Tampere University

TIINA KERÄNEN

The MAP Kinase / MAP Kinase Phosphatase-1 Pathway in the Anti-Inflammatory Effects of β_2-Agonists and Dexamethasone

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ACADEMIC DISSERTATION
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Responsible supervisor	Docent Riku Korhonen Tampere University Finland	
Supervisor	Professor Eeva Moilanen Tampere University Finland	
Pre-examiners	Professor Raimo Tuominen University of Helsinki Finland	Professor Markku Koulu University of Turku Finland
Opponent	Professor Kirsi Vähäkangas University of Eastern Finland Finland	
Custos	Professor Eeva Moilanen Tampere University Finland	

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications:

I Tiina Keränen, Tuija Hömmö, Mari Hämäläinen, Eeva Moilanen, Riku Korhonen. Anti-inflammatory effects of β_2-receptor agonists salbutamol and terbutaline are mediated by MKP-1. PLoS One. (2016) 11(2):e0148144. doi: 10.1371/journal.pone.

II Tiina Keränen, Eeva Moilanen, Riku Korhonen. Suppression of cytokine production by glucocorticoids is mediated by MKP-1 in human lung epithelial cells. Inflammation Research, (2017) 66(5):441-44 doi: 10.1007/s00011-017-1028-4.

III Tiina Keränen, Tuija Hömmö, Eeva Moilanen, Riku Korhonen. β_2-receptor agonists salbutamol and terbutaline attenuated cytokine production by suppressing ERK pathway through cAMP in macrophages. Cytokine. (2017) 94:1-7. doi: 10.1016/j.cyto.2016.07.016.

In addition, some unpublished data are presented.

ABBREVIATIONS

cAMP	cyclic adenosine 3′-5′-monophosphate
8-Br-cAMP	8-bromoadenosine 3′-5′-cyclic monophosphate
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DUSP	dual specificity phosphatase
ERK	extracellular signal regulated kinase
ELISA	enzyme-linked immunosorbent assay
Epac	exchange protein directly activated by cAMP
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
cGMP	cyclic guanosine 3′-5′-monophosphate
GPCR	G protein-coupled receptor
IL	interleukin
JNK	c-Jun N-terminal kinase
KO	knockout
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MEK	MAPK/ERK kinase

MIP	macrophage inflammatory protein
MKK	mitogen-activated protein kinase kinase
MKKK	mitogen-activated protein kinase kinase kinase
MKP	mitogen-activated protein kinase phosphatase
MMP	matrix metalloproteinase
PBS	phosphate buffered saline
PDE	phosphodiesterase
PKA	protein kinase A
PM	peritoneal macrophage
RANKL	receptor activator of nuclear factor κB ligand
RNA	ribonucleic acid
miR	microRNA
mRNA	messenger RNA
siRNA	small interfering RNA
qRT-PCR	quantitative reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
S.E.M.	standard error of the mean
TAK1	TGFβ (transforming growth factor beta)-activated kinase-1
TBS/T	tris-buffered saline/ tween-20
Th1	T helper cell type 1
TNF	tumor necrosis factor
WT	wild-type

ABSTRACT

Chronic inflammatory diseases, such as arthritis, asthma, COPD or psoriasis, are characterized by excessive inflammatory response, leading to tissue injury, impaired organ function, pain, disability and co-morbidity. Inflammatory cells, including macrophages, granulocytes and lymphocytes, are present in the inflamed tissue, and together with epithelial and parenchymal cells produce inflammatory factors, such as cytokines (e.g., TNF, IL-1, IL-6, IL-17, RANKL), chemokines (e.g., MCP-1, IL-8), other inflammatory mediators (e.g., prostaglandins, nitric oxide) and growth factors (e.g., GM-CSF) that maintain and amplify the inflammation. The role of increased cytokine production in chronic inflammatory diseases is evidenced by the clinical efficacy of cytokine antagonists (e.g., anti-TNF, anti-IL-6) in chronic inflammatory diseases.

Mitogen-activated protein kinases (MAP kinases, MAPKs) are an evolutionary conserved family of intracellular signaling proteins that regulate many physiological cellular processes, such as cell growth, proliferation, differentiation, motility, stress response, survival and apoptosis. The three main MAP kinase pathways are p38 MAPK, JNK (c-Jun N-terminal kinase) and ERK (extracellular signal-regulated kinase). MAPKs are activated by phosphorylation of their threonine and tyrosine residues, and their targets include transcription factors and other cellular regulatory proteins. p38 MAPK and JNK are involved in the regulation of inflammation and immune response. They regulate the production of inflammatory cytokines and other mediators, such as prostaglandins and nitric oxide, during inflammatory response, and they augment the Th1 type immune response.

Mitogen-activated protein kinase phosphatases (MKPs) are a group of dual specificity phosphatases (DUSPs) that dephosphorylate tyrosine and threonine residues of their target proteins. Currently, 11 MKPs have been characterized. MKPs are endogenous suppressors of MAPK pathways. MKP-1 is a nuclear phosphatase that regulates mainly p38 MAPK, and in some cells, JNK activity. MKP-1 suppresses inflammatory gene expression (such as TNF and IL-6) and attenuates acute inflammatory response by inhibiting p38 MAPK. Glucocorticoids, anti-rheumatic

gold compounds and phosphodiesterase 4 (PDE4) inhibitors increase MKP-1 expression, and MKP-1 partly mediates their anti-inflammatory effects.

β_2-agonists and glucocorticoids form the corner stone of the treatment of asthma and COPD. Glucocorticoids have strong anti-inflammatory effects through regulation of inflammatory gene expression. β_2-agonists are mainly used as bronchodilating drugs, but they seem to have anti-inflammatory effects also. PDE4 inhibitors are used as an anti-inflammatory treatment of COPD, psoriasis and psoriatic arthritis. β_2-receptor agonists and PDE4 inhibitors increase cAMP levels in cells, but through different molecular mechanisms. In addition to its other cellular effects, cAMP regulates gene expression through activating the transcription factor CREB (cAMP-responsive element binding protein).

The aim of this thesis was to identify drugs that increase the expression of MKP-1 and modulate the activity of MAPK pathways and further, to investigate the role of MKP-1 in their anti-inflammatory effects. We focused on the β_2-agonist salbutamol and the glucocorticoid dexamethasone because the MKP-1 promoter has been identified to contain cAMP and glucocorticoid responsive elements.

The result of this thesis evidences that β_2-agonists and dexamethasone inhibit inflammatory gene expression and have anti-inflammatory properties which are mediated by enhanced MKP-1 expression and dephosphorylation / inactivation of the p38 MAPK pathway. Furthermore, β_2-agonists alone and in combination with PDE4 inhibitors suppress cytokine TNF and chemokine MCP-1 in activated macrophages in a manner associated with the inhibition of ERK pathway.

The results of the present thesis project extend our understanding on the regulation of inflammation in chronic inflammatory diseases and on the role of MKP-1 in inflammation and anti-inflammatory drug effects. Novel findings on the regulation of inflammatory genes and the mechanism behind it provide targets for the development of improved anti-inflammatory treatment strategies.

TIIVISTELMÄ

Kroonisissa tulehdussairauksissa, kuten nivelreumassa, astmassa, keuhkoahtaumataudissa ja psoriaasiksessa on tyypillistä liian voimakas ja pitkittynyt tulehdusvaste, joka voi johtaa kudoksen heikentyneeseen toimintaan ja vaurioihin, aiheuttaa kipua, heikentää toimintakykyä ja lisätä riskiä sairastua liitännäissairauksiin. Tulehtuneessa kudoksessa on monia tulehdussoluja mm. makrofageja, granulosyyttejä ja lymfosyytteja. Nämä solut tuottavat yhdessä epiteeli- ja kudossolujen kanssa tulehdustekijöitä, kuten sytokiinejä (esim. TNF, IL-1, IL-6, IL-17, RANKL), kemokiineja (esim. MCP-1, IL-8), muita tulehduksen välittäjäaineita (esim. prostaglandiinit, typpioksidi) ja kasvutekijöitä (esim. GM-CSF), jotka ylläpitävät ja voimistavat tulehdusta. Monet tulehdusta rauhoittavat lääkeaineet estävät sytokiinien tuottoa. Myös tiettyjä sytokiineja tai niiden reseptoreja estävien vasta-aineiden (esim. anti-TNF, anti-IL-6) teho on osoitettu kliinisissä tutkimuksissa ja nykyään niitä käytetään kroonisten tulehdussairauksien hoidossa.

Mitogeeniaktivoidut proteiinikinaasit (MAP-kinaasit) ovat signalointiproteiineja, jotka säätelevät solun monia fysiologisia toimintoja, kuten jakautumista, erilaistumista, liikkumista, stressivastetta ja apoptoosia. Kolme tärkeintä MAP-kinaasiryhmää ovat p38 MAP-kinaasi, JNK (engl. c-Jun N-terminal kinase) ja ERK (engl. extracellular signal-regulated kinase). MAP-kinaasit aktivoituvat, kun niiden treoniini- ja tyrosiinitähteet fosforyloituvat. Tämä johtaa MAP-kinaasien kohteena olevien transkriptiotekijöiden ja muiden solujen toimintaa säätelevien proteiinien aktivoitumiseen. Erityisesti p38 MAP-kinaasi ja JNK osallistuvat soluissa tulehduksen ja immuunivasteen säätelyyn. Ne säätelevät tulehdussytokiinien ja muiden välittäjäaineiden, kuten prostaglandiinien ja typpioksidin, tuottoa tulehduksen aikana. Ne lisäävät myös Th1-tyyppistä immuunivastetta.

MAP-kinaasifosfataasit (MPK:t) ovat spesifisiä fosfataaseja, jotka defosforyloivat kohdeproteiiniensa tyrosiini- ja treoniinitähteitä. Niitä tunnetaan tällä hetkellä 11 ja ne ovat tärkeitä MAP-kinaasien aktiivisuutta sääteleviä tekijöitä. Tulehdusreaktion kannalta MKP-1 on osoittautunut erityisen kiinnostavaksi proteiiniksi. Se on tumafosfataasi, joka säätelee erityisesti p38 MAP-kinaasin, ja joissakin soluissa myös

JNK:n, aktiivisuutta. MKP-1 vaimentaa tulehdustekijöiden tuottoa ja heikentää akuuttia tulehdusvastetta rajoittamalla p38 MAP-kinaasin aktivaatiota. MKP-1:n on osoitettu välittävän ainakin osan glukokortikoidien, reuman hoidossa käytettävien kultayhdisteiden ja fosfodiesteraasi 4:n (PDE4:n) estäjien tulehdusta rauhoittavista vaikutuksista.

β_2-agonistit ja glukokortikoidit ovat astman ja keuhkoahtaumataudin lääkehoidon kulmakivi. Glukokortikoidit rauhoittavat tulehdusta vaimentamalla tulehdusgeenien ilmentymistä. β_2-agonisteja käytetään keuhkoputkia laajentavina lääkkeinä, mutta niillä näyttää olevan myös tulehdusta lievittäviä vaikutuksia. PDE4:n estäjiä käytetään tulehdusta rauhoittavina lääkkeinä keuhkoahtaumataudin ja (nivel)psoriaasin hoidossa. β_2-agonistit ja PDE4:n estäjät lisäävät syklisen AMP:n pitoisuutta soluissa eri mekanismeilla. Muiden vaikutustensa ohella syklinen AMP säätelee geenien ilmentymistä CREB (cAMP responsive element binding protein) -transkriptiotekijän välityksellä.

Tämän väitöskirjatyön tarkoituksena oli löytää lääkeaineita, jotka lisäävät MKP-1:n ilmentymistä ja säätelevät MAP-kinaasien aktiivisuutta, ja edelleen selvittää MKP-1:n merkitystä kyseisten tulehdusta rauhoittavien lääkeaineiden vaikutuksissa ja vaikutusmekanismeissa. Väitöskirjatyössä tutkittiin erityisesti β_2-agonisteihin kuuluvaa salbutamolia ja glukokortikoidi deksametasonia, koska MKP-1-geenin säätelyalueella on tunnistettu sekä sykliselle AMP:lle että glukokortikoideille reagoivia alueita.

Väitöskirjani tulokset osoittavat, että β_2-agonistit ja deksametasoni estävät tulehdusgeenin ilmentymistä ja että niillä on tulehdusta lievittäviä vaikutuksia. Osoitin myös, että nämä vaikutukset välittyvät ainakin osittain lisääntyneen MKP-1:n ilmentymisen ja p38 MAP-kinaasireitin vaimentumisen kautta. Tämän lisäksi β_2-agonistit hillitsevät sekä yksinään että yhdessä PDE4-estäjien kanssa sytokiini TNF:n ja kemokiini MCP-1:n ilmentymistä estämällä ERK-reitin toimintaa syklisestä AMP:stä riippuvalla mekanismilla.

Tämän väitöskirjan tulokset tuottivat uutta tietoa tulehdusreaktion mekanismeista ja MKP-1:n merkityksestä tulehduksessa ja tulehdusta vähentävien lääkkeiden vaikutuksissa. Uudet löydökset tulehdusgeenien säätelystä ja mekanismeista niiden takana tuovat esiin uusia tulehdusta rauhoittavien lääkehoitojen kehitystyön kohteita.

INTRODUCTION

Inflammation is a defense reaction of the body against external (pathogens, chemical or physical factors) or internal (inflammatory mediators, cellular factors or tissue debris) threats. The purpose of the inflammatory reaction is to remove causing pathogens and other contributing factors, limit tissue damage and start regenerative processes to repair tissue damage. One task of inflammatory mediators is to transmit information about inflammation and start regenerative processes. If the inflammatory reaction is too weak, too strong or directed incorrectly, it can lead to illness or even a life-threatening medical condition. In autoimmune diseases, such as rheumatoid arthritis, the inflammatory reaction is typically too strong, dysregulated and directed against host tissues. In such a case, leukocytes are activated against the body's own organs and tissues, causing tissue damage and leading to tissue injury and organ dysfunction. Often, in such diseases, the quality of life of patients is impaired, their ability to work is deteriorated, and morbidity and mortality increase as compared to the general population.

Over the last decades, several new drugs have been developed to treat inflammatory diseases and they have improved treatment outcomes of chronic inflammatory diseases significantly. Such drugs are, e.g., cytokine antagonists and Janus kinase (JAK) inhibitors, developed for the treatment of rheumatoid arthritis and phosphodiesterase 4 (PDE4) inhibitors for the treatment of COPD, psoriasis and psoriatic arthritis. Although the treatment of chronic inflammatory diseases has improved and drug treatment options have become more diverse, there is still unmet medical need for effective anti-inflammatory medication for different patient subpopulations. (Chong, et al., 2011, Cludts, et al., 2018, Fragoulis, et al., 2019, Jazayeri, et al., 2010, Lipari, et al., 2013, Mogul, et al., 2019, Moreland, et al., 1999, Papp, et al., 2012, Schett, et al., 2012)

Mitogen-activated protein kinases (MAPKs) are involved in the intracellular signal transmission system of inflammatory response (Kim and Choi, 2015). They regulate the production and release of inflammatory factors, such as cytokines and chemokines. In particular, p38 MAPK and c-Jun N-terminal kinase (JNK) are significant regulators in inflammatory reactions and in immune response. MAP

kinase phosphatases (MKPs) inactivate MAPKs by dephosphorylation (Caunt and Keyse, 2013). Thus MKPs regulate the expression of cytokines and other inflammatory factors and, in general, act as suppressors of the inflammatory reaction and immune responses. MKP-1 is one of the key MKPs (Wancket, et al., 2012). MKP-1 suppresses inflammatory gene expression and attenuates acute inflammatory response by inhibition of p38 MAPK (Korhonen, et al., 2011, Zhao, et al., 2006). Glucocorticoids, anti-rheumatic gold compounds and phosphodiesterase 4 (PDE4) inhibitors increase MKP-1 expression, and MKP-1 mediates, at least partly, the anti-inflammatory effects of these drugs (Abraham, et al., 2006, Korhonen, et al., 2013, Nieminen, et al., 2010). Other members of MKP family have been found to regulate inflammatory response (Jeffrey, et al., 2006, Qian, et al., 2009), but the role of other MKPs in anti-inflammatory drug effects is not known.

The results of the present thesis will extend our understanding of the regulation of inflammation in chronic inflammatory diseases and of the role of MKP-1 in inflammation and anti-inflammatory drug effects. Novel findings of the regulation of inflammatory genes and the mechanism behind it provide targets for the development of more selective and effective anti-inflammatory treatment strategies.

1 REVIEW OF LITERATURE

1.1 Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are an evolutionarily conserved family of intracellular signaling proteins that regulate many physiological cellular processes in eukaryotic cells, such as cell growth, proliferation, differentiation, motility, stress response, survival, and apoptosis. MAPKs are also an important part of the intracellular signal transmission system in inflammatory cell response. MAPKs affect the production of inflammatory factors, such as cytokines, growth factors, and chemokines (Kim and Choi, 2015, Plotnikov, et al., 2011, Rincon and Davis, 2009). MAPKs are also involved in the cognitive brain functions, such as memory, learning, and neural development (Abel, et al., 1998, Kornhauser and Greenberg, 1997, Lisman, 1994).

The three main MAPK pathways (Figure 1) are p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), which all consist of several isoforms. In principle, MAPK pathways are three-tier kinase cascades that are activated in response to several extracellular signals. These signals include cytokines, growth factors, chemokines and bacterial substances, and they activate MAP kinase pathways through G protein-coupled and/or kinase-linked receptors. At the first step, MAPK kinase kinase (MKKK) is activated, which then phosphorylates the second cascade level, MAPK kinase (MKK). At the second phase, MAPK kinase phosphorylates threonine and tyrosine residues (Thr-X-Tyr) of MAPK, which leads to activation of MAPK. Targets of activated MAPKs include transcription factors and other regulatory proteins, and they control many important cellular processes through a number of downstream effector proteins, such as c-Jun, and p53 (Kim and Choi, 2015, Kyriakis and Avruch, 2012, Plotnikov, et al., 2011, Rincon and Davis, 2009).

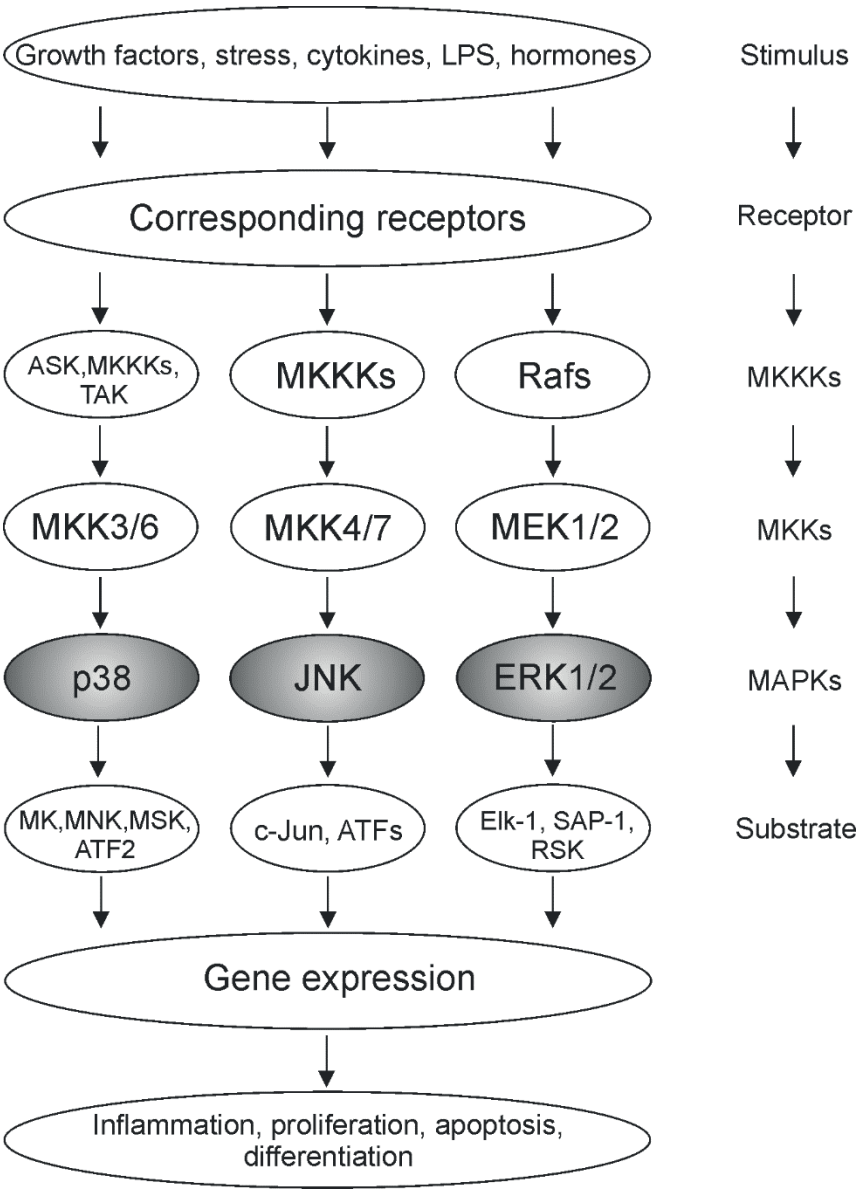


Figure 1. MAPK pathways. LPS (lipopolysaccharide), ASK (apoptosis signal-regulating kinase), MKKK (mitogen-activated protein kinase kinase kinase), TAK (TGF-beta-activated kinase), MKK (mitogen-activated protein kinase kinase), MEK (MAPK/ERK Kinase), JNK (c-Jun N-terminal kinase), ERK (extracellular signal regulated kinase), MAPK (mitogen-activated protein kinase), MK (MAPK-activated protein kinase), MNK (MAPK-interacting kinase), MSK (mitogen- and stress-activated kinase), ATF (activating transcription factor), SAP (SRF accessory protein), RSK (ribosomal s6 kinase). Modified from Plotnikov, et al. 2011.

