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The MNK-1/eIF4E pathway as a new therapeutic pathway to target inflammation and remodelling in asthma

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ABSTRACT

Therapeutic targets in asthma are reduction of airway inflammation and remodelling, the latter is not affected by available drugs. Here we present data that inhibition of MAPK-activated protein kinase (MNK)-1 reduces inflammation and remodelling. MNK-1 regulates protein expression by controlling mRNA stability, nuclear export and translation through the eukaryotic initiation factor 4E (eIF4E). Airway smooth muscle cells were derived from asthmatic and non-asthmatic donors. Cells were pre-treated with CGP57380 (MNK-1 inhibitor) or MNK-1 siRNA, before TNF-α stimulation. Cytokine and protein expression was analysed by ELISA, real time PCR and immunoblotting. Proliferation was monitored by cell counts. TNF-α activated MNK-1 phosphorylation between 15 and 30 min. and subsequently eIF4E between 15 and 60 min. EIF4E activity was inhibited by CGP57380 dose-dependently. Inhibition of MNK-1 by CGP57380 or MNK-1 siRNA significantly reduced TNF-α induced CXCL10 and eotaxin mRNA expression and secretion, but had no effect on IL-8. However, CXCL10 mRNA stability or NF-κB activity were not affected by MNK-1 inhibition. Furthermore, eIF4E was detected in the cytosol and the nucleus, but TNF-α did not affected its export from the nucleus. Cytokine array assessment showed that in addition to eotaxin and CXCL10, asthma relevant GRO α and RANTES were down-regulated by MNK-1 inhibition. In addition, MNK-1 inhibition significantly reduced FCS and PDGF-BB induced cell proliferation. We are the first to report that MNK-1 controls chemokine secretion and proliferation in human airway smooth muscle cells. Therefore we suggest that MNK-1 inhibition may present a new target to limit inflammation and remodelling in asthmatic airways.

1. Introduction

Asthma is characterized by chronic inflammation, hyper-responsiveness (AHR), and airway remodelling. Airway smooth muscle cells (ASMC) are important remodelling relevant cells and also secret pro-inflammatory chemokines such as CXCL10 or eotaxin that subsequently attract immune cells into the airway to increase inflammation and remodelling in asthma [1]. Increased CXCL10 expression was reported in ASMC in vitro and in airway biopsies derived from asthma patients [2,3]. Furthermore, CXCL10 derived from ASMC induced the infiltration of mast cells into asthmatic airway smooth muscle bundles [3,4]. The activation of mast cells leads to the activation of airway smooth muscle cells and increases the secretion of further pro-inflammatory cytokines such as eotaxin [5].

Eotaxin expression correlated with asthma severity [6] and it was expressed at higher levels in ASMC derived from asthma patients [7,8]. Eotaxin is a chemoattractant for eosinophils and elevated numbers of infiltrated eosinophils in the airway is a prominent pathological feature of asthma [9,10]. Several pro-inflammatory conditions increase both CXCL10 and eotaxin through the mitogen activated protein kinases (MAPK), which is followed by the activation of MAPK-activated protein kinase (MNK)-1 in different cell types [11] but its contribution to asthma has not been investigated yet.

In regard to its stimulating role in inflammation toll-like receptor-agonist-stimulated TNF-α, monocyte chemoattractant protein (MCP)-1 or IL-6 production in macrophages, and this effect was significantly reduced by inhibition of MNK-1 [12]. Similarly, TNF-α, IL-6 and IL-1β expression was down regulated by MNK-1 inhibition in human keratinocytes [13]. Interestingly the action of MNK-1 does not seem to be mediated via transcription, but is also regulated through translation control as it was reported in two other cell types. MNK-1 up-regulated the expression of IFN-γ and IL-4 by natural killer T cells [14] and that of CCL3 and CCL4 by neutrophils [15] via increased translation not

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transcription, both effects were reduced in the presence of the MNK-1 inhibition.

MNK-1 is activated by both p38 MAPK and ERK 1/2 MAPK [16] and directly phosphorylates the eukaryotic initiation factor 4E (eIF4E) in response to external stimuli [17]. Whereas MNK-2 mediates the constitutive phosphorylation of eIF4E, which is not further increased by activation of p38 or ERK 1/2 MAPK [17,18]. Interestingly, in MNK- 1/2 double knockout mice the phosphorylation of eIF4E is absent, which suggests that MNK-1 and MNK-2 are the only kinases phosphorylating eIF4E [19]. Furthermore, eIF4E is over-expressed in many forms of human cancer and regulates protein expression through protein translation control, mRNA nuclear export or mRNA stability [20]. In asthma, eIF4E is required for ASMC hypertrophy [21]; however, its role in ASMC cytokine secretion or airway inflammation had not yet been investigated.

In this study, we investigated whether the MNK-1/eIF4E pathway is activated by TNF-α in asthmatic and non-asthmatic ASMC. Furthermore, we determined the effect of the MNK-1 inhibitor CGP57380 or MNK-1 siRNA on chemokine expression and on proliferation.

2. Material and methods

2.1. Culture of human ASMC

Primary human ASMC were isolated from airway tissue obtained by endobronchial biopsy or therapeutic lung resection, characterized and grown as previously described [22]. The use of human primary ASMC was approved by the local ethical committee of the University Hospital, Basel, Switzerland (EKB05/06) and written consent was given by each patient.

