin and MCP-1 expression and release in IL-1β stimulated HASMC. H-89, a known inhibitor of PKA, was able to prevent the observed inhibition of IL-1β-induced eotaxin and MCP-1 protein release by forskolin,

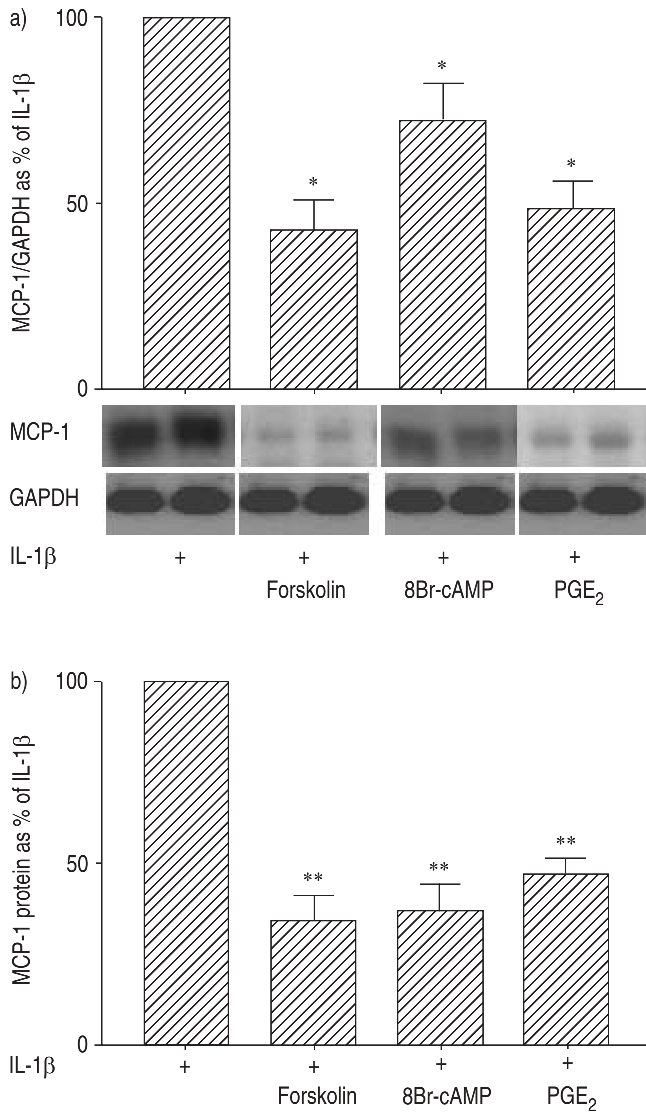

Fig. 2. – Effect of intracellular cyclic adenosine monophosphate (cAMP)-increasing agents on monocyte chemotactic protein (MCP)-1 expression and release. Human airway smooth muscle cells were pretreated with forskolin (10 µM), 8Bromo (8Br)-cAMP (1 mM) or prostaglandin (PG)E_2 (1 µM), 30 min later interleukin (IL)-1β (10 ng·mL^{-1}) was added for 4 h. a) MCP-1 expression, presented as the ratio of MCP-1 messenger ribonucleic acid (mRNA) to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data are expressed as a percentage of the control response to IL-1β alone and are represented as mean±SEM of four independent experiments, carried out in duplicate using cells cultured from four different donors, cell passages three to six. Duplicate blots from one representative experiment are also shown. b) MCP-1 release after 24 h. *: $p < 0.05$; **: $p<0.01$.