1.1.1 Family of p38 MAPK

p38 MAPK is an important regulator of the production of inflammatory mediators and immune response. It regulates the activity of many intracellular signal proteins and many cell types of innate immunity, for example, macrophages and neutrophils. Further, p38 MAPK regulates adaptive immunity by regulating maturation of T lymphocytes and helper T cells and distribution of B lymphocytes. p38 MAPK is activated in response to cytokines, bacterial products or cell stress, e.g., osmotic shock, heat or ultraviolet radiation. (Liu, et al., 2007, Martin-Blanco, 2000, Murphy, 2012, Plotnikov, et al., 2011, Wancket, et al., 2012, Yang, et al., 2014)

There are four identified isoforms in the p38 MAPK family; p38α, p38β, p38γ and p38δ. p38α and p38β are expressed in most tissues. The p38β gene is more than 70 % similar to the p38α gene. p38δ is found particularly in the lungs, pancreas, testes, kidneys, small intestine, and CD4+ T lymphocytes. p38γ is mainly expressed in skeletal muscle. p38δ and p38γ genes are about 60 % similar to p38α. (Ashwell, 2006, Kumar, 2010, Plotnikov, et al., 2011, Rincon and Davis, 2009)

Distinct isoforms have specific biological functions to a certain extent, possibly due to substrate selectivity (Ashwell, 2006, Thornton and Rincon, 2009). Homozygote $p38\alpha^{-/-}$ mice are embryonically lethal at day 10. Heterozygote $p38\alpha^{+/-}$ mice have a normal phenotype. p38β, p38γ and p38δ KO (knockout) mice are viable and they do not have any obvious phenotype as compared to WT (wild-type) mice (Tamura, et al., 2000).

Depending on the cell type, the first component of p38 MAPK pathway may be either MKKK 1-4, apoptosis signal related kinase-1 (ASK1) or TGFβ-activated kinase-1 (TAK1), which all phosphorylate the second component of the cascade, namely MKK3 or MKK6. Their activation leads to phosphorylation of p38 MAPK (Thornton and Rincon, 2009). MKK3 specifically activates p38α and p38β, while MKK6 activates all p38 MAPK isoforms (Roux and Blenis, 2004).

p38 MAPK has a significant role in the regulation of inflammation and immune response. It regulates the production of inflammatory cytokines [for instance, tumor necrosis factor (TNF) and interleukin-6 (IL-6)] and other mediators, such as prostaglandins, nitric oxide and vascular cell adhesion molecule 1 (VCAM-1). It also augments Th1 type immune response and supports the activation and functions of

Th1 cells (Craxton, et al., 1998, Da Silva, et al., 1997, Liu, et al., 2007, Pietersma, et al., 1997, Plotnikov, et al., 2011, Rincon and Davis, 2009, Wancket, et al., 2012).

Table 1. Ongoing clinical trials with p38 MAPK inhibitors

p38 MAPK inhibitor	*Medical condition*	*Sponsor*
ARRY-371797	Cardiomyopathy	Array BioPharma
AZD7624	Corticosteroid-Resistant Asthma	AstraZeneca
CHF6297	COPD	Chiesi Farmaceutici S.p.A.
RO4402257	Rheumatoid arthritis	F. Hoffmann-La Roche Ltd
Losmapimod (GW856553)	Neuropathic pain, major depressive disorder and atherosclerosis	GSK R&D Ltd
SB681323	Neuropathic pain	GSK R&D Ltd
Neflamapimod (VX745)	Alzheimer's disease	EIP Pharma, LLC
LY2228820	Glioblastoma, epithelial ovarian cancer, fallopian tube cancer, and primary peritoneal cancer	Eli Lilly and Co
LY3214996	Advanced cancer and metastatic melanoma	Eli Lilly and Co

https://www.clinicaltrialsregister.eu, https://clinicaltrials.gov/ (updated 6.4.2019)

Experiments with selective p38 MAPK inhibitors have provided data on the role of p38 MAPK in inflammation and immune responses. p38 MAPK inhibitors attenuate the production of inflammatory mediators (Chen, et al., 2007, Huang and Zhang, 2003, Korhonen, et al., 2011, Turpeinen, et al., 2010), and they have clear anti-inflammatory effects in experimental models of inflammatory diseases

(Badger, et al., 2000, Burnette, et al., 2009, Underwood, et al., 2000). p38 MAPK is involved in the pathophysiology of chronic inflammatory diseases, such as rheumatoid arthritis, COPD and inflammatory bowel disease (Charron, et al., 2017, Genovese, et al., 2011, Goldstein, et al., 2010, Hollenbach, et al., 2004, Meng, et al., 2016), and interestingly, p38 MAPK signaling has been associated with the development of Alzheimer's disease (Johnson and Bailey, 2003). p38 MAPK has also been shown to be involved in the development of myocardial hypertrophy. The p38α pathway controls the apoptosis of cardiac muscle cells (Wang, et al., 1997). It is also possible that the decrease in the activity of p38 MAPK is one of the factors contributing to the development of heart failure (Arabacilar and Marber, 2015, Tenhunen, et al., 2006). p38 MAPK inhibitors have been investigated in clinical trials in several human diseases (Table 1). So far, p38 MAPK inhibitors have not been approved for therapy in human diseases.

1.1.2 Family of ERK

ERK1 and ERK2 are the prototypes of the eight isoforms of ERK (Torii, et al., 2006). ERK1 and ERK2 are 83 % similar and they are expressed in many tissues, particularly in the brain, skeletal muscle, thymus, and heart (Boulton, et al., 1990). ERK3 and ERK4 are 73 % identical in their kinase domain and they are atypical MAPK because their activation loop contains the Ser-Glu-Gly motif instead of Tyr residue. ERK7 (ERK8 in humans) has the Thr-Glu-Tyr motif, and it has autophosphorylation activity. ERK5, also known as BMK1 (big MAPK 1), is expressed in high levels in the brain, thymus, and spleen. ERK5 regulates cell survival, proliferation and differentiation via several mechanisms (Cargnello and Roux, 2011).

The ERK1/2 pathway can be activated by different extracellular signals, such as growth factors, osmotic stress, mitotic signals, hormones and inflammatory cytokines. First, a ligand-receptor engagement leads to the activation of GTPase Ras, which in turn activates MKKK of the ERK1/2 pathway and it is usually A-Raf, B-Raf or Raf-1. It can also be the protein kinases MEKK1, Mos or Tpl2 (also known as Cot) in some cell types. MKK of the ERK1/2 pathway is MAPK/ERK kinase (MEK)1/2. This pathway is called the Ras-Raf-MEK1/2-ERK1/2 pathway, and it is important for cell proliferation and metastasis, e.g., in cancer development (Cargnello and Roux, 2011). Phosphorylated ERK translocates to the nucleus, where it activates

transcription factors, for example, activator protein-1 (AP-1), and regulates gene expression to promote growth, differentiation and mitosis. In addition, phosphorylated ERK is involved in the regulation of cell functions through cytosolic target proteins, such as ELK-1, SAP-1, and RSK (Plotnikov, et al., 2011, Raman, et al., 2007, Rincon and Davis, 2009, Roskoski, 2012, Torii, et al., 2006). Further, it has been found that in yeast ERK1/2 could bind directly to DNA and affect several cytokine-induced genes as a transcriptional repressor (Hu, et al., 2009, Pokholok, et al., 2006).

ERK1 KO mice are viable, fertile and normal in size (Nekrasova, et al., 2005, Pages, et al., 1999). ERK1 KO mice have abnormalities in thymocyte development and neuronal synaptic plasticity and they have mild osteopetrosis (Mazzucchelli, et al., 2002, Saulnier, et al., 2012). ERK1 KO mice have also impaired adipocyte functions and, for example, they do not develop high-fat-diet-induced obesity and insulin resistance (Bost, et al., 2005). ERK2 KO mice have severe placental defects leading to early embryonic lethality (Hatano, et al., 2003, Saba-El-Leil, et al., 2003, Yao, et al., 2003). ERK1 and ERK2 double KO mice have embryonic lethality due to generalized impairment of organ development (Blasco, et al., 2011).

Pharmacological modulations of the ERK pathway show therapeutic potential in animal models of inflammation. The MEK1/2 inhibitor PD 184352, which inhibits ERK1/2 activation, inhibited the development of arthritis in a mouse collagen-induced arthritis model (Thiel, et al., 2007). MEK inhibitor UO126 has been shown to reduce cytokine production, inflammatory cell recruitment into the lung tissue, and airway hyper-reactiveness in an ovalbumin-induced airway inflammation model (Duan, et al., 2004). MEK inhibitor U0126 also prevents ERK phosphorylation and ear swelling in an ear edema model (Jaffee, et al., 2000).

In patients with COPD-related emphysema, chronically increased ERK activity has been observed in airway lining and alveolar cells. Cigarette smoke has been shown to induce a rapid, strong and prolonged ERK activation in airway epithelial cells, and chronically elevated ERK activity has been demonstrated in airway and alveolar epithelial cells of emphysema patients (Mercer, et al., 2004, Mercer and D'Armiento, 2006). Also, increased expression of MMP-1 by cigarette smoke is dependent on active ERK signaling (Mercer, et al., 2004). MMP-1, as well as many other matrix metalloproteinases, is associated with the development of emphysema (Ostridge, et al., 2016). Aberrant ERK activation has been observed in some chronic inflammatory diseases in humans, also. For example, the ERK pathway is chronically

activated in the synovial tissue of rheumatoid arthritis patients (Schett, et al., 2000, Thiel, et al., 2007).

ERK has been identified as a drug target in many cancer types (Martin-Liberal, et al., 2014, Neuzillet, et al., 2014, Pratilas and Solit, 2010). There are several ongoing clinical drug development programs with ERK inhibitors for the treatment of cancers (Table 2).

Table 2. Ongoing clinical trials with MEK and ERK inhibitors

Inhibitor	*Medical condition*	*Sponsor*
RAF/MEK RO5126766	Solid tumor	Institute of Cancer Research
MEK WX-554	Solid tumor	WILEX AG
MEK PD-0325901	Solid tumor and colorectal cancer	University of Oxford
MEK Selumetinib (AZD6244)	Malignant peripheral nerve sheath tumors	Sarcoma Alliance for Research through Collaboration
MEK Selumetinib (AZD6244)	Neurofibromatosis type 1	National Cancer Institute
MEK Selumetinib (AZD6244)	Lung cancer	AstraZeneca
ERK1/2 BVD-523	Pancreatic cancer	Washington University School of Medicine
ERK1/2 BVD-523	Uveal melanoma	Dana-Farber Cancer Institute
ERK ASTX029	Solid tumor	Astex Pharmaceuticals
ERK1/2 LY3214996	Advanced cancer and metastatic melanoma	Eli Lilly and Co

MEK 1/2 GSK2256098	Pancreatic cancer and adenocarcinoma	University Health Network
MEK162	Low-grade gliomas	Children's Hospital Los Angeles
TAK-580 (MLN2480)	Low-grade gliomas	Karen D. Wright MD
ONC201	Endometrial cancer	Fox Chase Cancer Center

https://www.clinicaltrialsregister.eu, https://clinicaltrials.gov/ (updated 6.4.2019)

1.1.3 Family of JNK

There are three identified JNK isoforms and they are 85 % identical (Johnson and Lapadat, 2002, Kyriakis and Avruch, 2012). JNK1/2 are widely expressed, while JNK3 is located primarily in neuronal tissue, testes and cardiac myocytes (Bogoyevitch, 2006). They differ from each other also in substrate specificity and activation routes. JNKs are activated in response to inflammatory cytokines and cellular stresses (Bogoyevitch, 2006, Kyriakis and Avruch, 2012). MKKKs of the JNK pathway include MEKK1-4, MLK1-3 (mixed lineage kinase), Tpl2, DLK (dual leucine zipper kinase), TAO1/2 (thousand-and-one amino acid), TAK1 (TGFβ-activated kinase), and ASK1/2 (apoptosis signal related kinase) (Johnson and Nakamura, 2007, Kyriakis and Avruch, 2012). They activate MKK4 or MKK7, which are the second-level kinases in the signaling cascade. MKK4/7 phosphorylate their target proteins JNK1-3. JNKs can stimulate many transcription factors in the nucleus, but certain target proteins are found in the cytoplasm also (Lawler, et al., 1998). JNKs can regulate transcription factors c-Jun, ATF-2, p53, Elk-1, and the nuclear factor of activated T cells (NFAT). JNK1/2 have an important role in the control of cytokine production, inflammatory response, cell proliferation, differentiation and apoptosis (Bogoyevitch, 2006).

Both JNK1 and JNK2 KO mice are viable and fertile (Coffey, 2014). JNK1 KO mice have impaired differentiation of CD4+ T helper cells, and they have a skewed Th2 response (Dong, et al., 1998, Dong, et al., 2000). JNK1 seems to be important also in obesity and insulin resistance. Lack of JNK1 leads to decreased adiposity and resistance to high-fat-diet-induced obesity (Hirosumi, et al., 2002). Lack of the JNK2 gene partially protects non-obese diabetic mice from destructive insulitis (Jaeschke,

et al., 2005). Th2 cells protect against type 1 diabetes. JNK2 KO mice have a Th2 pancreatic environment that protects against insulitis and diabetes (Tournier, et al., 2000). JNK1 KO mice and JNK2 KO mice were reported to be resistant to concanavalin A (ConA)-induced hepatitis (Maeda, et al., 2003). Embryonic fibroblasts deficient in both JNK1 and JNK2 are resistant to stress-induced apoptosis (Tournier, et al., 2000). JNK1/2 KO mice are hard to investigate, because lack of JNK1/2 induces embryonic lethality by day 11 and mice have severe deregulation of apoptosis in the brain (Kuan, et al., 1999).

1.2 Family of MAP kinase phosphatases

Mitogen-activated protein kinase phosphatases (MKPs) belong to a larger family of dual specificity phosphatases (DUSPs) that dephosphorylate tyrosine and threonine residues of their target proteins. MKPs are endogenous suppressors of MAPK pathways. MKPs control MAPK activity by dephosphorylating the activation domain (Thr-X-Tyr) of MAPK, thereby inactivating the kinase. MKPs regulate the inflammatory response as well as other cellular functions, including apoptosis, cell cycle, proliferation and differentiation (Boutros, et al., 2008, Korhonen and Moilanen, 2014, Lang, et al., 2006, Patterson, et al., 2009, Salojin and Oravecz, 2007).

Eleven MKPs have been characterized, and they display differences in substrate specificity among MAPKs, as well as in tissue distribution, cellular location and expressional pattern (Boutros, et al., 2008, Caunt and Keyse, 2013). MAPK phosphatases are divided into three groups according to their intracellular location, tissue distribution and substrate specificity (Lang, et al., 2006, Salojin and Oravecz, 2007). The first group includes the nuclear proteins DUSP1/MKP-1, DUSP2, DUSP4/MKP-2 and DUSP5. The second group includes cytosolic ERK-specific phosphatases DUSP6/MKP-3, DUSP7 and DUSP9/MKP-4. The rest of the MPK are both cytosolic and nuclear phosphatases that regulate p38 MAPK and JNKs activity (Korhonen and Moilanen, 2014, Rios, et al., 2014).

1.2.1 MKP-1

MKP-1 [mitogen-activated protein kinase phosphatase 1, also called dual specific phosphatase 1 (DUSP1)] is the best characterized member of the MAP kinase phosphatase family (Wancket, et al., 2012). The DUSP1 gene, which encodes human MKP-1, is located at chromosome five. It consists of four exons and three introns (Kwak, et al., 1994). MKP-1 is expressed in most cell types and tissues in the human body. It is a nuclear tyrosine/threonine phosphatase of 39 kDa in size, and its expression is induced in response to many external signals, including inflammatory cytokines, growth factors and microbe-derived substances (Liu, et al., 2007).

MKP-1 is a regulator in inflammation and immune responses. Mainly, MKP-1 dephosphorylates p38, and in certain cell types, JNKs (Chi, et al., 2006, Zhao, et al., 2006). MKP-1 has been shown to regulate ERK1/2 in certain conditions e.g., in skeletal muscle and myoblasts and in the lungs of LPS -challenged mice, in a situation where MKP-1 is strongly overexpressed (Shi, et al., 2010, Wang, et al., 2009, Wu, et al., 2006).

1.2.1.1 Regulation of MKP-1 expression

Expression/activity of MKP-1 is regulated at transcriptional, post-transcriptional and post-translational levels. Extracellular stimuli, such as growth factors and pro-inflammatory cytokines induce transcription of the MKP-1 gene (Boutros, et al., 2008). The MKP-1 promoter contains binding sites for many transcription factors, including AP-1 (activator protein), NF-κB (nuclear factor kappa B), SP-1 (specificity protein 1) and CAAT-binding transcription factor/nuclear factor 1, CREB (cAMP-responsive element binding protein), E-box, GRE (glucocorticoid responsive element) and VDRE (vitamin D receptor element) (Kwak, et al., 1994, Noguchi, et al., 1993, Shipp, et al., 2010, Sommer, et al., 2000, Wang, et al., 2008, Zhang, et al., 2012).

Stability and translation of a newly synthesized MKP-1 mRNA is regulated by RNA-binding proteins. Factors that bind MKP-1 mRNA and regulate its stability and translation are, e.g., TTP (tristetraprolin), HuR (Hu antigen R) and NF90 (nuclear factor 90) (Kuwano, et al., 2008, Prabhala, et al., 2015). TTP is an important RNA binding protein and it makes a complex with MKP-1 (Prabhala, et al., 2015),

suggesting that TTP is associated with increased MKP-1 protein levels. RNA binding proteins HuR and NF90 have been described as controlling the stabilization and translation of MKP-1 mRNA (Kuwano, et al., 2008). Also, miR-101 inhibits MKP-1 expression, leading to prolonged activation of p38 MAPK and JNKs in LPS-challenged mice. Dexamethasone treatment prevented the expression of miR-101 and subsequently enhanced the expression of MKP-1 after LPS-stimulation (Zhu, et al., 2010). More recently, many other miRNAs including miR-708, miR-200c, and miR-210 have been found to regulate MKP-1 levels (Dileepan, et al., 2014, Jin, et al., 2015, Singh, et al., 2017).

MKP-1 seems to have regulatory functions beyond inflammation. Other physiological and pathophysiological processes where MKP-1 has been suggested to play a role include energy metabolism, muscle cell metabolism, cancer development and atherosclerosis (Boutros, et al., 2008, Flach and Bennett, 2010, Shi, et al., 2010, Wu, et al., 2006). MKP-1 has been linked to the regulation of mood and depression. In a rodent model, increased MKP-1 levels in the hippocampus were associated with depressive behavior, and interestingly, treatment with antidepressants normalized MKP-1 expression in the hippocampus and improved behavior. In addition, mice lacking MKP-1 were resistant to stress-induced depression (Duric, et al., 2010). Further, patients with depression had higher MKP-1 levels in the hippocampus post-mortem (Duric, et al., 2010). In a pain-induced rodent depression model, certain cortical areas displayed increased MKP-1 levels (Barthas, et al., 2017). Genetic disruption of MKP-1 inhibited the development of depressive behavior, and moreover, anti-depressive medication attenuated MKP-1 levels and improved depressive behavior (Barthas, et al., 2017). These results imply that the modulation of MAP kinase pathways may provide interesting drug targets in neurological and psychiatric diseases. Important questions here are which MAP kinases and their isoforms are expressed in the different areas of the brain and how their activity is regulated by MKPs and neuronal mediators.

1.2.1.2 Role of MKP-1 in inflammation and immune responses

MKP-1 is an important regulator in inflammation and immune responses. MKP-1 dephosphorylates p38 MAPK and JNK and thereby deactivates those signaling cascades in cells. Normally, MKP-1 expression is increased after the onset of

inflammatory response and it, in delay, attenuates p38 MAPK signaling and suppresses expression of pro-inflammatory cytokines (such as TNF, IL-1β and IL-6) and chemokines [e.g., MCP-1 (monocyte chemotactic protein-1), MIP-1α (macrophage inflammatory protein-1α), MIP-1β and MIP-2α] thus resulting in the suppression of the acute and chronic inflammatory response (Chen, et al., 2002, Chi, et al., 2006, Hammer, et al., 2006, Korhonen, et al., 2011, Salojin, et al., 2006, Shepherd, et al., 2004, Turpeinen, et al., 2010, Wang, et al., 2007, Zhao, et al., 2006).

MKP-1 KO mice are fertile, they develop normally and they do not differ from ordinary WT counterpart mice in their phenotype in resting conditions. By studying MKP-1 KO mice, it has been found that MKP-1 limits the production of inflammation factors (cytokines and chemokines) and acute inflammation. The inflammatory response in MKP-1 KO mice has been shown to be significantly increased and mortality in different infection and sepsis disease models is clearly increased (Hammer, et al., 2006, Salojin, et al., 2006, Wang, et al., 2007). For example, after LPS-exposure, MKP-1 KO mice have been shown to be more sensitive to the septic shock syndrome than WT mice and they display severe hypotension and respiratory failure, nitric oxide over-production and multi-organ failure, e.g., pulmonary damage (Zhao, et al., 2006). After an LPS challenge, MKP-1 KO mice develop severe acute inflammation and also increased infiltration of leukocytes into inflamed tissues; microvascular damage; failure of kidney, hepatic and pulmonary function; and mortality. LPS injection also causes more severe bone loss and inflammation in MKP-1 KO mice than in WT mice in the periodontitis mouse model (Sartori, et al., 2009).