2.2. Chemokine secretion by ASMC (ELISA and human cytokine array)

Confluent ASMC were growth arrested, then pre-treated (1 h) with either CGP57380 (1.25–20 μM; Merck, Darmstadt, Germany) or SB203580 (10 μM), or PD98059 (30 μM; both: Calbiochem, Luzern, Switzerland) or MNK-1 siRNA before being stimulated with TNF-α (10 ng/ml; R&D Systems, Minneapolis, USA) for 24 h. Cell supernatants were collected and CXCL10, eotaxin and IL-8 protein was measured by enzyme-linked immunosorbent assay (ELISA, Duo Set, R&D Systems).

Four human cytokine array membranes, each detecting 36 different cytokines relevant to asthma, (Proteome Profiler™ Antibody Arrays Human Cytokine Array, R&D Systems, Minneapolis, MN) following the procedure as described by the manufacturer's instructions. Conditioned cell culture medium was collected from ASMC under two conditions: 1) after stimulation with TNF-α alone or 2) from cells that were pre-treated for 72 h with MNK1 siRNA as described below.

2.3. MNK-1 siRNA treatment

For further experiments investigating the role of MNK-1, **corresponding** small interfering RNA (siRNA) was purchased from Qiagen and ASMC were transfected with MNK1 and control siRNA (20 nM) for 72 h according to manufacturer's protocols. Transfection reagent was HiPerFect Transfection Reagent (Qiagen). ASMC were then starved for 24 h before being treated with TNF-α for 3 h (RNA) or 24 h (protein secretion).

2.4. Real-time RT-PCR

Growth arrested ASMC were pre-treated with the inhibitors (1 h) and then stimulated with TNF-α. After 3 h, total RNA was extracted with a Quick-RNA MiniPrep Kit (ZymoResearch, Orange, CA) and reverse transcription (RT) was performed with an Omniscript® RT kit (Qiagen, Hombrechtikon, Switzerland). The levels of cytokine mRNA were determined by real-time PCR using a CXCL10 (Hs00171042_m1), eotaxin (CCL11, Hs00237013_m1) or GAPDH (Hs03929097_g1) TaqMan® Gene Expression Assays (all Applied Biosystems, Foster City, CA). Samples were run at 50 °C for 2 min, 1 cycle; 95 °C for 10 min, 1 cycle; 95 °C for 15 s, 60 °C for 1 min, 40 cycle. mRNA expression was quantified by the delta delta Ct calculation method.

2.5. Immunoblotting

Growth arrested ASMC were treated as described above and total cell lysates were collected between 0 and 120 min, size-fractionated by SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes as described previously [23]. Membranes were blocked and then incubated with antibodies: phospho-MNK-1, MNK-1eIF4E, phospho-eIF4E, Lamin A/C (all Cell Signaling Technology, Beverly, MA), or to α-Tubulin (Santa Cruz Biotechnology, Santa Cruz, USA). Membranes were then incubated with horseradish peroxidase-conjugated IgG antibodies (anti-rabbit IgG, or anti-mouse IgG; Santa Cruz Biotech.) and protein bands were visualized by enhanced chemiluminescence (Pierce Biotechnology, Rockford, USA).

2.6. ASMC viability (MTT assay)

Growth arrested ASMC were either treated with CGP57380 (10, 20 μM), a positive control (Triton X-100, 0.5 mg/ml, Sigma) or left untreated (negative control). After 24 h, the cells were incubated with 3-(3,4-dimethylthiazol-2yl)-,5-diphenyl tetrazolium bromide (MTT, Sigma) for 6 h before being lysed by DMSO (Sigma) and the optical density was measured at 550 nm on a microplate reader.

2.7. mRNA stability

ASMC (growth arrested) were pre-treated with CGP57380 (10 μM, 1 h) and then stimulated with TNF-α for 3 h. In order to stop new mRNA synthesis, ASMC were washed and actinomycin D (5 μg/ml) in low serum medium was added. Total RNA was extracted after 0, 2, 4, 6, or 24 h, followed by reverse transcription and quantification of CXCL10 mRNA expression as described earlier.

2.8. Nuclear and cytosolic distribution of eIF4E

ASMC were stimulated with TNF-α or left un-stimulated for 30 and 60 min. ASMC were then trypsinised and incubated in low-salt buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.25% Nonidet P-40 and 1× protease inhibitor cocktail) to extract the cytosolic protein fraction. Nuclei were re-suspended in high-salt buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1× protease inhibitor cocktail). Cytosolic and nuclear protein extracts were analysed by immunoblotting as described in Section 2.4 earlier.

2.9. NF-κB EMSA

ASMC were pre-treated with CGP57380 (10 μM, 1 h), stimulated with TNF-α for 30 min, and then fractionated into cytosolic and nuclear extracts as described above. NF-κB p65/DNA binding was determined in the nuclear protein extract by using the NF-κB p65 TransAm™ transcription factor assay kit (Active Motif, Carlsbad, CA).

2.10. ASMC proliferation

ASMC were pre-treated with CGP57380 (1–10 μM for 1 h) and then stimulated with either FCS (5%) or PDGF-BB (10 ng/ml). After 72 h, ASMC were trypsinized and cell numbers were determined using a Beckman Coulter particle counter Z1 (Beckman, Nyon, Switzerland).