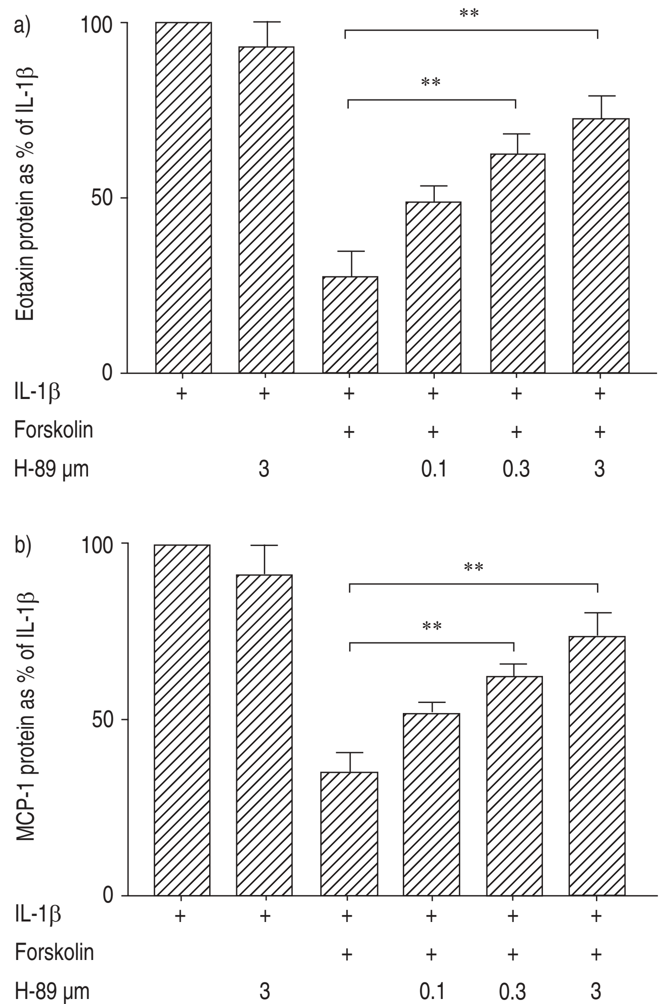

Fig. 3. – a) Eotaxin and b) monocyte chemotactic protein (MCP)-1 release from human airway smooth muscle cells stimulated with interleukin (IL)-1β (10 ng·mL^{-1}) and forskolin (10 µM) after increasing concentrations (0.1, 0.3 and 3 µM) of H-89, an inhibitor of cyclic adenosine monophosphate-dependent protein kinase, had been added 30 min before. Data are expressed as a percentage of the control response to IL-1β alone and are represented as mean±SEM of duplicate values from independent experiments using cells cultured from four different donors, cell passages three to six. **: $p<0.01$.

showing that these effects are PKA dependent. The authors also demonstrated that an increase in $[cAMP]_i$ did not modulate the IL-1β-induced p38 MAPK, ERK or JNK activation.

It is widely known that cAMP is able to regulate the expression of several genes. To investigate whether the IL-1β-induced eotaxin and MCP-1 release could be modulated by cAMP, HASMC were stimulated with IL-1β in combination with forskolin, a direct stimulator of AC, or with 8Br-cAMP, an analogue of cAMP, or PGE_2, which is known to activate the prostanoid receptors EP_2 and EP_4. It is known that all these agents increase $[cAMP]_i$ [17, 18]. This increase in $[cAMP]_i$ resulted in a decrease of eotaxin and MCP-1 mRNA expression. The release of eotaxin and MCP-1 protein was also decreased (after 4 h of stimulation). These data are consistent with the findings by Jocks *et al.* [19], who found that PGE_2 reduces the glomerular mRNA expression of MCP-1 in antithymocyte antibody-induced glomerular injury. In HASMC it has been shown by Pang and Knox [20] that an increase in $[cAMP]_i$ significantly inhibits TNF-α-induced eotaxin release. Moreover, the same group has shown that salbutamol and forskolin, two $[cAMP]_i$ increasing agents, together with corticosteroids produce more inhibition than steroids alone on TNF-α-induced IL-8 release [21]. In addition, in human and mouse mesangial cells incubated with TNF-α, it has been shown that an increase in $[cAMP]_i$ inhibits TNF-α-induced MCP-1 gene expression [10, 22]. Ammit *et al.* [23] have demonstrated that RANTES (regulated upon activation, normal T-cell expressed and secreted) release is decreased after an increase of $[cAMP]_i$ in HASMC. The current results confirm those of Hallsworth *et al.* [24], demonstrating that cAMP is able to decrease IL-1β-induced eotaxin production. However, the current study was broader and to the best of the authors' knowledge it is the first time that a decrease in eotaxin and MCP-1 mRNA expression and MCP-1 release has been observed after an