In ovalbumin-induced airway inflammation, a common experimental asthma model, mice were found to have increased activation of the p38 MAPK pathway and downregulation of MKP-1 expression in the lung tissue. In these mice, treatment with dexamethasone and p38 MAPK inhibitor effectively reduced lung inflammation and inactivated p38 MAPK and upregulated MKP-1 in the lung tissue (Liang, et al., 2013).

Despite excessive inflammatory response, MKP-1 KO mice have reduced IL-12 production and display defective antibacterial responses and impaired Th1 response (Huang, et al., 2011, Korhonen, et al., 2012). In response to LPS and heat-killed Gram-negative staphylococcus aureus, MKP-1 KO mice displayed increased inflammatory cytokine production and mortality (Frazier, et al., 2009). MKP-1 KO

mice had increased production of TNF, IL-6 and IL-10, greater bacterial burden, substantial defects in energy metabolism, and higher mortality in response to Escherichia coli exposure as compared to the WT mice. Treatment of Escherichia coli infection with the bactericidal antibiotic protected WT mice from septic shock but had no effect in MKP-1 KO mice. In bacterial sepsis, MKP-1 has been shown to be an important regulator in the production of cytokines, but also in the control of bactericidal activities of the innate immune system and thus it is part of the control of the metabolic stress response.

MKP-1 has also been shown to be important in arthritis. After collagen immunization, MKP-1 KO mice were reported to develop more severe arthritis than WT mice (Salojin, et al., 2006).

1.2.2 MKP-1 as a drug target

MKP-1 seems to mediate therapeutic effects of certain anti-inflammatory drugs. MKP-1 expression has been shown to be increased by glucocorticoids in some cell types and MKP-1 mediates, at least partly, the anti-inflammatory effects of glucocorticoids (Abraham, et al., 2006, Kassel, et al., 2001, Shipp, et al., 2010). Synthetic glucocorticoid dexamethasone inhibits the phosphorylation of p38 and JNK in bone marrow-derived macrophages from WT mice, while the effect is attenuated in cells from MKP-1 KO mice (Abraham, et al., 2006). Using the KO mice model, MKP-1 has also been shown to mediate the suppression of macrophage functions and systemic inflammation by glucocorticoids (Vandevyver, et al., 2012). Attenuation of ozone-induced airway constriction by glucocorticoids has also been shown to be mediated by MKP-1 in mice (Li, et al., 2011).

The promoter of the MKP-1 gene has been described to contain two binding sites for the transcription factor CREB (cAMP-responsive element binding protein) (Kwak, et al., 1994). cAMP (cyclic adenosine 3´-5´-monophosphate) is an important intracellular second messenger and it has been reported to increase the expression of MKP-1 by activating the PKA (protein kinase A)-CREB pathway (Brion, et al., 2011, Kaur, et al., 2008, Korhonen, et al., 2013, Lee, et al., 2012, Zhang, et al., 2008). Like many G-protein-coupled receptors, the β_2-receptor affects cellular responses through cAMP signaling, and therefore it is possible that a β_2-receptor agonist might enhance MKP-1 expression. Even though β_2-agonists are primarily used as

bronchodilators, they may also have anti-inflammatory effects (Theron, et al., 2013). Earlier it was reported that a long-acting β_2-agonist, formoterol, increased MKP-1 levels in a cAMP-mediated manner via the β_2-adrenergic receptor-PKA pathway in airway smooth muscle cells (Manetsch, et al., 2012).

Our group has previously reported that the disease-modifying anti-rheumatic gold compound aurothiomalate increases the expression of MKP-1, suppresses the phosphorylation of p38 MAPK and reduces the expression of IL-1β-induced IL-6, COX-2 and MMP-3 production in mouse chondrocytes and in the cartilage from rheumatoid arthritis patients. All these effects were impaired in cells where MKP-1 had been silenced by siRNA (small interfering RNA) or in cartilage from MKP-1 KO mice. These results suggest that anti-inflammatory effects of aurothiomalate are mediated, at least partly, by MKP-1 (Nieminen, et al., 2010).

Our research group found that the unselective PDE4 inhibitor rolipram increases MKP-1 levels and suppresses acute inflammatory response in WT mice, but the inhibition of the inflammatory response was severely impaired in MKP-1 KO mice (Korhonen, et al., 2013). This is interesting, because two PDE4 inhibitors have been approved for clinical use. Roflumilast has been approved for the treatment of severe COPD as an add-on therapy to prevent disease exacerbations. Roflumilast is used as an anti-inflammatory drug in COPD and it increases cAMP levels by abrogating the enzymatic degradation of cAMP to 5'AMP like all PDE4 inhibitors (Bateman, et al., 2011, Fabbri, et al., 2009, Patel, et al., 2014). The other PDE4 inhibitor apremilast has been approved for the treatment of plague psoriasis and psoriatic arthritis.

In conclusion, MKP-1 is an important endogenous anti-inflammatory factor included in inflammation. It affects the p38 MAPK and JNK pathways and inflammatory response and is associated with the anti-inflammatory effect of some drugs. Based on published research results, MKP-1 is an interesting target in anti-inflammatory drug development.

1.3 β_2-receptor signaling pathway

Adrenergic receptors couple with catecholamine neurotransmitters (noradrenaline, adrenaline, and dopamine) and mediate their effects in the body. Main adrenergic receptor types are α-adrenoceptors (α_1 and α_2) and β-adrenoceptors (β_1, β_2, and β_3),

and they all belong to the family of G-protein-coupled receptors. Catecholamines are important mediators in the autonomic nervous system, and α- and β-receptors are found in many peripheral tissues throughout the body. All β-receptors activate adenylyl cyclase (AC) and increase cAMP synthesis in target cells, while second messenger pathways for α-receptors typically include $Ca^{2+,}$ DAG, and IP3, and some α-receptor subtypes may also decrease cAMP formation (Strosberg, 1993, Wachter and Gilbert, 2012). More detailed description of the pharmacology of the autonomic nervous system can be found in the pharmacology text books and is out of the scope of this thesis.

1.3.1 β-receptors

β-receptors are divided in three subtypes: β_1, β_2, and β_3. β_1-receptors are found mainly in the heart and there they increase the heart rate and force of contraction (Wallukat, 2002). β_3-receptors are located mainly in adipose tissue and they are involved in the regulation of lipolysis and thermogenesis. β_3-receptors are also found in the gallbladder and urinary bladder. In the urinary bladder, activation of β_3-receptors cause relaxation of the detrusor muscle and β_3-receptor agonists can be used to treat overactive bladder syndrome (Schena and Caplan, 2019). β_2-receptors are found in smooth muscle cells in many organs, such as the bronchi, blood vessels, GI tract, and uterus; and β_2-receptor activation leads to smooth muscle relaxation (Wallukat, 2002). β_2-receptors are also present in the liver, heart and skeletal muscles. β_2-receptors, like all β-receptors, are G protein-coupled receptors, and they activate cAMP production.

Structurally the β_2-receptor is formed of extracellularly located ligand-binding domain, 7-TM (transmembrane) domain and intracellular domain interacting with G-proteins. The β_2-receptor is associated with G_s-protein that activates adenylyl cyclase and increases cAMP production (Cazzola, et al., 2012, Cazzola, et al., 2013, Deupi and Kobilka, 2007). β-subunits of the β_2-receptor-associated G-proteins interact with the β-arresting activating ERK pathway (Rosenbaum, et al., 2009).

1.3.2 cAMP

3´,5´-cyclic adenosine monophosphate (cAMP) is an important intracellular second messenger and a key mediator in many physiological regulatory systems, such as neurotransmission, muscle contraction, intracellular transport mechanisms, and ion fluxes as well as in the regulation of metabolic processes, such as glycogenolysis and lipolysis. It regulates various downstream signaling pathways through direct interaction with proteins. There are three main pathways which can be activated by cAMP: protein kinase A (PKA), exchange protein directly activated by cAMP (Epac) and cyclic nucleotide-gated ion channel (CNG) pathways, which are described below. cAMP is hydrolysed to non-cyclic 5'AMP by phosphodiesterases (PDE), and this is a mechanism to cease cAMP signaling (Serezani, et al., 2008).

1.3.3 PKA-CREB

Protein kinase A (PKA) was the first characterized direct effector of cAMP (Walsch 1968). Inactive PKA consists of two regulatory and two catalytic subunits. cAMP binds to the regulatory subunits of PKA, causing a conformational change which leads to dissociation of catalytic and regulatory subunits enabling PKA catalytic subunits to phosphorylate its target proteins (Serezani, et al., 2008, Taylor, et al., 2008).

PKA catalyzes phosphorylation of serine and threonine residues and regulates several physiological and pathological processes, e.g., cell metabolism and proliferation, differentiation, memory, inflammation and carcinogenesis (Taylor et al., 2013, Vandamme et al., 2012). Activation of PKA also causes hydrolysis of inositol phosphates and the reduction of Na^+/Ca^{2+} exchange, which leads to decreased intracellular Ca^{2+} concentration. PKA also activates Na^+/K^+-ATPase and K^+ channels and inactivates the activity of myosin light chain kinase (MLCK) (Taylor, et al., 2013).

cAMP response element binding protein (CREB) is one the main substrates of PKA (Mayr and Montminy, 2001). PKA can activate CREB by phosphorylation of serine and threonine residues. Phosphorylated CREB can bind to cAMP-responsive elements (CRE) in the promoter regions of responsive genes and activate gene transcription.

1.3.4 Epac

One of the latest effectors which have been found to be regulated by cAMP is the exchange protein directly activated by cAMP (Epac). Epacs were discovered in 1998, when it was found that the activation of Rap1 was insensitive to the inhibition of PKA (de Rooij, et al., 1998). Two isoforms, Epac1 and Epac2, were identified (Schmidt, et al., 2013, Shirshev, 2011). The main downstream effectors of Epacs are the Ras-like small G-protein Rap1/2 and RRas (de Rooij, et al., 1998, Enserink, et al., 2002, Rehmann, et al., 2003). Epac1 is expressed in several inflammatory cells, such as macrophages, B cells, T cells and eosinophils. Lower Epac1 expression has been observed in the lung tissue in COPD patients. Epac2 is expressed in the adrenals, brain and pancreatic β cells (Oldenburger, et al., 2012).

1.3.5 Cyclic nucleotide-gated ion channels

Cyclic nucleotide-gated (CNG) ion channels are nonselective cation channels, which are present in the membranes of many cell types. They play a role in sensory nerve transduction, e.g., vision and olfaction as well as other cellular functions, such as hormone release and chemotaxis. They belong to the class of ligand-gated cation channels and are activated by direct binding of cAMP or cGMP (cyclic guanosine monophosphate) and induce either depolarization or hyperpolarization in the target cell. CNG channels are multi-subunit channels and they are important in the cellular control of the membrane potential and intracellular Ca^{2+} concentration. (Grandoch, et al., 2010, Shabb, 2001)

1.3.6 β_2-receptor agonists

β_2-receptor agonists are used as bronchodilator drugs for the treatment of obstructive lung diseases, such as asthma and COPD. Short-acting β_2-agonists (salbutamol, terbutaline, and fenoterol) are used for acute bronchospasm to treat and prevent asthma attacks and exercise-induced bronchoconstriction and for the long-term treatment of symptoms of mild to moderate asthma. Long acting β_2-agonists (formoterol, salmeterol and indacaterol) are used in addition to glucocorticoid therapy in moderate to severe asthma and COPD. β_2-agonists prevent

and reduce bronchoconstriction regardless of its cause. (Cazzola, et al., 2013, Theron, et al., 2013)

Besides the smooth muscle relaxing effects of β_2-agonists, they have also been reported to have anti-inflammatory effects on inflammatory cells (Manetsch, et al., 2012, Theron, et al., 2013). β_2-agonists also reduce the degranulation of mast cells and eosinophils (Ezeamuzie and al-Hage, 1998, Hallsworth, et al., 2001). They reduce the release of inflammatory mediators and the exudation of plasma contributing to the formation of the inflammatory edema (Vida, et al., 2011).

β_2-agonists have beneficial effects, e.g., attenuation of pro-inflammatory activity and recruitment of neutrophils in an experimental acute lung injury model (Bosmann, et al., 2012, Theron, et al., 2013). In an experimental study, they have been reported to inhibit the expression of inflammatory mediators and to decrease capillary permeability and formation of plasma exudate and tissue edema (Baouz, et al., 2005, Vida, et al., 2011). Salbutamol has also been shown to reduce carrageenan-induced paw edema in rats and this effect was attenuated by a non-selective β-receptor antagonist, propranolol (Uzkeser, et al., 2012).

In addition, pretreatment with inhaled salmeterol reduced inhaled LPS-induced inflammatory response, e.g., neutrophil influx and degranulation, and TNF release in human lungs (Maris, et al., 2005). Isoproterenol/isoprenaline is a non-selective β-agonist, and it reduced early inflammation markers in human. Stimulated production of MIP-1 and plasma sCD40L were inhibited by isoproterenol infusion (Edwards, et al., 2007). Also, inhaled salbutamol reduced inflammatory mediators in exhaled breath condensates in acute lung injury patients (Nabe, 2014). The long-acting β_2-agonist olodaterol attenuated the influx of neutrophils and release of pro-inflammatory mediators in murine and guinea pig models of lung inflammation induced by cigarette smoke. Additionally, olodaterol attenuated inflammation through mechanisms that are not directly related to inhibition of bronchoconstriction (Wex, et al., 2015).

It has been previously shown that β_2-receptor activation can lead to the activation of the ERK signaling pathway (Azzi, et al., 2003, Lefkowitz and Shenoy, 2005, Rosenbaum, et al., 2009, Shenoy, et al., 2006). Repetitive or prolonged G-protein-receptor activation suppresses receptor signaling through activation of the G-protein-β-arrestin pathway, an intracellular accessory protein associated with

GPCRs. The G protein-coupled receptor kinase phosphorylates the β_2-receptor, leading to its coupling with β-arrestin. β-arrestin directs the β_2-receptor to inactivation and internalization from the cell surface, but it also serves as an activation signal for the ERK pathway leading to ERK phosphorylation. Receptor-β-arrestin complex binds Raf kinase leading to the activation of MEK and subsequently to ERK phosphorylation. These events are referred as G-protein independent signaling of GPCRs (Lefkowitz and Shenoy, 2005, Rosenbaum, et al., 2009). Therefore, modulation of ERK phosphorylation by β_2-agonists may contribute to their observed clinical efficacy.

The anti-inflammatory mechanisms of β_2-agonists are yet not fully clarified. Combinations of β_2-agonist bronchodilatory and anti-inflammatory properties improve the value of these drugs in the treatment of acute and chronic lung diseases**.**

1.3.7 Phosphodiesterase 4 (PDE4)

PDEs are a large family of enzymes that hydrolyze cAMP and cGMP into inactive non-cyclic mononucleotides. There are 11 different PDE families, which each have different isoforms and a plurality of splicing variants (Bender and Beavo, 2006, Francis, et al., 2011). They differ from each other by their substrate specificities, cyclic nucleotide affinities, regulatory mechanisms and tissue distribution. Functionally, PDEs are divided into three groups by their substrate specificities. PDE4, PDE7and PDE8 are cAMP-specific while PDE5, PDE6 and PDE9 are specific for cGMP and the rest of PDE can metabolize both cAMP and cGMP.

PDE4 is a cAMP-specific phosphodiesterase and it is expressed in the brain, inflammatory cells, such as neutrophils, macrophages and T cells, cardiovascular tissues, and smooth muscle. Four PDE4 genes, namely PDE4A, PDE4B, PDE4C and PDE4D have been identified (Lugnier, 2006).

PDE4 also regulates inflammatory responses. PDE4 KO mice have delayed growth and reduced viability and female fertility (Jin, et al., 1999). Different functions for distinct PDE4 genes have been revealed in studies with PDE4 KO mice. For example, PDE4B seems to be an important factor for TNF production. In PDE4B KO mice, LPS caused only moderate TNF production (Jin and Conti, 2002). In PDE4D KO mice, it

was found that PDE4D plays a major role in β_2-receptor signaling (Bruss, et al., 2008). cAMP accumulation was clearly increased in mouse embryonic fibroblasts from PDE4D KO mice. Return to basal levels after cAMP accumulation was also frequently severely delayed in PDE4D KO cells. This suggests that PDE4 could be important in dissipation of the cAMP. In this study PDE4D was the major form contributing to the shape of β_2-receptor signals by controlling β_2-receptor coupling to G-protein. PDE inhibitors reduce cAMP inactivation and thus increase intracellular cAMP concentration and prolong cAMP signaling in cells.

PDE4 inhibitors suppress the activity of inflammatory cells by preventing cAMP degradation to non-cyclic mononucleotides. Theophylline is a weak and non-selective phosphodiesterase inhibitor, which has long been used in the treatment of lung diseases. Rolipram is a classical, selective PDE4 inhibitor that has been shown to have anti-inflammatory effects (Kanehiro, et al., 2001, Mendes, et al., 2009). Our research group reported that rolipram suppressed inflammatory response in wild-type mice, but the response was clearly impaired in MKP-1 KO mice (Korhonen, et al., 2013). Roflumilast was the first selective PDE4 inhibitor approved for clinical use and it is used as an anti-inflammatory drug in COPD. It reduces the number of neutrophils, eosinophils and lymphocytes and the levels of inflammatory mediators in the airways, and prevents the clinical exacerbations of COPD (Calverley, et al., 2007, Calverley, et al., 2009, Fabbri, et al., 2009, Rabe, et al., 2005). Another PDE4 inhibitor, apremilast, has been approved [by FDA (Food and Drug Administration) and EMA (European Medicines Agency)] for the treatment of psoriasis and psoriatic arthritis. Apremilast reduced the symptoms and was also found to inhibit the production of inflammatory factors in synovial tissue of rheumatoid arthritis patients (Crilly, et al., 2011).

2 AIMS OF THE STUDY

MKP-1 is a physiological suppressor of MAPK pathway, and it has important anti-inflammatory effects. For these reasons, treatment modalities that enhance MKP-1 expression and/or its function may provide possibilities for modulating inflammation and reducing its complications, such as tissue injury, pain and disability. The aim of the present thesis project was to identify compounds and mechanisms that modulate the expression and/or the function(s) of MKP-1 and to investigate the role of MAP kinases and MKP-1 in inflammatory responses *in vitro* and *in vivo*. Also, intracellular signaling pathways involved in the expression of MKP-1 and the pharmacological modulation of those cascades were investigated. In the studies, cAMP-enhancing β_2-agonists and glucocorticoids were of particular interest because the cAMP responsive element and the glucocorticoid response element have been identified in the MKP-1 promoter.

Hypotheses were:

1. β_2-agonists increase MKP-1 expression and attenuate inflammatory gene expression. (I, III)
2. β_2-agonists modulate inflammatory response by regulating the activity of MAPK pathways p38 and ERK. (I, III)
3. MKP-1 suppresses the development of experimental inflammation in mice. β_2-agonists (which enhance MKP-1 expression) attenuate experimental inflammation, and those effects are impaired in MKP-1 KO mice. (I)
4. Glucocorticoids increase MKP-1 levels and MKP-1 mediates the anti-inflammatory effects of glucocorticoids in human lung epithelial cells. (II)

3 METHODS

3.1 Materials

3.1.1 Chemicals

Axon Medchem BV (Groningen, the Netherlands)

- p38 MAPK inhibitor BIRB 769 [1-(5-tert-butyl-2-p-tolyl-2H-pyrazol-3-yl)-3(4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl)urea]
- MEK1/2 inhibitor PD 0325901 (N-((2R)-2,3-Dihyroxypropoxy)-3,4-difluoro-2-((2-fluoro-4-iodophenyl)amino)-benzamide)
- PDE4 inhibitor Rolipram [4-(3-cyclopentyloxy-4-methoxy-phenyl)-pyrrolidin-2-one]

R&D systems Inc. (Minneapolis, Mass, USA)

- Recombinant human IL-1β

All other chemicals were provided by Sigma-Aldrich Inc. (St. Louis, MO, USA) unless otherwise indicated.

3.1.2 Antibodies

The following antibodies were used in the Western blot analysis:

Santa Cruz Biotechnology, CA, USA

- MKP-1 sc-1102
- Actin sc-1616 and sc-1615
- Polyclonal anti-goat sc-2020
- Polyclonal anti-rabbit sc-2004

Sigma-Aldrich Inc., St. Louis, MO, USA

- MKP-1 SAB2500331

Abcam, Cambridge, UK

- p38 MAPK ab27986

Cell Signaling Technology Inc., Beverly MA, USA

- phospho-p38 MAPK #9211
- ERK1/2 #9102
- phospho-ERK1/2 #9101

3.2 Experimental animals

Animal experiments were carried out in WT (wild-type) and MKP-1 KO (knockout) C57BL/6-mice (Scanbur, Copenhagen, Denmark). The MKP-1 KO mice were originally generated in the laboratory of R. Bravo at Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ, USA) (Dorfman, et al., 1996) and those as well as corresponding WT mice were bred at the University of Tampere School of Medicine animal facilities under conditions of optimum light (12:12 light-dark cycle), temperature (+22 ± 1 °C) and humidity (50-60 %), and food and water provided *ad libitum*. The study was carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and the Finnish Act on Animal Experimentation. The studies were approved by the National Animal Experiment Board (ESAVI/5019/04.10.03/2012).