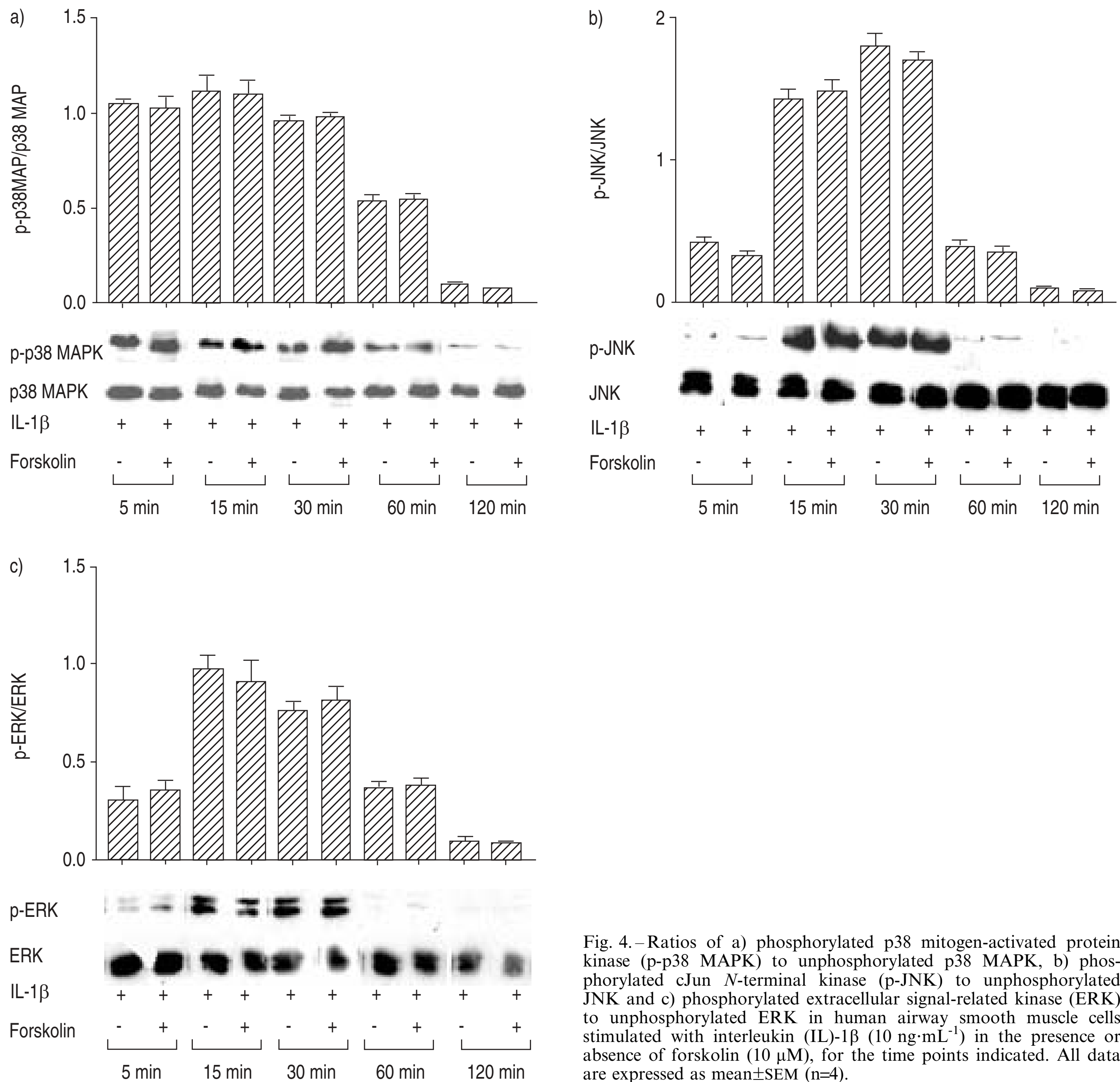

Fig. 4. – Ratios of a) phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK) to unphosphorylated p38 MAPK, b) phosphorylated cJun *N*-terminal kinase (p-JNK) to unphosphorylated JNK and c) phosphorylated extracellular signal-related kinase (ERK) to unphosphorylated ERK in human airway smooth muscle cells stimulated with interleukin (IL)-1β (10 $ng \cdot mL^{-1}$) in the presence or absence of forskolin (10 μM), for the time points indicated. All data are expressed as mean±SEM (n=4).

increase in $[cAMP]_i$ in HASMC stimulated with IL-1β. Furthermore, the authors have demonstrated that this effect is PKA dependent.