3.2.1 Carrageenan-induced paw edema

MKP-1 deficient and wild-type C57BL/6 mice (20-25 g) were divided into groups of six mice and treated with 200 µl of PBS or salbutamol (5 mg/kg in PBS) (Saleh, et al., 1996, Zarrindast, et al., 2002) by an intraperitoneal (i.p.) injection 2 h before applying carrageenan. Before the administration of carrageenan, the mice were anaesthetized by i.p. injection of 0.5 mg/kg of medetomidine (Domitor® 1 mg/ml; Orion Oyj, Espoo, Finland) and 75 mg/kg of ketamine (Ketalar® 10 mg/ml; Pfizer Oy Animal Health, Helsinki, Finland). The mice received a 45 µl intradermal (i.d.) injection of λ-carrageenan (2 % dissolved in normal saline) in one hind paw. The contralateral paw received 45 µl of the saline and it was used as a control. Paw volume was measured before and after (2 h, 4 h and 6 h) the carrageenan injection with a plethysmometer (Ugo Basile, Comerio, Italy). Edema is expressed as the

difference between the volume changes of the carrageenan-treated paw and the control paw.

3.3 Cell culture

3.3.1 J774 and A549 cell lines

J774 mouse macrophages [ATCC (American Type Culture Collection), Rockville Pike, MD, USA] were cultured at +37 °C in 5 % CO_2 atmosphere using Dulbecco´s Modified Eagle´s Medium containing glutamax-1 (DMEM; Invitrogen, Paisley, UK) supplemented with 10 % (v/v) heat-inactivated FBS (fetal bovine serum), 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (all from Gibco, Wien, Austria). For experiments, cells (2.5 x 10^5 cells/well) were seeded on 24-well plates and the cell monolayers were grown for 72 h before the experiments were started.

A549 human lung epithelial cells (ATCC, Manassas, VA, USA) were cultured at +37 °C in 5 % CO_2 atmosphere in Ham´s F-12K (Kaighn´s modification) medium supplemented with 5 % (v/v) heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (all from Invitrogen, Paisley, UK). For experiments, cells (4 x 10^5 cells/well) were seeded on 24-well plates and the cell monolayers were grown for 48 h before the experiments were started.

For siRNA experiments, A549 human lung epithelial cells were seeded at the density of 5 x 10^4 cells/well on a 24-well plates in 500 µl of Ham´s F-12K (Kaighn´s modification) medium with 5 % (v/v) heat-inactivated FBS without antibiotics followed by transfection with non-targeting control siRNA (siNEG) or MKP-1 siRNA pool (siMKP-1). Cells were incubated for 24 h before transfection and for additional 24 h before the experiments were started.

Salbutamol, terbutaline, rolipram, BIRB 769 and PD 0325901 were dissolved in dimethyl sulfoxide (DMSO), and 8-Br-cAMP (8-bromoadenosine 3´-5´-cyclic monophosphate), dexamethasone, LPS and IL-1β in PBS (phosphate buffered saline). LPS (10 ng/ml) and/or the other compounds of interest at the concentrations indicated or the solvent (DMSO, 0.1 % v/v) were added to the cells

in fresh culture medium containing 10 % (J774) or 5 % (A549) FBS and the supplements. Cells were then incubated for the time indicated.

3.3.2 Isolation and culture of peritoneal macrophages

Mice were killed by suffocation with CO_2, followed by an immediate cervical dislocation. Primary mouse peritoneal macrophages (PMs) were obtained by intraperitoneal (i.p.) lavage with sterile PBS supplemented with 0.2 mM EDTA (ethylenediaminetetraacetic acid). Cells were washed, resuspended in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 2 % heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and seeded on 24-well plates (5×10^5 cells per well). The cells were incubated overnight and washed with PBS to remove non-adherent cells before the experiments. Pharmacological compounds or the solvent (DMSO, 0.1 % v/v) were added to the cells in fresh culture medium containing 2 % heat-inactivated FBS and the antibiotics (see above), and cells were stimulated with LPS (10 ng/ml) for the time indicated.

3.3.3 Cell viability

Cell viability was evaluated by modified XTT test (Cell Proliferation Kit II; Roche Diagnostics, Mannheim, Germany).

3.4 siRNA

MKP-1 siRNA SMARTpool (5´-AGGAGGAUACGAAGCGUUU-3´, 5´-UUUGUGAAGCAGAGGCGAA-3´, 5´-GCUUACCUUAUGAGGACUA-3´ and 5´-CCAACCAUUUUGAGGGUCA-3´) was purchased from Dharmacon (Lafayette, CO, USA). Nontargeting control siRNA (5´-AATTCTCCGAACGTGTCACGT-3´) was obtained from Qiagen (Valencia, CA, USA).

A549 cells were transfected with siRNA using Dharmafect transfection reagent (Dharmacon) according to the manufacturer´s instruction. Fresh cultured medium was changed after 24 h transfection, and cytokines and dexamethasone were added into the culture medium. Cells were further incubated for the time indicated and

gene expression was analyzed. Transfection efficacy was monitored with green fluorescent siRNA oligos (siGLO green transfection indicator; Dharmacon RNA Technologies). Approximately 90 % of the cells emitted a green fluorescence signal when transfected with siGLO and Dharmafect.

3.5 Preparation of cell lysates and western blotting

At the indicated time points, culture medium was removed. Cells were rapidly washed with ice-cold PBS and solubilized in a cold cell lysis buffer containing 10 mM Tris-HCL, 5 mM EDTA, 50 mM NaCl, 1 % Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 µM n-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged (12,000 g, 10 min, +4 °C) and supernatants were collected and mixed in a 1:4 ratio with a SDS (sodium dodecyl sulfate) loading buffer (62.5 mM Tris-HCL, pH 6.8, 10 % glycerol, 2 % SDS, 0.025 % bromophenol blue and 5 % β-mercaptoethanol) and stored at -20 °C until analyzed. Protein concentrations in the samples were measured by the Coomassie Brilliant Blue method (Coomassie Protein Assay Reagent Kit; Pierce, Rockford, IL, USA) (Bradford 1976).

Before Western blot analysis, the samples were boiled for 5 min. Equal aliquots of protein (20 µg) were loaded on 12 % SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred by semi-dry electroblotting to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) or by iBlot® Dry Blotting System (Life Technologies, Carlsbad, California) following manufacturer's instructions. After transfer the membrane was blocked in TBS/T [20 mM Tris-base (pH 7.6), 150 mM NaCl, 0.1 % Tween-20] containing 5 % nonfat milk for 1 h at room temperature. For detection of phosphorylated proteins, membranes were blocked in TBS/T containing 5 % bovine serum albumin (BSA). Membranes were incubated overnight at +4 °C with the primary antibody and at room temperature for 1 h with the secondary antibody, and the chemiluminescent signal was detected by ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The chemiluminescent signal was quantified with FluoChem program (version 3.1) and Image Quant TL 7.0 Image Analysis software.

3.6 RNA extraction and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

At the indicated time points, the culture medium was removed, and cell homogenization and total RNA extraction were carried out by using GenEluteTM Mammalian Total RNA Miniprep Kit according to the manufacturer´s instructions. Reverse transcription of RNA to cDNA was performed by TaqMan® Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA, USA), according to the supplier´s instructions. The primer and probe sequences and concentrations were optimized according to the manufacturer´s guidelines in TagMan® Universal PCR Master Mix Protocol part number 4304449 revision C (Applied Biosystems, Branchburg, NJ, USA). The primer and probe sequences are given in Table 3.

Primers and probes (Table 3) were obtained from Metabion (Martinsried, Germany). Expression of mouse TNF and MCP-1 mRNA and human MKP-1, TNF and MIP-3α mRNA were measured using TagMan gene expression assays (Applied Biosystems, Foster City, CA, USA).

PCR reaction parameters were as follows: incubation at +50 °C for 2 min and at +95 °C for 10 min, and thereafter 40 cycles of denaturation at +95 °C for 15 s and annealing and extension at +60 °C for 1 min. A standard curve method was used to estimate the relative mRNA levels. When calculating the results, MKP-1, TNF, MCP-1 and MIP-3α mRNA levels were first normalized against GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Table 3. Primers and probes

	Gene	*Sequence 5´-..-3´*	*Oligonucleotide*
Mouse	MKP-1	AAGGATGCTGGAGGGAGAGT	forward
		TGAGGTAAGCAAGGCAGATGGT	reverse
		TTTGTTCATTGCCAGGCCGGCAT	probe
	TNF	AATGGCCTCCCTCTCATCAGTT	forward
		TCCTCCACTTGGTGGTTTGC	reverse
		CTCAAAATTCGAGTGACAAGCCTGTAGCCC	probe
	GAPDH	GCATGGCCGGCCGTGTTC	forward
		GATGTCATCATACTTGGCAGGTTT	reverse
		TCGTGGATCTGACGTGCCGCC	probe
Human	GAPDH	AAGGTCGGAGTCAACGGATTT	forward
		GCAACAATATCCACTTTACCAGAGTTAA	reverse
		CGCCTGGTCACCAGGGCTGC	probe

3.7 Enzyme-linked immunosorbent assay (ELISA)

Culture medium samples and cell lysates were kept at -20°C until assayed.

Cytokine concentrations in the culture medium and cAMP levels in cell lysates were measured by

R&D Systems Europe Ltd., Abindgon, UK Duoset® ELISA Development System

- Mouse TNF
- Mouse MCP-1
- Human TNF
- Human MIP-3α

Cell Biolabs, Inc. San Diego, CA, USA

- Mouse cAMP

3.8 Statistics

Results are expressed as mean ± standard error of the mean (S.E.M.). Statistical analyses (one-way ANOVA with Dunnett´s or Bonferroni´s post-test or two-way ANOVA with Bonferroni´s post-test) were performed with GraphPad Prism-5 (GraphPad Software Inc., La Jolla, CA, USA). P values less than 0.05 were considered significant.

4 RESULTS

4.1 β_2-receptor agonists and dexamethasone inhibited the phosphorylation of p38 MAPK (I-II)

Mitogen-activated protein kinase (MAPK) pathways are regulators of immune response and inflammation and they control the production of several inflammatory factors. Therefore, the effect of β_2-agonists and dexamethasone on p38 MAPK phosphorylation (i.e. activation) was investigated.

The phosphorylation of p38 MAPK was increased in response to LPS in J774 cells and it was inhibited by the β_2-agonists salbutamol (Figure 2A) and terbutaline (Figure 2B). Similarly, p38 MAPK phosphorylation was increased in by IL-1β in A549 cells and inhibited by dexamethasone (Figure 3).

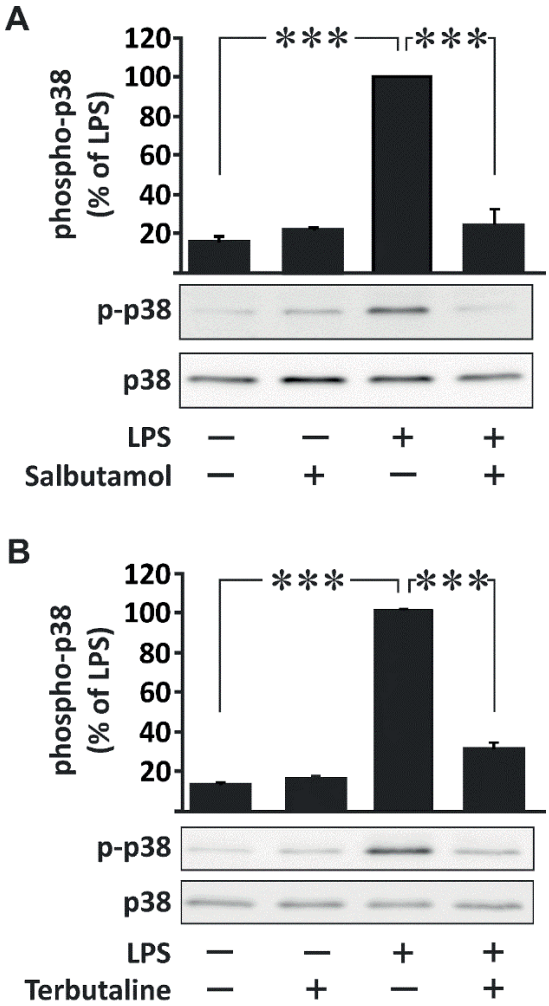


Figure 2. Salbutamol and terbutaline reduced the phosphorylation of p38 MAPK in activated J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of salbutamol (100 nM, A) or terbutaline (100 nM, B) for 1 h, and the phosphorylation of p38 MAPK was detected by Western blot. The chemiluminescent signal was quantified, and phosphorylated p38 MAPK was normalized against total p38 MAPK. Results are expressed as percentages in comparison with LPS. Values are presented as mean ± S.E.M, n=8. One-way ANOVA with Bonferroni´s post-test was performed, and statistical significance is indicated with *** $p < 0.001$. Reprinted with permission from Keränen et al. Plos One. (2016) 11(2):e0148144.

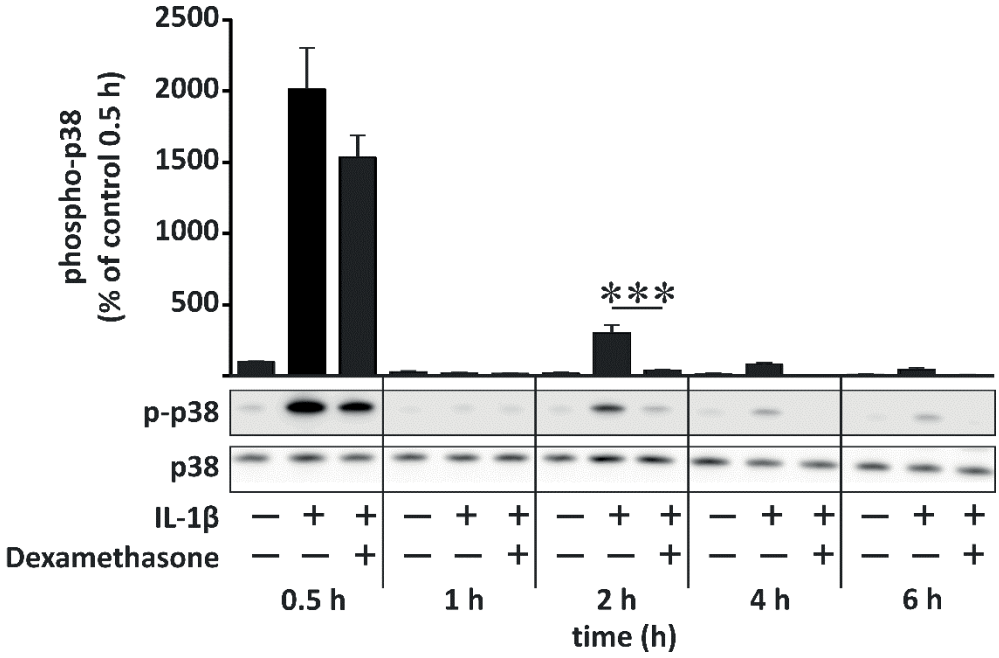


Figure 3. Dexamethasone reduced the phosphorylation of p38 MAPK in A549 lung epithelial cells. A549 cells were stimulated with IL-1β (10 ng/ml) in the absence or in the presence of dexamethasone (1 μM) for the time indicated, and the phosphorylation of p38 MAPK was detected by Western blot. The chemiluminescent signal was quantified, and phosphorylated p38 MAPK was normalized against total p38 MAPK. Results are expressed as percentages in comparison with control at 0.5 h. Values are presented as mean ± S.E.M, n=4. One-way ANOVA with Bonferroni´s post-test was performed, and statistical significance is indicated with *** $p < 0.001$. Reprinted with permission from Keränen et al. Inflammation Research, (2017) 66(5):441-44.

4.2 β_2-receptor agonists and dexamethasone enhanced MKP-1 expression (I-II)

The MKP-1 promoter contains cis-regulatory cAMP-responsive element (CRE) sequences (Kwak, et al., 1994, Noguchi, et al., 1993) and therefore cAMP-elevating compounds would potentially regulate MKP-1 expression.

In this study, we first investigated the effects of the β_2-agonist salbutamol on the intracellular cAMP levels in J774 macrophages, and an increasing effects was found as expected (Table 4).

Table 4. Salbutamol increased intracellular cAMP levels in J774 macrophages. J774 macrophages were incubated with salbutamol and stimulated with LPS for 1 min. Cells were then lysed and cAMP levels were measured by ELISA. Results are expressed as mean ± S.E.M, n=5. One-way ANOVA with Bonferroni´s post-test was performed, and statistical significance is (a) $p < 0.001$ between untreated cells and cells treated with salbutamol and (b) $p < 0.001$ between LPS-treated cells and cells treated with the combination of LPS and salbutamol. Reprinted with permission from Keränen et al. Plos One. (2016) 11(2):e0148144.

The effect of salbutamol on cAMP levels in J774 macrophages		
treatment	**cAMP (pg/ml)**	
Control	1.08 ± 0.8	
Salbutamol 100 nM	1230.9 ± 251.8	a
LPS 10 ng/ml	20.6 ± 11.1	
LPS + Salbutamol	730.5 ± 246.5	b

Thereafter, the effect of salbutamol was investigated on MKP-1 expression in J774 macrophages. MKP-1 expression was transiently increased following exposure to LPS, reaching its maximum level at 1 h and declining near to the basal level at 2 h. Salbutamol increased MKP-1 expression in a statistically significant manner at 0.5-1.5 h time points. (Figure 4)

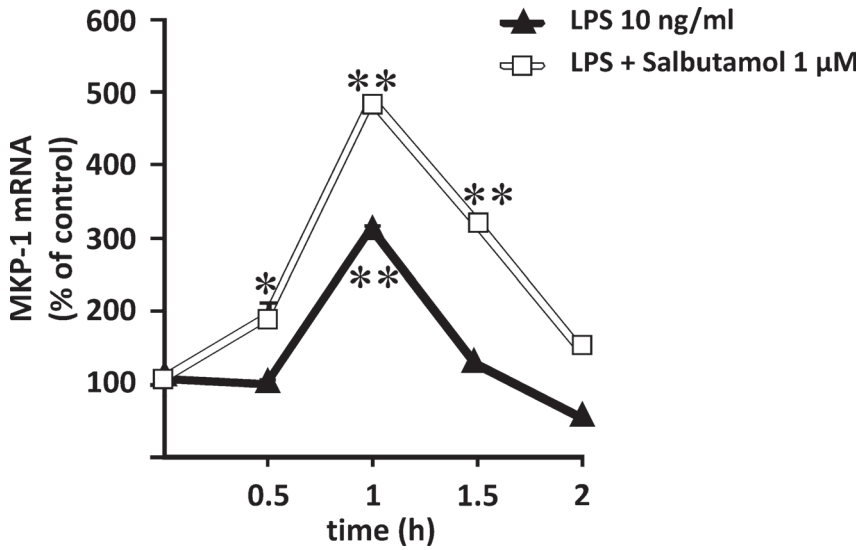


Figure 4. Salbutamol enhanced MKP-1 expression in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the presence or in the absence of salbutamol for the time indicated. MKP-1 mRNA was measured by quantitative RT-PCR, and MKP-1 mRNA expression levels were normalized against GAPDH mRNA levels. Results are expressed as mean ± S.E.M., n=3. One-way ANOVA with Dunnett´s post-test was performed, and statistical significance is indicated with * $p < 0.05$ and ** $p < 0.01$ between LPS and control or LPS + Salb and LPS. Reprinted with permission from Keränen et al. Plos One. (2016) 11(2):e0148144.

The MKP-1 promoter has been reported to contain glucocorticoid response element regions (Shipp et al., 2010). Therefore we aimed to investigate the effects of dexamethasone on MKP-1 expression in A549 human lung epithelial cells. As shown in Figure 5, dexamethasone increased and prolonged MKP-1 expression in A549 cells exposed to the inflammatory cytokine IL-1β.

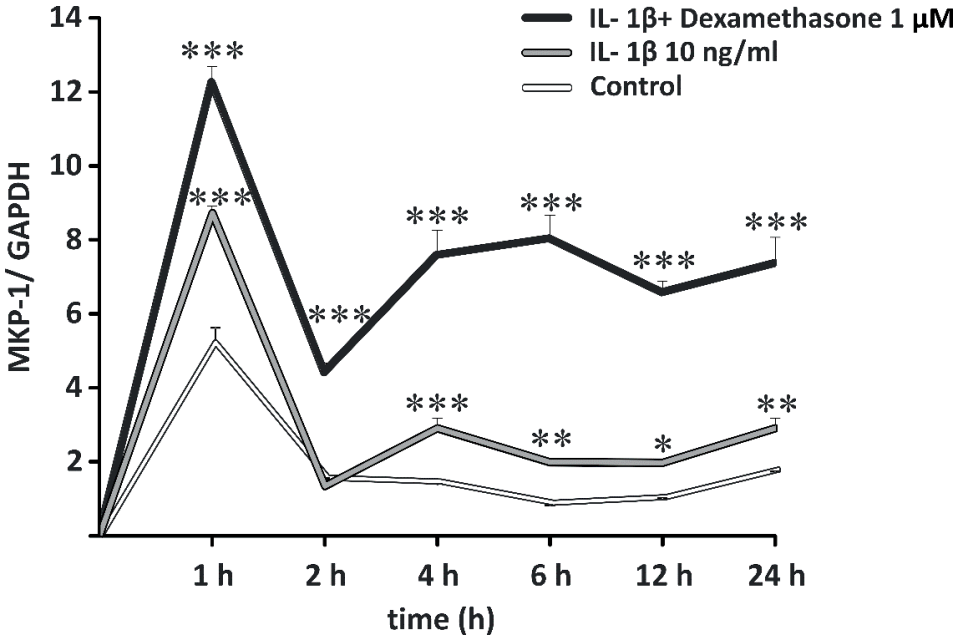


Figure 5. Dexamethasone enhanced MKP-1 mRNA expression in A549 cells. A549 cells were stimulated with IL-1β in the presence or in the absence of dexamethasone for the time indicated. MKP-1 mRNA was measured by quantitative RT-PCR, and MKP-1 mRNA expression levels were normalized against GAPDH mRNA levels. Results are expressed as mean ± S.E.M., n=4. One-way ANOVA with Bonferroni´s post-test was performed, and statistical significance is indicated with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between IL-1β and control or IL-1β + Dexa and IL-1β. Reprinted with permission from Keränen et al. Inflammation Research, (2017) 66(5):441-44.