This current study enhances the present knowledge about the role of cAMP in IL-1β-induced airway inflammation. IL-1β is a pro-inflammatory cytokine in asthma, which may intensify inflammation by increasing the release of chemokines and, therefore, the attraction of inflammatory cells. At the same time, however, there seems to be a counterbalance, a mechanism that diminishes the inflammatory reaction, by activating cyclo-oxygenase-2 and hence intracellular PGE_2 release, resulting in the activation of the prostanoid receptors EP_2 and EP_4. This increases $[cAMP]_i$ [25], which decreases the chemokine release, as is shown in this study. Therefore, an increase in $[cAMP]_i$ might be considered as a kind of auto-brake mechanism, which prevents inflammation from being excessive and resulting in manifest damage.

It is classically believed that the effects of cAMP are mediated by its receptor PKA, which can phosphorylate a diverse number of target proteins both in the cytoplasmic and the nuclear compartments [26]. Some authors speculate that AC, responsible for the production of cAMP and PKA, is arranged in such a way that cAMP is "channelled" from the AC to PKA by a molecular mechanism [27]. Conversely, in a few cell types, some effects of cAMP have been described to be PKA independent. In INS-1, a pancreatic islet beta cell line, it has been shown that inhibition of PKA with H-89 does not reverse the forskolin-induced suppression of insulin transcription [12]. BRYCE *et al.* [25] showed that elevation of cAMP activity by db-cAMP and forskolin induced significant effects on stimulated peripheral blood mononuclear cell proliferation. These antiproliferative effects persisted even after co-incubation with the selective PKA inhibitiors [28]. As a consequence of this, the current authors investigated whether it was possible to block the forskolin-induced decrease in eotaxin and MCP-1 production with H-89, a specific inhibitor

of PKA. A concentration-dependent inhibition of the decrease in MCP-1 production was observed following stimulation with forskolin. This suggests that PKA is involved in the decrease in eotaxin and MCP-1 production that is observed after increasing $[cAMP]_i$. Recently, it has been observed in HASMC that treatment with H-89 could prevent the inhibitory effect of salbutamol on release of granulocyte-macrophage colony-stimulating factor, RANTES and eotaxin [24]. It has been shown that blocking PKA results in a decrease of the synergistic inhibitory effect of adding salmeterol (which is know to increase $[cAMP]_i$) to fluticasone [21], conversely, it has been shown by the same group that blocking PKA does not alter TNF-α-induced eotaxin release [20]. These data show how complex the role of cAMP may be and how it may depend on the cytokines that are used to stimulate the cells.

To the present authors′ knowledge, they are the first to describe that blocking PKA can prevent the decrease observed in IL-1β-induced eotaxin and MCP-1 production in HASMC.

It has been shown previously that there is a modulation of p38 MAPK activation by cAMP in some cell types [29, 30]. In a previous study [13] the present authors showed that p38 MAPK, ERK and JNK are important pathways in IL-1β-induced eotaxin and MCP-1 expression and production. Therefore, the effect on the IL-1β-induced activation of these MAPKs of increasing $[cAMP]_i$ was investigated. However, when increasing $[cAMP]_i$ no effect was seen in IL-1β-induced p38 MAPK, ERK or JNK activation. The mechanism of the observed cAMP-induced inhibition of eotaxin and MCP-1 expression is not clear and needs further investigation.

In conclusion, it has been demonstrated that the interleukin-1β-induced protein release and messenger ribonucleic acid expression of eotaxin and monocyte chemotactic protein-1 in human airway smooth muscle cells are modulated by intracellular cyclic adenosine monophosphate levels; an increase in intracellular cyclic adenosine monophosphate decreases the release and messenger ribonucleic acid expression of these chemokines. Cyclic adenosine monophosphate protein kinase seems to be a key-element in the decrease in chemokine expression that is observed after an increase in intracellular cyclic adenosine monophosphate. These findings could support the importance of human airway smooth muscle cells, not only as contractile cells, but also as pro-inflammatory cells that might induce and enhance, and even control the airway inflammation in conditions such as asthma. These findings could be important with regards the future development of new anti-inflammatory drugs.

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