4.3 β_2-receptor agonists and dexamethasone inhibited cytokine TNF and chemokine MIP-3α production (I-II)

TNF is a key inflammatory cytokine regulated by MKP-1 and p38 MAPK (Korhonen, et al., 2013, Lasa, et al., 2002). MIP-3α is a potent chemokine present in inflamed tissue, and its expression is increased by inflammatory signals (Power, et al., 1997, Reibman, et al., 2003, Schutyser, et al., 2003). Therefore, the effects of β_2-agonists and dexamethasone on TNF and MIP-3α production were investigated. Salbutamol, terbutaline and dexamethasone inhibited the production of TNF and MIP-3α in J774 (Figure 6) and A549 (Figure 7) cells. TNF production was further inhibited when salbutamol or terbutaline was combined with the PDE4 inhibitor rolipram (Figure 6). cAMP analog 8-Br-cAMP also inhibited TNF release (Figure 6). In addition, the p38 MAPK inhibitor BIRB 796 inhibited LPS- or IL-1β-induced TNF (Figure 7A) and MIP-3α (Figure 7B) release.

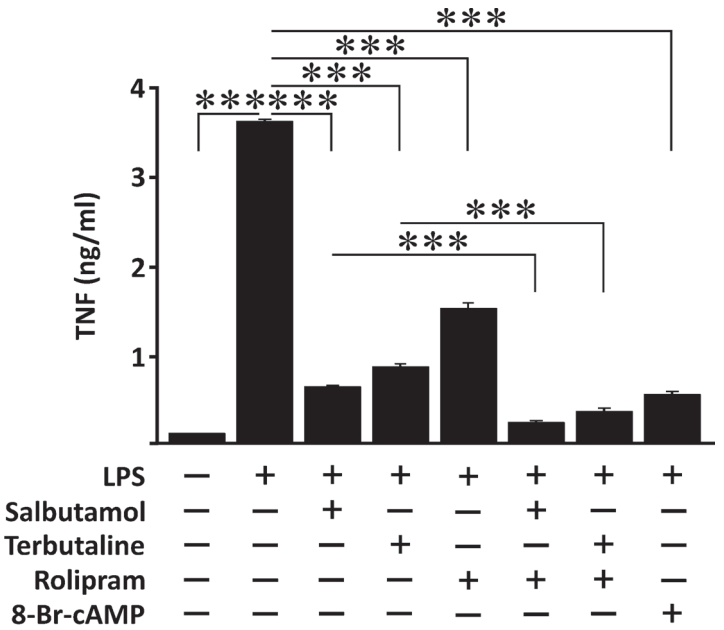


Figure 6. Salbutamol, terbutaline, rolipram and 8-Br-cAMP inhibited TNF production in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of salbutamol (100 nM), terbutaline (100 nM), rolipram (1 μM) or 8-Br-cAMP(1000 μM) for 24 h. TNF protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M., n=8. One-way ANOVA with Bonferroni´s post-test was performed and statistical significance is indicated with *** $p < 0.001$.

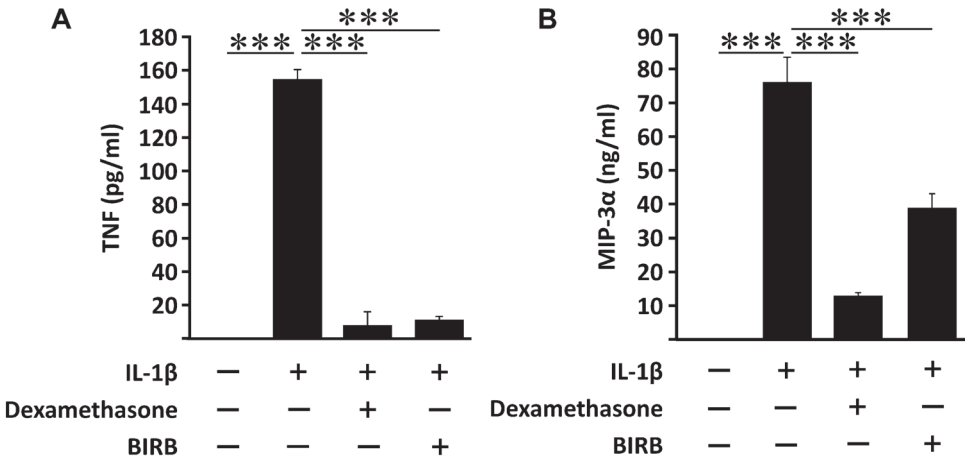


Figure 7. Dexamethasone and the p38 MAPK inhibitor BIRB 769 inhibited TNF (A) and MIP-3α (B) production in A549 cells. A549 cells were stimulated with IL-1β (10 ng/ml) in the absence or in the presence of dexamethasone (1 μM) or the p38 MAPK inhibitor BIRB 769 (100 nM) for 24 h. TNF (A) and MIP-3α (B) protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M., n=4. One-way ANOVA with Bonferroni´s post-test was performed and statistical significance is indicated with *** $p < 0.001$. Reprinted with permission from Keränen et al. Inflammation Research, (2017) 66(5):441-44.

4.4 The inhibition of carrageenan-induced paw inflammation by salbutamol was mediated by MKP-1 (I)

The effect of salbutamol was tested on the severity of carrageenan-induced paw inflammation in wild-type (Figure 8A) and MKP-1 KO (Figure 8B) mice. Carrageenan-induced paw edema was increased in MKP-1 KO mice as compared to WT mice (AUC values were 186.7 ± 13.1 µL/h and 236.7 ± 15.9 µL/h in WT and MKP-1 KO mice, respectively, p=0.0352). Carrageenan-induced paw edema was clearly attenuated by salbutamol in WT mice (73 % reduction in AUC value), while salbutamol was less effective to reduce paw edema in MKP-1 KO mice (43 % reduction in AUC value) (Figure 8C). This result indicates that MKP-1 is involved in the anti-inflammatory effects of salbutamol.

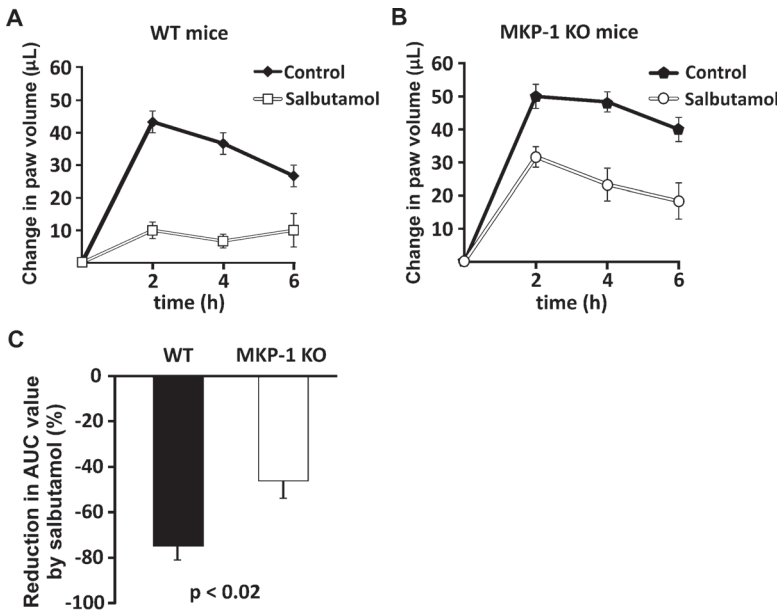


Figure 8. Salbutamol attenuated carrageenan-induced paw inflammation in mice. Mice were treated with salbutamol (5 mg/kg, i.p.) 2 h before the experiment. In the beginning of the experiment (0 h), hind paw volumes were measured with a plethysmometer. After that, carrageenan (2 %; index paw) or vehicle (contralateral paw) was injected into the paw and the paw edema was measured with plethysmometer at the time points indicated. Edema is expressed as the difference between the volume changes of the carrageenan-treated paw and the vehicle-treated paw. Mean ± S.E.M., n=6. The figure 8C presents the percent decrease in AUC values by salbutamol in WT vs. MKP-1 KO mice. Reprinted with permission from Keränen et al. Plos One. (2016) 11(2):e0148144.

4.5 MKP-1 regulated the inhibition of TNF production by dexamethasone (II)

To investigate the role of MKP-1 in the inhibition of TNF production by dexamethasone, MKP-1 was silenced by siRNA in A549 cells. MKP-1 protein levels in A549 human lung epithelial cells stimulated with IL-1β, or a combination of IL-1β and dexamethasone, were effectively reduced with MKP-1 siRNA (Figure 9A). Further, IL-1β-induced p38 MAPK phosphorylation was inhibited by dexamethasone in cells treated with control siRNA, but the inhibition of p38 MAPK phosphorylation was attenuated in cells treated with MKP-1 siRNA (Figure 9B). In cells transfected with control siRNA, dexamethasone had an inhibitory effect on TNF mRNA levels, while in MKP-1 siRNA transfected cells, TNF mRNA expression was not reduced by dexamethasone (Figure 10). These results suggest that MKP-1 is present and functional in A549 cells and that it mediates the inhibition of p38 MAPK phosphorylation and TNF expression by dexamethasone.

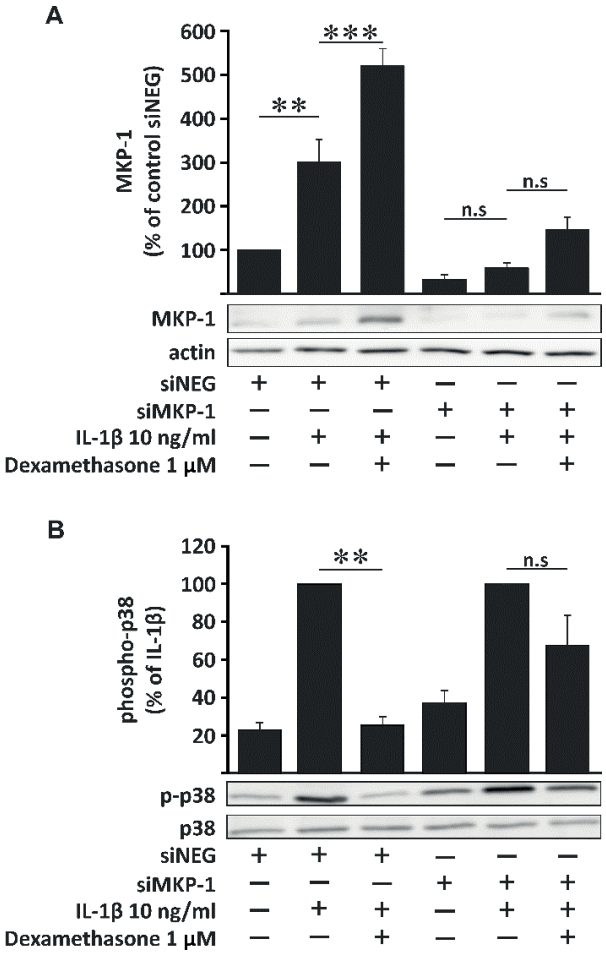


Figure 9. MKP-1 siRNA effectively down-regulated MKP-1 expression. A549 cells were transfected with MKP-1 siRNA (siMKP-1) or control siRNA (siNEG) for 24 h. Cells were stimulated with IL-1β in the absence or in the presence of dexamethasone for 1 h (A) or preincubated 1 h with dexamethasone and stimulated for 30 min with IL-1β (B). MKP-1 protein was detected by Western blot (A). The chemiluminescent signal was quantified, and MKP-1 was normalized against actin. Phosphorylated p38 MAPK was detected by Western blot (B). The chemiluminescent signal was quantified, and pp38 was normalized against total p38. Results are expressed as percentages in comparison with (A) control siNEG or (B) IL-1β + siNEG or IL-1β + siMKP-1. Values are presented as mean ± S.E.M, n=4. One-way ANOVA with Bonferroni´s post-test was performed and statistical significance is indicated with n.s. = not significant, ** $p < 0.01$ and *** $p < 0.001$. Reprinted with permission from Keränen et al. Inflammation Research, (2017) 66(5):441-44

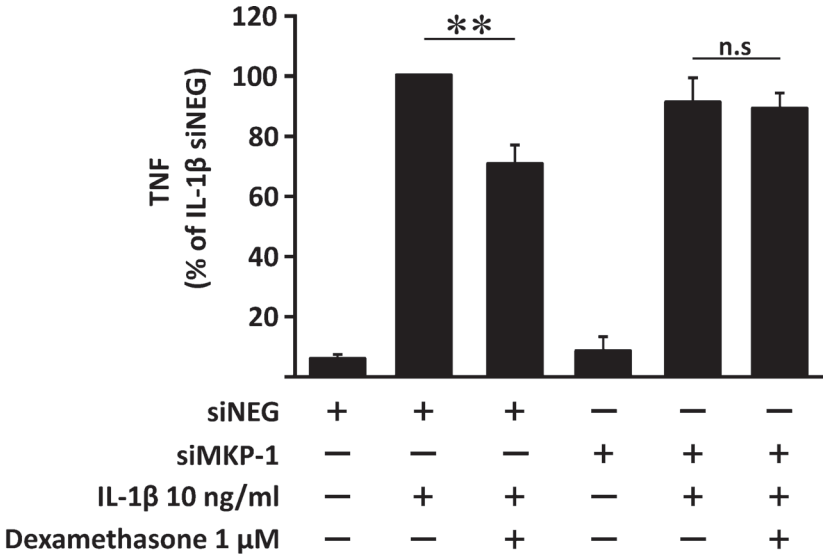


Figure 10. Dexamethasone inhibited TNF production in A549 cells transfected with control siRNA, but not in cells transfected with MKP-1 siRNA. A549 cells were transfected with MKP-1 siRNA (siMKP-1) or control siRNA (siNEG) for 24 h. Thereafter the cells were preincubated with dexamethasone for 1 h and stimulated with IL-1β for 4 h. TNF mRNA was measured by quantitative RT-PCR and TNF mRNA expression levels were normalized against GAPDH mRNA levels. Results are expressed as mean ± S.E.M, n=6. Two-way ANOVA with Bonferroni´s post-test was performed and statistical significance is indicated with n.s. = not significant, ** $p < 0.01$. Reprinted with permission from Keränen et al. Inflammation Research, (2017) 66(5):441-44

4.6 β_2-receptor agonists inhibited chemokine MCP-1 expression (III)

MCP-1 is an important inflammatory chemokine and its expression is regulated by the MAPK pathway (Wang, et al., 2019). Therefore, the effects of the β_2-agonists salbutamol and terbutaline on MCP-1 production were investigated in J774 macrophages. β_2-agonists inhibited LPS-induced MCP-1 expression (Figure 11). In addition, the inhibitory effect of the β_2-agonists on MCP-1 expression was enhanced in the presence of the PDE4 inhibitor rolipram. Also, cAMP chemical analog 8-Br-cAMP inhibited the expression of MCP-1.

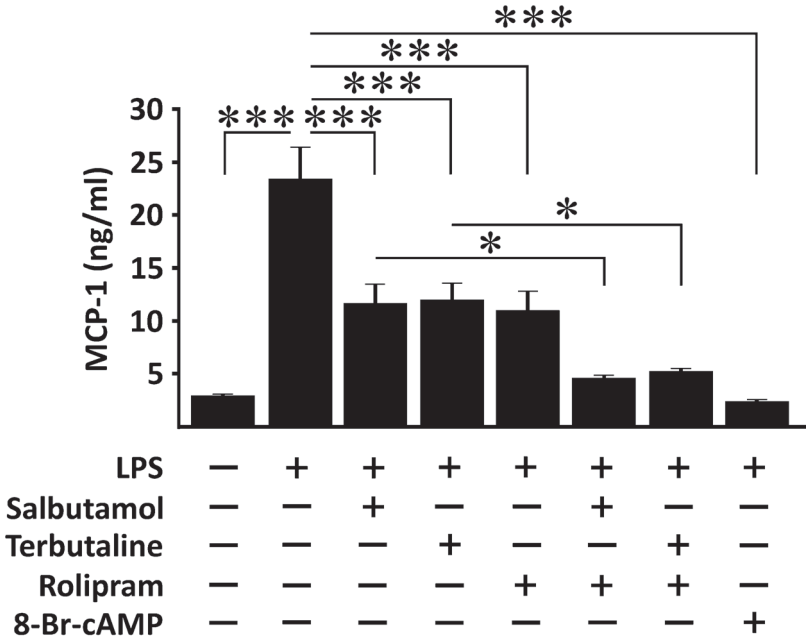


Figure 11. β_2-receptor agonists salbutamol and terbutaline, PDE4 inhibitor rolipram and cAMP analog 8-Br-cAMP inhibited MCP-1 production in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of salbutamol (100 nM), terbutaline (100 nM), rolipram (1 μM) or 8-Br-cAMP (1000 μM) for 24 h. MCP-1 protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M., n=8. One-way ANOVA with Bonferroni´s post-test was performed and statistical significance is indicated with * $p < 0.05$ and *** $p < 0.001$. Reprinted with permission from Keränen et al. Cytokine. (2017) 94:1-7.

4.7 β_2-receptor agonists inhibited ERK phosphorylation (III)

The phosphorylation of ERK was investigated in J774 macrophages in response to LPS and the combination of LPS and the β_2-agonists salbutamol and terbutaline over time (Keränen, et al., 2017). ERK phosphorylation was increased at 15 min and reached maximal level at 30 min after LPS stimulation, and then reduced near basal level at 60 min. β_2-agonists inhibited ERK phosphorylation at the time points of 15 min and 30 min and cAMP chemical analog 8-Br-cAMP was reported to have a similar effect in a previous study (Jin, et al., 2015). Combining salbutamol with the PDE4 inhibitor rolipram further attenuated ERK phosphorylation levels (Figure 12). 8-Br-cAMP also inhibited ERK phosphorylation in J774 macrophages (Keränen, et al., 2017).

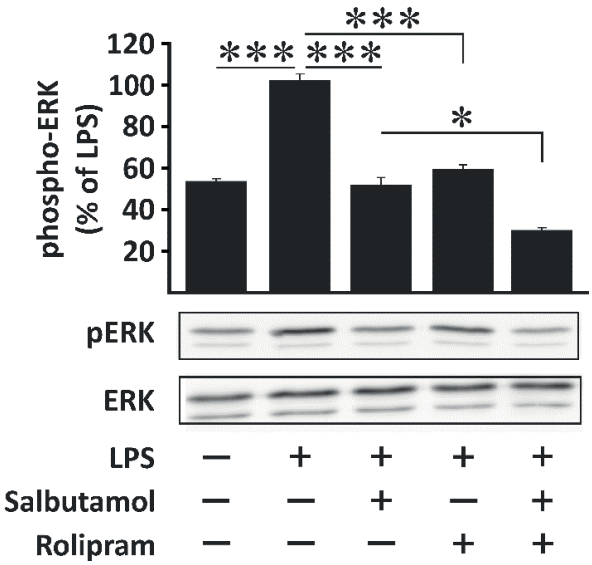


Figure 12. Salbutamol, rolipram and their combination reduced ERK phosphorylation in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of salbutamol (100 nM), rolipram (1 μM), or their combination for 30 min, and the phosphorylation of ERK was detected by Western blot. The chemiluminescent signal was quantified, and phosphorylated ERK was normalized against total ERK. Results are expressed as percentages in comparison with LPS. Values are presented as mean ± S.E.M, n=4. One-way ANOVA with Bonferroni´s post-test was performed, and statistical significance is indicated with * $p < 0.05$ and *** $p < 0.001$. Reprinted with permission from Keränen et al. Cytokine. (2017) 94:1-7.

4.8 MEK1/2 inhibitor reduced TNF and MCP-1 expression (III)

The effect of inhibition of the ERK pathway was also investigated on TNF and MCP-1 production. MAPK/ERK kinase (MEK)1/2 directly phosphorylates and thereby activates ERK. PD 0325901 is an inhibitor of MEK1/2 and it has been reported to prevent ERK phosphorylation and activity (Barrett, et al., 2008). PD 0325901 inhibited LPS-induced TNF (Figure 13A) and MCP-1 (Figure 13B) expression in J774 macrophages and in mouse peritoneal macrophages (Keränen, et al., 2017).

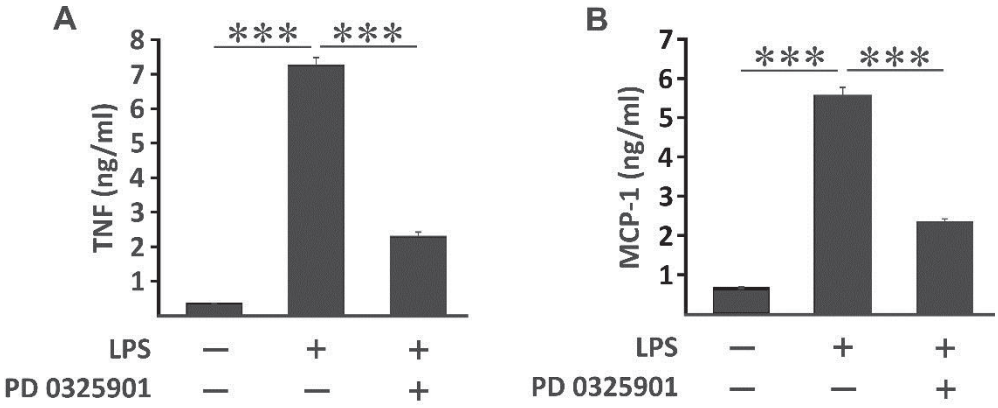


Figure 13. MEK1/2 inhibitor PD 0325901 inhibited TNF and MCP-1 production in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of PD 0325901 (100 nM) for 4 h. TNF (A) or MCP-1 (B) protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M., n=6. One-way ANOVA with Bonferroni´s post-test was performed and statistical significance is indicated with *** $p < 0.001$. Reprinted with permission from Keränen et al. Cytokine. (2017) 94:1-7.

5 DISCUSSION

The role of MKP-1 in inflammation and anti-inflammatory drug effects *in vitro* and *in vivo* were investigated. The aim of the present thesis project was to identify compounds and mechanisms that modulate the expression and/or the function(s) of MKP-1, and to investigate the role of MKP-1 in their anti-inflammatory effects *in vitro* and *in vivo*.

In this thesis, it was found that β_2-agonists increase MKP-1 expression and attenuate inflammatory gene expression. They also modulate inflammatory response by regulating the activity of MAPK pathways p38 and ERK. Glucocorticoid dexamethasone increased MKP-1 levels and MKP-1 mediated anti-inflammatory effects of glucocorticoids in human lung epithelial cells.

5.1 Methodology

The methods in this thesis are commonly used and well standardized laboratory methods in biomedical and pharmacological research. This simplifies the interpretation of the results and enables the comparison of the results to previously published reports.

5.1.1 J774 and A549 cell lines

In the *in vitro* part of this thesis, two immortalized cell lines were used; the mouse J774 macrophages and the human A549 lung epithelial cells (Ralph, et al., 1975, Smith, 1977). Immortalized cells are stable and durable and they produce relatively reproducible results over time. Using immortalized cell lines allows large series of experiments, such as time and concentration series to be conducted. Immortalization and cell growth under *in vitro* conditions may, on the other hand, modify cell function and cellular responses. In general, the variation in cell culture results can be controlled by parallel assays, positive and negative controls, and

repeating the experiment. Some experiments were repeated using mouse primary peritoneal macrophages (PMs). The justification to use primary cells is that they are considered to better reflect those cell responses that take place *in vivo*.

Macrophages are important cells in inflammation with both regulatory and effector functions, which makes them a relevant cell type in inflammation research (Schultze, et al., 2015). Epithelial cells are also important in first-line defense against different pathogens and other foreign substances. They also produce many cytokines, chemokines, other inflammatory mediators and growth factors that contribute to the development of local inflammatory response.

5.1.2 Laboratory methods and pharmacological inhibitors

In this thesis standard molecular and cellular biology methods to study protein (Western blot), and mRNA (qRT-PCR) expression and cytokine (ELISA) production were used. These methods are standardized and reagents, such as antibodies, oligonucleotides (PCR primes and probes) and enzymes, are commercially available. In the Western blot method, the proteins are separated from each other by size, and the target protein is identified with antibody. The advantages of Western blot are its relatively low cost and its ability to be used to identify a wide range of proteins. The method, however, is crucially dependent on the specificity of the antibodies used to identify target proteins; low quality antibodies may cause non-specific signals or underestimation in the appropriate signal intensity. qRT-PCR is a simple, sensitive, fast, effective and reliable method for exponential amplification of DNA. The PCR method allows detection of very low DNA concentrations in the sample. ELISA is a sensitive immunological method based on specific antibody binding for determining the target antigen. The advantage of the method is that it can quantitatively determine the target antigen while Western blot is, at best, semi-quantitative and results are expressed in relative changes.

siRNA is a practical method to investigate gene function in cells by down-regulating protein expression (Kong, et al., 2007). This method is based on the phenomenon of RNA interference, in which short targeted RNA oligonucleotides with complementary base sequence cause the controlled degradation of the target mRNA and, consequently, the reduction of the functional protein levels. siRNA is a relatively simple and effective method to silence a specific gene of interest.

However, there are some disadvantages as well: transfection efficacy of siRNA oligonucleotides can vary between cell types, and importantly, siRNA oligonucleotides may have off-target effects, which may have a significant impact on the effects observed. In this study, the transfection efficacy was tested with a fluorescent indicator. Off-target effects were controlled and reduced by using non-targeting siRNA as a control and using a siRNA pool with four siRNAs.

Pharmacological inhibitors were used to modulate intracellular signaling in cell culture experiments. In this thesis the p38 MAPK inhibitor BIRB 796 and the MEK1/2 inhibitor PD 0325901 were used. 8-Br-cAMP, a chemical analog of cAMP, was also used. Inhibitors and analogs are easy-to-use tools, but at the same time they may inhibit other pathways or have other pleiotropic effects, which may impact the effects observed. This should be taken into account when interpreting the results.

5.1.3 Animal model

In i*n vivo* studies effects of (any) intervention can be investigated in different tissues or even in the whole organism in the physiological context. In this study, *in vivo* studies gave an opportunity to confirm *in vitro* findings in a physiological context.

For ethical reasons, the number of animals used in experiments should be limited to the lowest possible able to give statistically significant results. To control the inter-individual variation between experimental animals, the environment must be standardized to a reasonable amount (age, sex, growth conditions) and use matched strains. MKP-1 KO and corresponding WT mice were healthy and age- and sex-matched, and the KO mice did not have any obvious phenotype.

The effects of MKP-1 on inflammation and its role in the anti-inflammatory effects of salbutamol were investigated *in vivo* using MKP-1 KO and corresponding WT mice. This approach gives definitive information on the mediator role of MKP-1 in the effects observed. In these experiments, a carrageenan-induced paw edema model was used, because it is a well standardized model of acute inflammation (Posadas, et al., 2004). Carrageenan induces the production of many inflammatory factors, including COX-2, TNF, IL-6 and IL-1 as well as bradykinin, histamine and prostaglandins (Kalokasidis, et al., 2009, Loram, et al., 2007, Morris, 2003). Carrageenan also causes accumulation of inflammatory cells in the tissue.

5.2 MKP-1 in the control of the inflammatory response

MKP-1 KO mice studies have shown that MKP-1 has a role in the regulation of innate and adaptive immune responses, inflammation and infection control. It suppresses the production of inflammatory factors (e.g., TNF, IL-6, IL-10), and reduces acute and chronic inflammatory response by inhibiting p38 MAPK (Chi, et al., 2006, Korhonen, et al., 2011, Turpeinen, et al., 2010, Zhao, et al., 2006). Despite excessive inflammatory response, MKP-1 KO mice appear to have defective anti-microbial responses associated with reduced IL-12 production, impaired Th1 response and excessive IL-10 release (Chi, et al., 2006, Frazier, et al., 2009, Huang, et al., 2011, Korhonen, et al., 2012).

In an acute inflammation model, carrageenan-induced paw edema was more severe in MKP-1 KO than WT mice (Korhonen, et al., 2013), and that observation was seen in this study, also. In a bone inflammation model, MKP-1 KO mice have been reported to have a more severe inflammatory bone loss, increased inflammatory cell infiltration into the inflamed bone and enhanced generation of osteoclasts as compared to WT mice (Sartori, et al., 2009). In the collagen-induced arthritis (CIA) mouse model, MKP-1 KO mice were shown to have a higher disease incidence, and their arthritis was more serious and took a more destructive course than in their WT counterparts (Salojin, et al., 2006, Vattakuzhi, et al., 2012). Further, deficiency of MKP-1 has also been shown to be associated with increased osteoclast activity and bone erosions (Vattakuzhi, et al., 2012). Altered MKP-1 expression has also been linked to psoriasis. Skin biopsies from psoriatic lesions express less MKP-1 mRNA than samples from healthy skin (Kjellerup, et al., 2013). In an experimental autoimmune encephalomyelitis (EAE) model, which is used to study the neuroinflammatory events associated with multiple sclerosis (MS), MKP-1 KO mice had more severe EAE and higher expression of IL-17-dependent genes than their WT counterparts. These and other data suggest that MKP-1 is associated with autoimmune inflammation which is caused by T helper 17 (Th17) cells that secrete the pro-inflammatory cytokine (IL-17) (Huang, et al., 2015). In a common experimental asthma model, ovalbumin-induced airway inflammation, increased activation of p38 MAPK was seen and at the same time MKP-1 expression was decreased (Liang, et al., 2013). It was also found that the MKP-1 level was reduced in the IMQ (imiquimod)-induced psoriasiform mouse skin. MKP-1 KO mice have higher sensitivity to IMQ-induced skin inflammation than their counterparts, which

was associated with increased production of inflammatory cytokines and chemokines (Zhao, et al., 2018). In the present study, β_2-agonists were found to suppress carrageenan-induced acute inflammatory response, and that effect was clearly attenuated in MKP-1 KO mice. This indicates that β_2-agonists have anti-inflammatory effects and those are mediated, at least partly, by MKP-1. In addition, β_2-agonists also suppressed p38 MAPK phosphorylation, thus providing a mechanism for the observed effects seen *in vivo*.

5.2.1 The role of MKP-1 and ERK in mediating the anti-inflammatory effects of the β_2-receptor agonists *in vitro* and *in vivo*

β_2-receptor agonists are primarily used as bronchodilator drugs for the treatment of obstructive lung diseases. Besides smooth muscle relaxing effects in the respiratory tract, they have also been reported to have anti-inflammatory effects in inflammatory and other cells (Manetsch, et al., 2012, Theron, et al., 2013) and they have been found to reduce the production of inflammatory factors, capillary permeability, and formation of plasma exudate and tissue edema (Vida, et al., 2011, Baouz 2005).

cAMP is an important intracellular second messenger that mediates many effects of β_2-agonists. The MKP-1 promoter contains two binding sites for the transcription factor cAMP-responsive element binding protein (CREB) (Kwak, et al., 1994). In this study it was found that β_2-agonists increased cAMP production and MKP-1 levels in macrophages. Also, the chemical analog of cAMP, 8-Br-cAMP, increased MKP-1 expression in a similar manner to β_2-agonists. Suppression of IL-8 production by β_2-agonists has previously been shown to occur concomitantly with increased MKP-1 expression (Mortaz, et al., 2008, Patel, et al., 2014). In this thesis, β_2-agonists were also found to suppress the production of the cytokine TNF and the chemokine MCP-1 in macrophages. β_2-agonists also limited the phosphorylation of p38 MAPK and ERK. 8-Br-cAMP had comparable effects on ERK phosphorylation and TNF and MCP-1 release also. β_2-agonists were more potent inhibitors of TNF production as compared to the p38 MAPK inhibitor. This suggests that the anti-inflammatory effects of β_2-agonists are mediated not only by inhibition of p38 MAPK activity by MKP-1, but that there are other anti-inflammatory mechanisms involved also. This is indirectly supported by the finding showing that the inhibition of carrageenan-

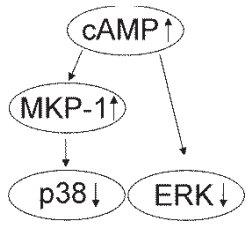
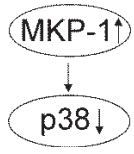
induced inflammatory response by salbutamol was partially, but not completely, impaired in MKP-1 KO mice.

Result of the this thesis suggest that cAMP signaling suppresses TNF production in macrophages by regulating two distinct MAPK pathways (Table 5). Firstly, β_2-agonists inhibit early ERK phosphorylation leading to partially impaired ERK signaling and reduced TNF production. Secondly, β_2-agonists increase MKP-1 expression leading to the dephosphorylation of p38 MAPK and hence to the suppression of the p38 MAPK pathway and TNF production.

β_2-agonists increase cAMP levels by a mechanism dependent on G_s-protein and adenylate cyclase. PDE4 inhibitors prolong increased cAMP levels by preventing degradation of cAMP to non-cyclic mononucleotides. PDE4 inhibitors have been reported to suppress cytokine production and reduce the inflammatory response (Page and Spina, 2012). In this thesis, it was found that when β_2-agonists were combined with the PDE4 inhibitor rolipram, ERK phosphorylation and the production of TNF and MCP-1 were further suppressed. This supports the hypothesis that the observed anti-inflammatory effects of β_2-agonists were due to changes in cAMP signaling.

Taken together, β_2-agonists have anti-inflammatory effects *in vitro* and *in vivo*. This study provides evidence for the idea that β_2-agonists have anti-inflammatory effects and those are mediated by cAMP. Results show that β_2-agonists clearly reduce TNF and MCP-1 release in macrophages and suppress acute inflammation *in vivo*. Effects of β_2-agonists on MKP-1 expression and inflammation may contribute to the therapeutic effect of the β_2-agonists in the treatment of asthma, when long-acting β_2-agonists are combined with inhaled glucocorticoids in the treatment of moderate or severe asthma. Interestingly, TNF levels are increased in the airways of patients with asthma (Keatings, et al., 1997), and anti-TNF treatment has been reported to reduce disease severity, for example, by lowering the incidence of disease exacerbations in patients with symptomatic moderate asthma (Erin, et al., 2006).

Table 5. Summary of the main result of the thesis

Drug	Effect	Mechanism	Methods
β_2-agonist	cytokines ↓ chemokines ↓ paw edema ↓		LPS-stimulated murine macrophages carrageenan-induced paw edema in MKP-1 KO vs. WT mice
Dexamethasone	cytokines↓ chemokines↓		IL-1β-stimulated A549 human lung epithelial cells

5.2.2 The role of MKP-1 in mediating the anti-inflammatory effects of the glucocorticoid dexamethasone

This thesis also investigated the link between MKP-1 and anti-inflammatory effects of glucocorticoids. The MKP-1 promotor contains functional glucocorticoid response element regions, where activated glucocorticoid receptor (GR) dimers can bind and activate MKP-1 gene transcription (Shipp, et al., 2010).

In this study, dexamethasone increased MKP-1 expression, reduced the levels of the phosphorylated p38 MAPK and suppressed the production of the cytokine TNF and the chemokine MIP-3α in IL-1β-treated human lung epithelial cells. Airway epithelium has been shown to produce many inflammatory factors and contribute to airway inflammation (Gao, et al., 2015). Inflammatory gene expression is suppressed by MKP-1 in human bronchial epithelial cells and in primary human airway smooth muscle cells (Manetsch, et al., 2012, Turpeinen, et al., 2010). In this study, the dephosphorylation of p38 MAPK and the inhibition of TNF production by dexamethasone was attenuated when MKP-1 was silenced with siRNA in airway epithelial cells. These results suggest that the anti-inflammatory effects of glucocorticoids in human lung epithelial cells are, in part, mediated by MKP-1. Hence, MKP-1 may contribute to the therapeutic effects of glucocorticoids in chronic inflammatory lung diseases.

It has been shown that dexamethasone increases MKP-1 expression and the anti-inflammatory effect of dexamethasone is, at least partly, mediated by MKP-1 (Abraham, et al., 2006, Kassel, et al., 2001, Shipp, et al., 2010). Dexamethasone also reduced the levels of phosphorylated p38 and JNK in bone marrow-derived macrophages from WT mice, while the effect is attenuated in cells from MKP-1 KO mice (Abraham, et al., 2006).

Inhaled glucocorticoids are widely used in the treatment of asthma and COPD. However, inhaled glucocorticoids are less effective in patients with severe asthma. Alveolar macrophages from patients with glucocorticoid-insensitive asthma displayed higher p38 MAPK phosphorylation and increased sensitivity to LPS stimulation. In addition, they had lower induction of MKP-1 by glucocorticoids in macrophages and peripheral blood mononuclear cells (Bhavsar, et al., 2008, Sutherland, et al., 2008). In the lung tissue of ovalbumin-sensitized mice, activation of the p38 MAPK pathway was reported to be increased and the expression of MKP-1 reduced. Dexamethasone and the p38 MAPK inhibitor inhibited p38 MAPK and attenuated lung inflammation, and dexamethasone increased MKP-1 levels in the lung tissue (Liang, et al., 2013). MKP-1 has also been shown to mediate attenuation of ozone-induced airway constriction by glucocorticoids (Li, et al., 2011).

5.3 MAPKs and MKPs as drug targets

p38 MAPK inhibitors have anti-inflammatory properties in various preclinical *in vitro* and *in vivo* models as discussed in chapter 1.1.1 and also as seen in the present study. So far, p38 MAPK inhibitors have not, however, been approved for clinical use. In the early studies, p38 MAPK inhibitors BIRB-796, SCIO-323 and VX-745 were tested in humans, but unfortunately, those studies reported adverse effects, such as hepatotoxicity, cardiotoxicity, CNS effects and infections (Chopra et al., 2008). It remains, however, unclear to which extend those unwanted effects were associated with the p38 MAPK inhibition and to which extend they were unrelated to the mechanism of action. Currently, there are clinical trials ongoing with p38 MAPK inhibitors, for example, in rheumatoid arthritis, asthma and COPD (Table 1).

The ERK pathway also contributes to inflammation, which was seen in the present study, as well. Pharmacological modulation of the ERK pathway shows

therapeutic effects in animal models of inflammation (Duan, et al., 2004, Jaffee, et al., 2000, Thiel, et al., 2007). ERK has also been identified as a drug target in many cancer types (Martin-Liberal, et al., 2014, Neuzillet, et al., 2014, Pratilas and Solit, 2010). There are several ongoing clinical drug development programs with ERK inhibitors, mainly for treatment of cancers (Table 2).

MAPK pathways have an important role in the regulation of inflammation as discussed in chapter 1.1., but so far, their inhibitors have not entered the clinics. Alternatively, MKP-1 may provide a potential anti-inflammatory drug target. MKP-1 mediates at least partly the anti-inflammatory effects of glucocorticoids (Abraham, et al., 2006, Kassel, et al., 2001, Shipp, et al., 2010). In addition, the anti-rheumatic gold compound aurothiomalate was found to have anti-inflammatory effects that were mediated by MKP-1 (Nieminen, et al., 2010). Also, the PDE4 inhibitor rolipram suppressed inflammatory response *in vivo* in WT mice, but the inhibition of the inflammatory response was clearly reduced in MKP-1 KO mice (Korhonen, et al., 2013). This is interesting, because two PDE4 inhibitors have been approved for clinical use. Roflumilast is approved for the treatment of severe COPD (Rabe, et al., 2017) and apremilast is approved for the treatment of plaque psoriasis (Reich, et al., 2017, Sobell, et al., 2016) and psoriatic arthritis (Cutolo, et al., 2016). The present study adds β_2-agonists to this list and shows that the anti-inflammatory effects of β_2-agonists and glucocorticoids are, at least partly, mediated by enhanced MKP-1 expression and suppressed p38 MAPK phosphorylation.

The role of MKP-1 as an anti-inflammatory drug target is further supported by the findings in MKP-1 KO mice. As discussed previously in this thesis, expression of various inflammatory genes is enhanced in cells from MKP-1 KO mice and, accordingly, those mice also develop more severe responses in models of arthritis and several other inflammatory diseases. It is of note that MKP-1 seems also to have regulatory functions beyond inflammation, including cancer and mood/depression. Further studies are needed to understand the potential contribution of those effects to the therapeutic and/or adverse effects of MKP-1 enhancing drugs.

In summary, MKP-1 is an important regulatory factor in inflammation. It suppresses inflammatory gene expression, attenuates inflammatory responses and is associated with anti-inflammatory drug effects. MKP-1/MAP kinase regulation is a potential research area for anti-inflammatory drug discovery.

SUMMARY AND CONCLUSIONS

The aim of the present thesis was to identify compounds that modulate the expression and/or the function(s) of MKP-1 and to investigate the role of MKP-1 in their anti-inflammatory effects focusing mainly on the β_2-agonist salbutamol and the glucocorticoid dexamethasone. The main findings and conclusions are as follows:

1. β_2-agonists and glucocorticoids reduced the production of inflammatory factors and attenuated the inflammatory response.
2. β_2-agonists and glucocorticoids increased MKP-1 expression and reduced p38 MAPK phosphorylation.
3. β_2-agonists reduced the levels of the phosphorylated ERK in activated macrophages.
4. Anti-inflammatory effects of β_2-agonists and glucocorticoids were mediated, at least partly, by MKP-1.

This study provides new information on the regulation of inflammation and production of inflammatory factors and reveals new potential targets for pharmacological therapies. In many diseases, inflammation drives the development of the disease and is also the reason for the patent's symptoms. In this study, it was found that β_2-agonists that are used as bronchodilating drugs in chronic inflammatory lung diseases have also anti-inflammatory effects both *in vitro* and *in vivo*. In addition, the glucocorticoid dexamethasone showed anti-inflammatory effects in human lung epithelial cells. Those effects of β_2-agonists and dexamethasone were mediated by MKP-1, emphasizing its role as an endogenous factor controlling inflammatory response. The anti-inflammatory effects of β_2-agonists observed in this study may also contribute to their therapeutic efficacy. Also, the finding that MKP-1 was a common pathway for two distinct anti-

inflammatory drug-induced pathways suggests that MKP-1 is a potential target for anti-inflammatory drug development.

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Tämä tutkimus on tehty Immunofarmakologian tutkimusryhmässä Tampereen yliopiston Lääketieteen ja terveysteknologian tiedekunnassa. Olen ollut myös opiskelijana Tuki- ja liikuntaelinsairauksien ja biomateriaalien tohtoriohjelmassa (TBDP).

Lukioikäisenä pääsin tutustumaan yliopistolle fysiikan tutkimukseen. Tutkimustyö näytti silloin lukiolaisen silmissä yksinäiseltä ja tylsältä. Tämä kokemus enemmän kauhistutti minua, kuin sai minut kiinnostumaan tutkimuksesta. Vannoin silloin, että en tule ikinä tekemään tutkimusta työkseni. No miten väärässä sitä ihminen voi olla. Immunofarmakologian tutkimusryhmä on näyttänyt minulle, mitä tutkimustyö on parhaimmillaan. Se on tiimityötä, toisten auttamista ja tukemista, välillä toki yksin puurtamista, mutta aina on saanut apua, kun sitä on vain osannut kysyä. Tulin ryhmään tekemään pro gradua, mutta erityisesti ryhmän hyvä yhteishenki sai minut jatkamaan tutkimustyötä. Tässä vuosien varrella olen saanut todella paljon apua monilta ihmisiltä ja ilman heidän panostaan tätä kirjaa ei olisi syntynyt.

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ORIGINAL COMMUNICATIONS

PUBLICATION
I

Anti-inflammatory effects of β_2-receptor agonists salbutamol and terbutaline are mediated by MKP-1

Tiina Keränen, Tuija Hömmö, Mari Hämäläinen, Eeva Moilanen, Riku Korhonen

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RESEARCH ARTICLE

Anti-Inflammatory Effects of β_2-Receptor Agonists Salbutamol and Terbutaline Are Mediated by MKP-1

Tiina Keränen, Tuija Hömmö, Mari Hämäläinen, Eeva Moilanen, Riku Korhonen*

The Immunopharmacology Research Group, University of Tampere School of Medicine, and Tampere University Hospital, Tampere, Finland

* riku.korhonen@staff.uta.fi

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Abstract

Mitogen-activated protein kinase phosphatase 1 (MKP-1) expression is induced by inflammatory factors, and it is an endogenous suppressor of inflammatory response. MKP-1 expression is increased by PDE4 inhibitor rolipram suggesting that it is regulated by cAMP-enhancing compounds. Therefore, we investigated the effect of β_2-receptor agonists on MKP-1 expression and inflammatory response. We found that β_2-receptor agonists salbutamol and terbutaline, as well as 8-Br-cAMP, increased MKP-1 expression. Salbutamol and terbutaline also inhibited p38 MAPK phosphorylation and TNF production in J774 mouse macrophages. Interestingly, salbutamol suppressed carrageenan-induced paw inflammation in wild-type mice, but the effect was attenuated in MKP-1(-/-) mice. In conclusion, these data show that β_2-receptor agonists increase MKP-1 expression, which seems to mediate, at least partly, the observed anti-inflammatory effects of β_2-receptor agonists.

Introduction

Mitogen-activated protein kinases (MAPKs) are important intracellular signaling pathways that regulate many physiological and pathophysiological events in cells. The three main MAPK pathways include p38 MAPK, Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) [1,2]. MAPK pathways are three-tier kinase cascades that are activated in response to several extracellular signals, such as cytokines, growth factors and bacterial substances through G-protein-coupled and/or kinase-linked receptors. Upon activation, threonine and tyrosine residues in the activation motif of the given MAPK are phosphorylated by the upstream kinase in the signaling cascade [3,4]. Targets of activated MAPKs include transcription factors and other regulatory proteins, and they regulate many physiological cellular processes, such as cell growth, proliferation, differentiation, motility, stress response, survival, and apoptosis [1,2]. p38 MAPK and JNK have also a marked role in inflammation and immune response. They regulate the production of inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-6 (IL-6) and other mediators, such as prostaglandins and nitric oxide. Also, p38 MAPK and JNK augment Th1 type immune response and support the activation and functions of Th1 cells [1,5,6].

Abbreviations: COPD, chronic obstructive pulmonary disease; CRE, cAMP responsive element; CREB, cAMP responsive element binding protein; DUSP, dual-specificity phosphatase; ERK, extracellular-regulated kinase; IL, interleukin; LPS, lipopolysaccharide; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKP, mitogen-activated protein kinase phosphatase; PDE, phosphodiesterase; TNF, tumor necrosis factor.

Dual specificity phosphatases (DUSPs) are endogenous factors that dephosphorylate tyrosine and threonine residues of their target proteins. Mitogen-activated protein kinase phosphatases (MKPs) are a subgroup of DUSPs, and they specifically dephosphorylate MAPKs, which makes them endogenous suppressors of activated MAPK pathways. MKP family of phosphatases has eleven members, and they display differences in substrate specificity among MAPKs, as well as in tissue distribution, cellular location and expressional pattern [7,8]. MAP kinase phosphatase 1 (MKP-1) is a nuclear phosphatase and it regulates p38 MAPK, and in some cells, JNK activity [6,9]. It has earlier been shown that hypoxia and inflammatory signals increase MKP-1 expression [10], and by inhibiting p38 MAPK, MKP-1 suppresses inflammatory gene expression and attenuates inflammatory response [11,12]. Interestingly, MKP-1 has also been reported to mediate certain anti-inflammatory drug effects. MKP-1 expression is increased by glucocorticoids and anti-rheumatic gold-compounds, and MKP-1 mediates, in part, the anti-inflammatory effects of these drugs [13,14]. Recently, we demonstrated that phosphodiesterase (PDE) 4 inhibitor rolipram increased MKP-1 levels and suppressed inflammatory response in wild-type mice, but the response was impaired in MKP-1(-/-) mice [15].

Salbutamol and terbutaline are β_2-receptor agonists used in the treatment of obstructive lung diseases as bronchodilating remedy. β_2-receptors are G protein-coupled receptors and their activation stimulates G_s-proteins leading to increased adenylate cyclase activity and elevation of cAMP levels in cells [16–18]. In addition to their bronchodilation effects, β_2-receptor agonists have been shown to possess certain anti-inflammatory properties in immune and inflammatory cells, which effects may contribute to the therapeutic drug effects in the treatment of inflammatory lung diseases. In experimental acute lung injury, β_2-receptor agonists have been reported for example to attenuate proinflammatory activity and neutrophil recruitment. Combinations of β_2-receptor agonists' bronchodilatory and anti-inflammatory properties improve the value of these drugs in the treatment of acute and chronic lung diseases [19]. Because MKP-1 promotor contains a cAMP response element CRE [20,21], we hypothesized that cAMP elevating β_2-receptor agonists may regulate the expression of this important endogenous anti-inflammatory factor. In the present study we investigated the effects of salbutamol on MKP-1 expression and further, whether MKP-1 is involved in the anti-inflammatory effects of this β_2-receptor agonist.

Methods

Materials

Reagents were obtained as follows. Salbutamol [α-((*tert*-butylamino)methyl)-4-hydroxy-*m*-xylene-α,α′-diol], terbutaline [5-(2-(*tert*-butylamino)-1-hydroxyethyl)benzene-1,3-diol], 8-Br-cAMP (8-bromoadenosine 3′,5′-cyclic monophosphate) and LPS from *Escherichia coli* strain 0111:B4 were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Rolipram [4-(3-cyclopentyloxy-4-methoxy-phenyl)-pyrrolidin-2-one] and BIRB 769 [1-(5-*tert*-butyl-2-*p*-tolyl-2H-pyrazol-3-yl)-3(4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl)urea] were obtained from Axon Medchem BV (Groningen, the Netherlands). All other reagents were purchased also from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless otherwise stated below.

Cell culture

J774 mouse macrophages (ATCC, Rockville Pike, MD, USA) were cultured at +37°C in 5% CO_2 atmosphere in Dulbecco′s Modified Eagle′s Medium supplemented with glutamax-1 (DMEM; Invitrogen, Paisley, UK) containing 10% (v/v) heat-inactivated FBS (fetal bovine serum), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (all from

Gibco, Wien, Austria). For experiments, cells (2.5 x 10^5 cells/well) were seeded on 24-well plates and the cell monolayers were grown for 72 h before the experiments were started.

Salbutamol, terbutaline and rolipram were dissolved in dimethyl sulfoxide (DMSO), and 8-Br-cAMP and LPS in phosphate buffered saline (PBS). LPS (10 ng/ml) and/or the compounds of interest at the concentrations indicated or the solvent (DMSO, 0.1% v/v) were added to the cells in fresh culture medium containing 10% FBS and the supplements. Cells were further incubated for the time indicated.

The effect of LPS and the tested chemicals on cell viability was evaluated by modified XTT test (Cell Proliferation Kit II; Roche Diagnostics, Mannheim, Germany). Neither LPS nor the other chemicals used in the experiments were observed to evoke cytotoxicity.

Preparation of cell lysates and Western blot analysis

At the indicated time points, culture medium was removed. Cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged (12 000 g, 10 min, +4°C) and supernatants were collected and mixed in a ratio 1:4 with SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol) and stored at -20°C until analyzed. Protein concentrations in the samples were measures by the Coomassie Brilliant Blue method (Coomassie Protein Assay Reagent Kit; Pierce, Rockford, IL, USA).

Before Western blot analysis, the samples were boiled for 10 min. Equal aliquots of protein (20 μg) were loaded on 12% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred to Hybond enhance chemiluminescence nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) by semi-dry electroblotting. After transfer the membrane was blocked in TBS/T [20 mM Tris-base (pH 7.6), 150 mM NaCl, 0.1% Tween-20] containing 5% non-fat milk for 1 h at room temperature. For detection of phosphorylated proteins, membranes were blocked in TBS/T containing 5% bovine serum albumin (BSA). Membranes were incubated overnight at +4°C with the primary antibody and at room temperature for 1 h with the secondary antibody, and the chemiluminescent signal was detected by ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The chemiluminescent signal was quantified with FluoChem program (version 3.1) and Image Quant TL 7.0 Image Analysis software.

Following antibodies were used in the Western blot analysis: MKP-1 (SAB2500331; Sigma-Aldrich Inc., St. Louis, MO, USA), p38 MAPK (ab27986; Abcam, Cambridge, UK) and phospho-p38 MAPK (#9211; Cell Signaling Technology Inc., Beverly, MA, USA), as well as actin (sc-1615), polyclonal anti-goat (sc-2020) and polyclonal anti-rabbit (sc-2004) (all three from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

At the indicated time points, the culture medium was removed, and cell homogenization and total RNA extraction was carried out by using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Inc., St. Louis, MO, USA) according to the manufacturer´s instructions. Reverse transcription of RNA to cDNA was performed by TaqMan® Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA, USA), according to the supplier´s instructions. The primer and probe sequences and concentrations were optimized according to the manufacturer´s guidelines in TagMan® Universal PCR Master Mix Protocol part number

4304449 revision C (Applied Biosystems, Branchburg, NJ, USA). The following primer and probe sequences were used: for mouse MKP-1 5′-AAGGATGCTGGAGGGAGAGT-3′ (forward), 5′-TGAGGTAAGCAAGGCAGATGGT-3′ (reverse) and 5′-TTTGTTCATTGC CAGGCCGGCAT-3′ (probe containing 6-FAM as the 5′-reporter dye and TAMRA as the 3′-quencher); for mouse TNF 5′-AATGGCCTCCCTCTCATCAGTT-3′ (forward), 5′-TCCT CCACTTGGTGGTTTGC-3′ (reverse) and 5′-CTCAAAATTCGAGTGACAAGCCTGTAGC CC-3′ (probe containing 6-FAM as the 5′-reporter dye and TAMRA as the 3′-quencher); for mouse GAPDH 5′-GCATGGCCGGCCGTGTTC-3′ (forward), 5′-GATGTCATCATACTT GGCAGGTTT-3′ (reverse) and 5′- TCGTGGATCTGACGTGCCGCC-3′ (probe containing 6-FAM as the 5′-reporter dye and TAMRA as the 3′-quencher). Primers and probes were obtained from Metabion (Martinsried, Germany).

PCR reaction parameters were as follows: incubation at +50°C for 2 min and at +95°C for 10 min, and thereafter 40 cycles of denaturation at +95°C for 15 s and annealing and extension at +60°C for 1 min. Each sample was determined in duplicate. A standard curve method was used to estimate the relative mRNA levels. When calculating the results, MKP-1 and TNF mRNA levels were first normalized against GAPDH.

Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium samples and cell lysates were kept at -20°C until assayed. The concentrations of mouse TNF (Duoset® ELISA Development System mouse TNF kit; R&D Systems Europe Ltd., Abindgon, UK) and mouse cAMP (cAMP ELISA Kit; Cell Biolabs, Inc. San Diego, CA, USA) were determined by ELISA according to the manufacturer´s instructions.

Animals

Carrageenan-induced paw edema was carried out in wild-type and MKP-1(-/-) C57BL/6 mice. The MKP-1 deficient mice were originally generated in the laboratory of R. Bravo at Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ, USA) and those as well as corresponding wild-type mice were bred at the University of Tampere School of Medicine animal facilities under conditions of optimum light (12:12 light-dark cycle), temperature (+22 ± 1°C) and humidity (50–60%), and food and water provided *ad libitum*. The study was approved by the National Animal Experiment Board. Female mice aged 10–12 weeks were used in the study.

Carrageenan-induced paw edema

MKP-1 deficient and wild-type C57BL/6 mice (20–25 g) were divided into groups of six mice and treated with 200 μl of PBS or salbutamol (5 mg/kg in PBS) [22,23] by an i.p. injection 2 h before applying carrageenan. Before the administration of carrageenan, the mice were anaesthetized by i.p. injection of 0.5 mg/kg of medetomidine (Domitor® 1 mg/ml; Orion Oyj, Espoo, Finland) and 75 mg/kg of ketamine (Ketalar® 10 mg/ml; Pfizer Oy Animal Health, Helsinki, Finland). The mice received a 45 μl i.d injection of λ-carrageenan (2% dissolved in normal saline) in one hind paw. The contralateral paw received 45 μl of saline and it was used as a control. Paw volume was measured before and 2 h, 4 h and 6 h after the carrageenan injection with a plethysmometer (Ugo Basile, Comerio, Italy). Edema is expressed as the difference between the volume changes of the carrageenan-treated paw and the control paw.

Statistics

Results are expressed as mean ± standard error of mean (S.E.M.). When appropriate, Student´s t-test, one-way ANOVA with Dunnett´s or Bonferroni´s post test or two-way ANOVA with

Bonferroni´s post test was performed using GraphPad Prism-5 version 5.04 for Window XP (GraphPad Software Inc., La Jolla, CA, USA). P values less than 0.05 were considered significant.

Results

β_2-receptor agonists salbutamol and terbutaline enhanced MKP-1 expression in activated mouse macrophages

MKP-1 promoter has been described to contain *cis*-regulator CRE sequences [20,21]. Therefore we hypothesized that cAMP-elevating compounds would regulate MKP-1 expression. As expected, β_2-receptor agonist salbutamol increased intracellular cAMP levels in J774 macrophages (Table 1). Next, we investigated the effects of salbutamol on MKP-1 expression in these cells. MKP-1 mRNA expression was increased by LPS and, interestingly, it was further enhanced by salbutamol and by a cAMP analog 8-Br-cAMP (Fig 1). Salbutamol and another β_2-receptor agonist terbutaline increased MKP-1 expression alone and in combination with LPS in J774 macrophages in a dose-dependent manner (Fig 2). MKP-1 is an endogenous suppressor of p38 MAPK activity. Therefore, we investigated the effect of β_2-receptor agonists salbutamol and terbutaline on p38 MAPK phosphorylation. The phosphorylation of p38 MAPK was increased in response to LPS and it was inhibited by β_2-receptor agonists in J774 macrophages (Fig 3).

β_2-receptor agonists salbutamol and terbutaline inhibited TNF production in activated mouse macrophages

TNF is a cytokine whose expression is known to be regulated by MKP-1 and p38 MAPK [15,24]. Therefore, we continued by investigating the effects of β_2-receptor agonists on TNF production. Salbutamol and terbutaline as well as cAMP analog 8-Br-cAMP inhibited LPS-induced TNF mRNA and protein expression, and maximal/submaximal inhibition of TNF protein release was observed with 100 nM drug concentration in J774 macrophages (Fig 4). TNF production was further inhibited when salbutamol or terbutaline was combined with a PDE4 inhibitor rolipram (Fig 4E). We also investigated the effect of p38 MAPK inhibitor on TNF production. As expected, p38 MAPK inhibitor BIRB 796 inhibited LPS-induced TNF release in macrophages (Fig 4F).

The inhibition of carrageenan-induced paw inflammation by salbutamol was mediated by MKP-1

As salbutamol increased MKP-1 expression, we wanted to investigate whether MKP-1 could mediate the anti-inflammatory effects of salbutamol, therefore we tested the effect of

Table 1. Salbutamol increased intracellular cAMP levels in J774 macrophages.

treatment	cAMP (pg/ml)	
Control	1.08 ± 0.8	
Salb 100 nM	1230.9 ± 251.8	a
LPS 10 ng/ml	20.6 ± 11.1	
LPS+ Salb	730.5 ± 246.5	b

J774 macrophages were incubated with salbutamol (Salb) and stimulated with LPS for 1 min. Cells were then lysed and cAMP levels were measured by ELISA. Results are expressed as mean ± S.E.M, n = 5. One-way ANOVA with Bonferroni´s post test was performed, and statistical significance is (a) $p < 0.001$ between untreated cells and cells treated with salbutamol and (b) $p < 0.001$ between LPS-treated cells and cells treated with the combination of LPS and salbutamol.

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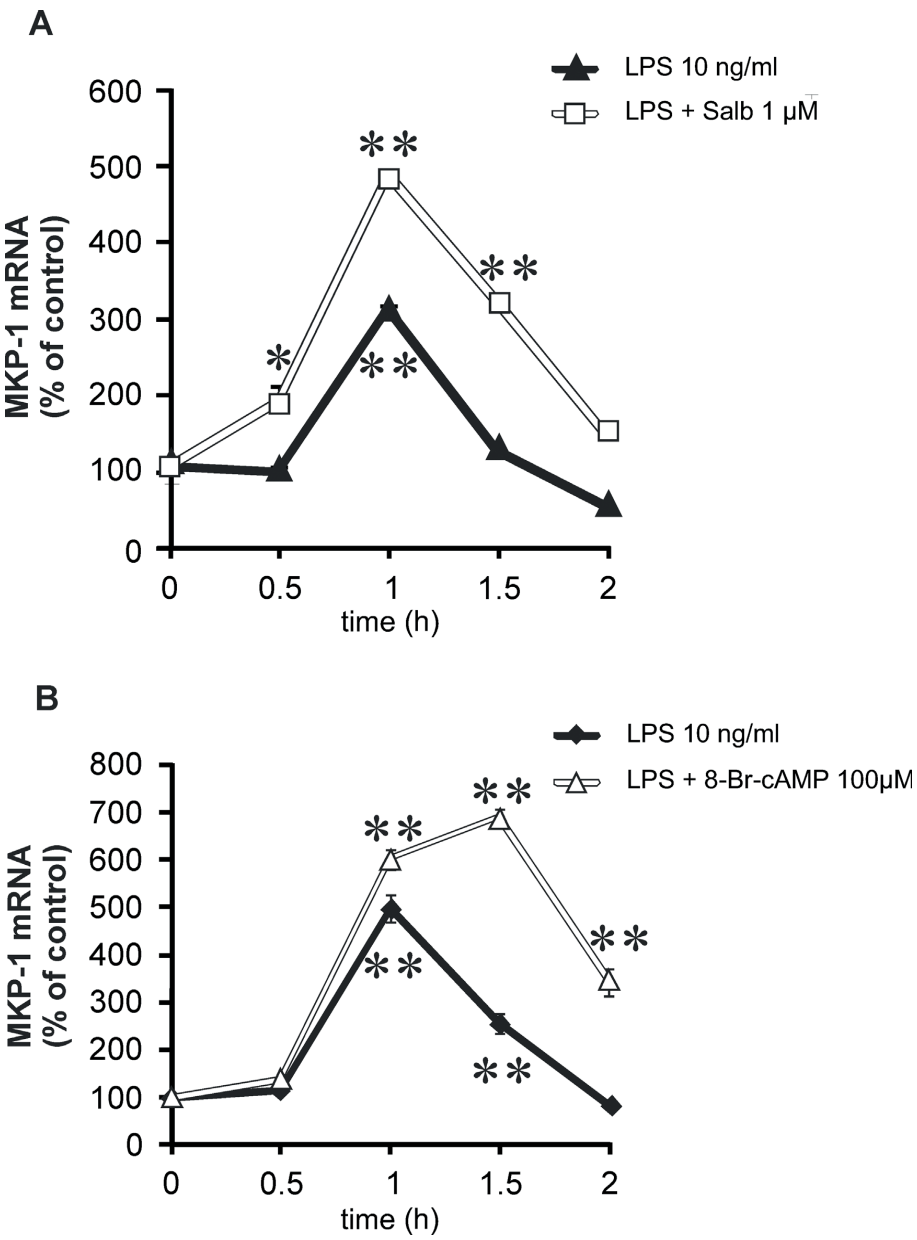

Fig 1. Salbutamol and cAMP analog 8-Br-cAMP enhanced MKP-1 expression in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the presence or in the absence of salbutamol (A) or 8-Br-cAMP (B) for the time indicated. MKP-1 mRNA was measured by quantitative RT-PCR, and MKP-1 mRNA expression levels were normalized against GAPDH mRNA levels. Results are expressed as mean ± S.E.M., n = 3. One-way ANOVA with Dunnett´s post test was performed, and statistical significance is indicated with * $p < 0.05$ and ** $p < 0.01$ as compared to control cells.

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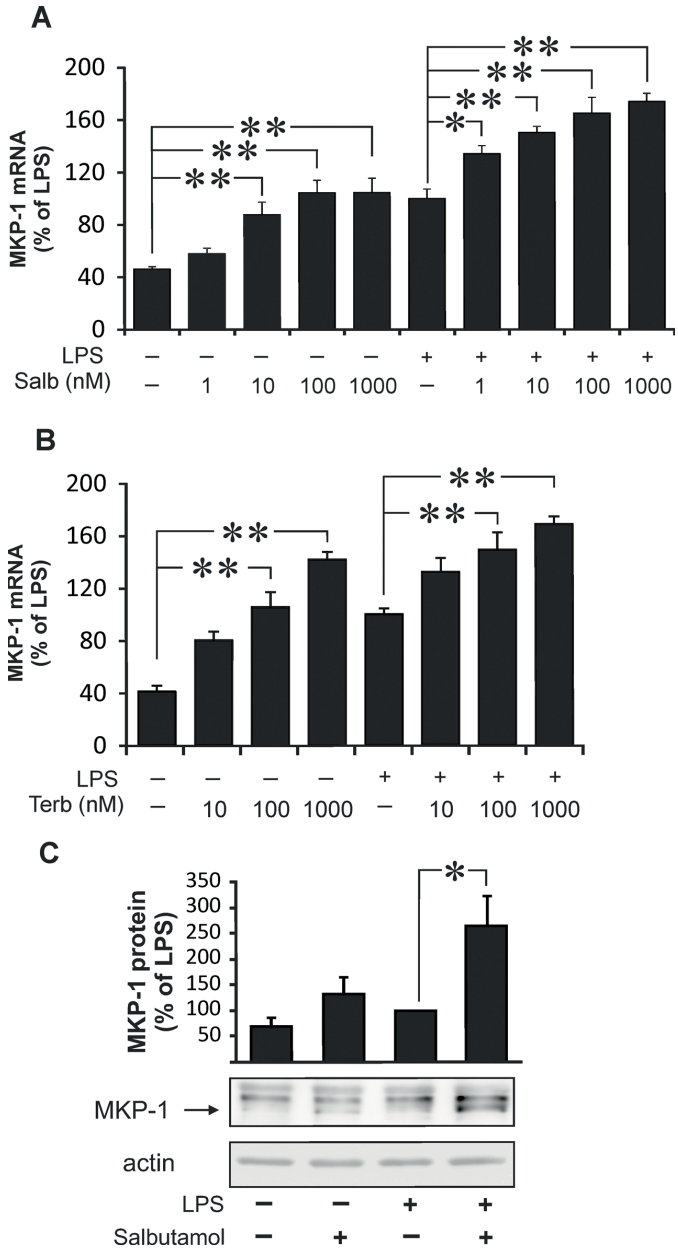

Fig 2. Salbutamol and terbutaline enhanced MKP-1 expression in J774 macrophages in a dose-dependent manner. (A and B) J774 macrophages were treated with increasing concentrations of salbutamol or terbutaline in the absence or in the presence of LPS (10 ng/ml) for 1 h. MKP-1 mRNA was measured by quantitative RT-PCR, and MKP-1 mRNA expression levels were normalized against GAPDH mRNA levels. (C) J774 cells were incubated with LPS (10 ng/ml) and salbutamol (100 nM) for 1 hour and MKP-1 protein was measured by Western blot. The chemiluminescent signal was quantified, and the amounts of MKP-1

were normalized against actin. Results are expressed as mean ± S.E.M., n = 6 (A and B) or n = 4 (C). One-way ANOVA with Dunnett´s (A and B) or Bonferroni´s (C) post test was performed, and statistical significance is indicated with * p < 0.05, ** p < 0.01 and *** p < 0.001 as compared to control or LPS-treated cells.

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salbutamol on the severity of carrageenan-induced paw inflammation in wild-type and MKP-1(-/-) mice. Carrageenan-induced paw edema was increased in MKP-1(-/-) mice as compared to wild-type mice (AUC values were 186.7 ± 13.1 μL/h and 236.7 ± 15.9 μL/h in WT and MKP-1(-/-) mice, respectively, p = 0.0352, n = 6, Fig 5). Carrageenan-induced paw edema was clearly attenuated by salbutamol in wild-type mice (73% reduction in AUC value), while salbutamol was less effective to reduce paw edema in MKP-1(-/-) mice (43% reduction in AUC value, p = 0,0148) (Fig 5).

Discussion

The aim of the present study was to investigate the effects of β_2-receptor agonist salbutamol on MKP-1 expression, on TNF production and on acute inflammatory response *in vivo*. We found that salbutamol increased MKP-1 expression, inhibited the phosphorylation of p38 MAPK and suppressed the production of TNF. We used another β_2-receptor agonist terbutaline as a reference compounds and its effects on p38 MAPK phosphorylation and TNF production were comparable to those of salbutamol. Salbutamol suppressed carrageenan-induced acute inflammation *in vivo* in wild-type mice, but that effect was attenuated in MKP-1(-/-) mice in a statistically significant manner. These results suggests that acute anti-inflammatory effects of β_2-receptor agonists are partly mediated by MKP-1.

β_2-receptor agonists are used as a bronchodilators in obstructive lung diseases [17,19]. β_2-receptor agonists have been reported to have anti-inflammatory effects in addition to their effects on smooth muscle relaxation in the airways. They have been shown to inhibit the expression of inflammatory mediators and to reduce capillary permeability and formation of plasma exudate and tissue edema [25,26]. Salbutamol also reduced carrageenan-induced paw edema in rats and that effect was attenuated when the β_2-receptors were blocked by a non-selective β-receptor antagonist propranolol [27]. In this study, we found that β_2-receptor agonists salbutamol and terbutaline inhibited the production of TNF in macrophages. We also found that carrageenan-induced paw edema was reduced by salbutamol, which is in line with the previous studies in rats [27]. These results show that β_2-receptor agonists have anti-inflammatory effects *in vitro* and *in vivo*, and these findings are in line with the previously published reports.

MKPs belong to a larger family of DUSPs, and they are endogenous suppressors of MAPK activity [28,29]. MKPs control MAPK activity by dephosphorylating the activation domain (Thr-X-Tyr, threonine and tyrosine residues) of MAPK, which inactivates the kinase. MKPs regulate the inflammatory response as well as other cellular functions including apoptosis, cell cycle, proliferation and differentiation [7,30].

MKP-1 is an interesting member of the MKP phosphatase family. MKP-1 is expressed in most cell types and tissues in human body. It is a nuclear tyrosine/threonine phosphatase that regulates primarily the activity of p38 MAPK, and in some cells, JNK [5,31]. p38 MAPK pathway is an important regulator of inflammation and immune response [2]. p38 MAPK inhibitors attenuate the production of inflammatory mediators [11,31–33], and they have clear anti-inflammatory effects in experimental models of inflammatory diseases [34–36]. Studies with MKP-1(-/-) mice have shown that MKP-1 suppresses inflammatory gene expression (such as TNF and IL-6), and attenuates acute and chronic inflammatory response by inhibiting p38 MAPK [11,12,31,37]. Despite excessive inflammatory response, MKP-1(-/-) mice display

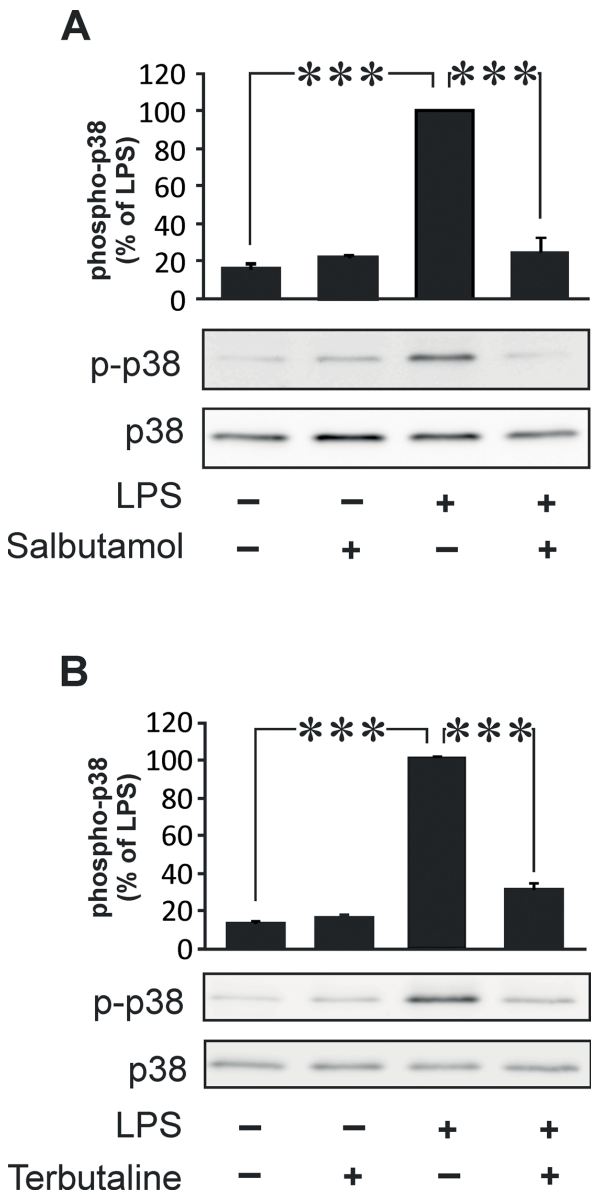

Fig 3. Salbutamol and terbutaline reduced the phosphorylation of p38 MAPK in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of salbutamol (100 nM, A) or terbutaline (100 nM, B) for 1 h, and the phosphorylation of p38 MAPK was detected by Western blot. The chemiluminescent signal was quantified, and phosphorylated p38 MAPK was normalized against total p38 MAPK. Results are expressed as mean ± S.E.M, n = 8. One-way ANOVA with Bonferroni´s post test was performed, and statistical significance is indicated with *** $p < 0.001$ as compared to LPS-treated cells.

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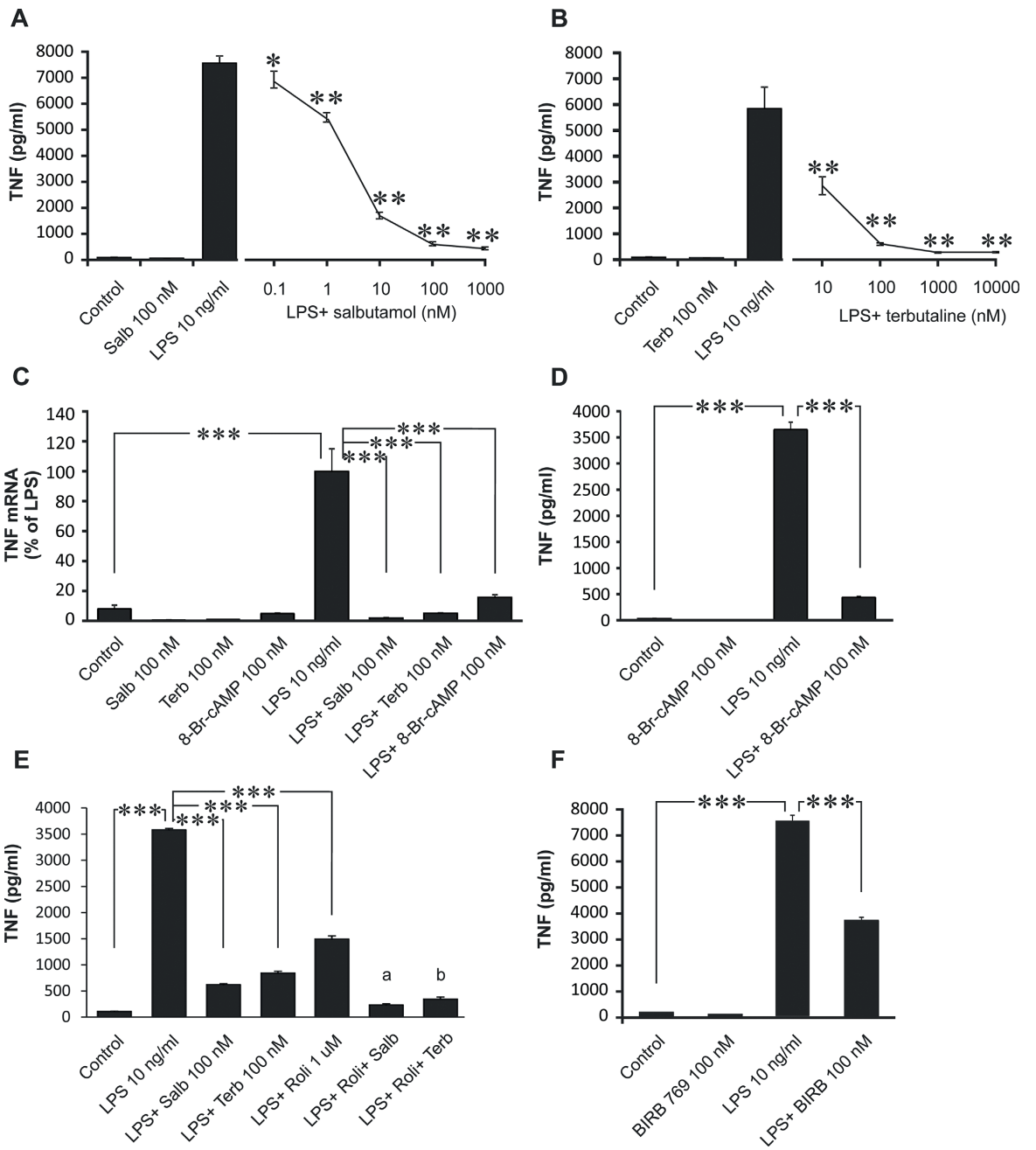

Fig 4. Salbutamol, terbutaline, 8-Br-cAMP and the p38 MAPK inhibitor BIRB 769 inhibited TNF production in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of salbutamol (A, C and E), terbutaline (B, C and E), 8-Br-cAMP (C and D), rolipram (E) or the p38 MAPK inhibitor BIRB 769 (F) for 24 h (A, B, D, E and F; TNF protein) or for 4 h (C; TNF mRNA). TNF mRNA was measured by quantitative

RT-PCR and TNF mRNA expression levels were normalized against GAPDH mRNA levels. TNF protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M., n = 8 (A-C) or n = 4 (D, E and F). One-way ANOVA with Dunnett´s post test (A and B) or Bonferroni´s post test (C-F) was performed and statistical significance is indicated with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared to LPS-treated cells. In the Panel 4E, statistical significance (a) is $p < 0.001$ between LPS+ salbutamol and LPS+ salbutamol+ rolipram treated cells, and (b) is $p < 0.001$ between LPS + terbutaline and LPS+ terbutaline+ rolipram treated cells.

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defective anti-microbial responses due to reduced IL-12 production, impaired Th1 response and excessive IL-10 release [38–40]. Interestingly, in ovalbumin-induced airway inflammation model, a common experimental asthma model, the increased activation of p38 MAPK coincide with the decreased expression of MKP-1 [41].

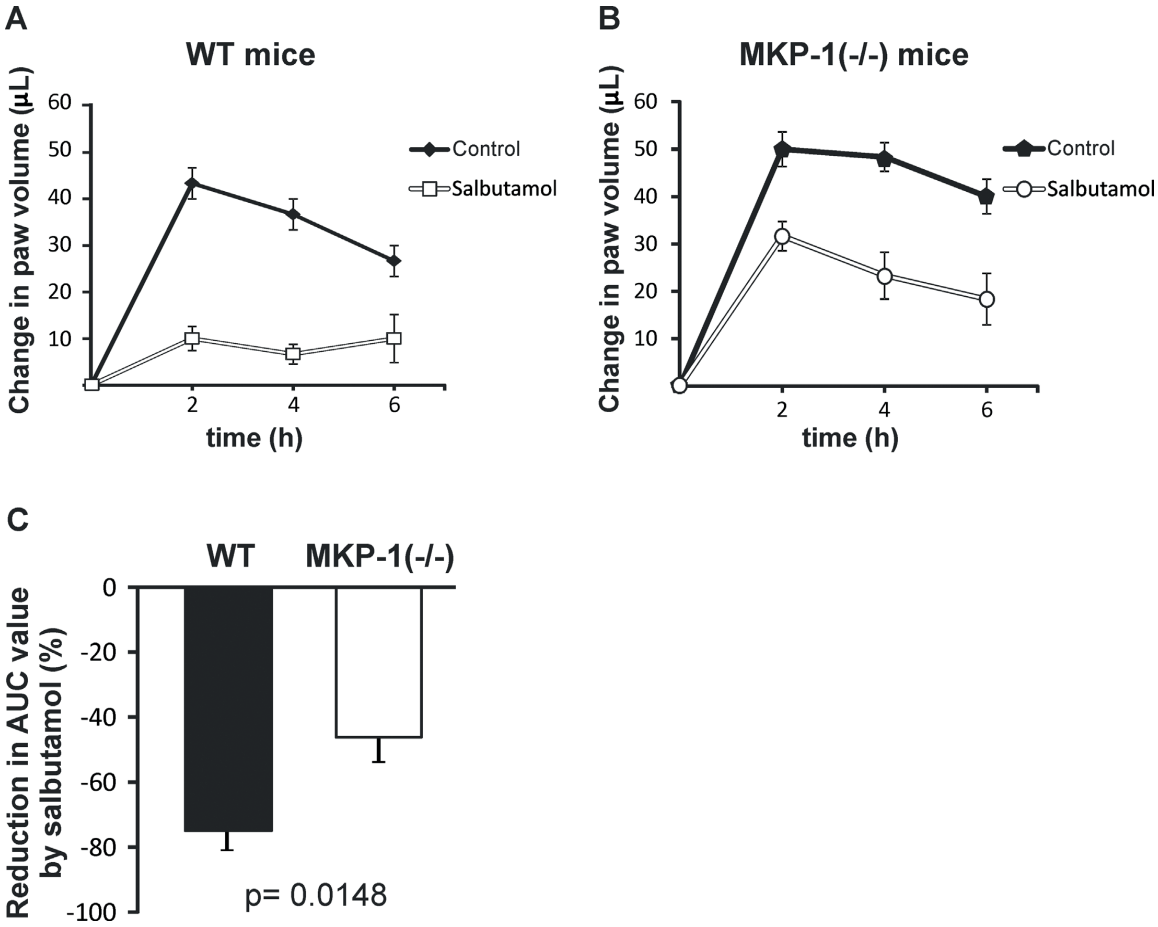

Fig 5. Salbutamol attenuated carrageenan-induced paw inflammation in mice. Mice were treated with salbutamol (5 mg/kg, i.p.) 2 h before the experiment. In the beginning of the experiment (0 h), hind paw volumes were measured with a plethysmometer. After that, carrageenan (2%) or vehicle (control) was injected into the paw and the paw edema was measured with plethysmometer at the time points indicated. Edema is expressed as the difference between the volume changes of the carrageenan-treated paw and the control paw. Mean ± S.E.M., n = 6. The Fig 5C presents the percent decrease in AUC values by salbutamol.

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cAMP is an important intracellular second messenger that mediates many effects of β_2-receptor agonists. MKP-1 promoter contains two binding sites for the transcription factor cAMP responsive element binding protein (CREB) [20]. We found that salbutamol enhanced cAMP level in macrophages, and that cAMP analog 8-Br-cAMP enhanced MKP-1 expression as did β_2-receptor agonists. cAMP has been reported to increase the expression of MKP-1 by activating protein kinase A-CREB pathway [15,42–45].

Suppression of IL-8 production by β_2-agonists has previously been shown to occur concomitantly with increased MKP-1 expression [46,47]. Accordingly, we found here that salbutamol suppressed TNF production in macrophages along with increased MKP-1 expression and decreased p38 MAPK phosphorylation. More interestingly, the current findings extend the previous data by providing *in vivo* evidence that MKP-1 mediates the anti-inflammatory effects of salbutamol, at least partly. Carrageenan-induced acute inflammatory response was inhibited by salbutamol in wild-type mice, but that effect was impaired in MKP1(-/-) mice. This strongly suggests that MKP-1 participates in the anti-inflammatory effects of β_2-receptor agonists. In this study, we found that β_2-agonists were more potent inhibitors of TNF production as compared to that seen with p38 MAPK inhibitor. This suggests that the anti-inflammatory effects of β_2-agonists are mediated not only through inhibition of p38 MAPK activity by MKP-1 but there are other anti-inflammatory mechanisms involved, also. This is also supported by the finding showing that the inhibition of carrageenan-induced inflammatory response by salbutamol was partially but not completely, impaired in MKP-1(-/-) mice. For instance, cAMP has been reported to inhibit macrophage phagocytosis through a cAMP effector Exchange protein activated by cAMP-1 [48].

Importantly, MKP-1 is linked to certain other important anti-inflammatory drug effects. The expression of MKP-1 is increased by glucocorticoids, and MKP-1 mediates, at least partly, the anti-inflammatory effects of glucocorticoids [13,49]. Earlier it has been reported that long-acting β_2-agonists increased MKP-1 levels in airway smooth muscle cells [50].We have shown that disease-modifying anti-rheumatic gold compounds enhance MKP-1 expression along with their inhibitory effects on the production of IL-6, cyclooxygenase-2 and matrix metalloproteinase 3, and those effects were mediated by MKP-1 [14]. Recently, we have also demonstrated that a PDE4 inhibitor rolipram increased MKP-1 levels and suppressed inflammatory response in wild-type mice, but the inhibition of the inflammatory response was severely impaired in MKP-1(-/-) mice [15]. Interestingly, we found here that combining rolipram to β_2-agonist further inhibited TNF production when compared to that with β_2-agonist alone. This is interesting because PDE4 inhibitor roflumilast is used as an anti-inflammatory remedy in COPD and it increases cAMP levels by abrogating the enzymatic degradation of cAMP to 5'AMP [46,51,52]. This further supports the idea that the anti-inflammatory effects of β_2-agonists are mediated by cAMP and that combination of β_2-agonist and PDE4 inhibitor would have improved anti-inflammatory effect through enhanced MKP-1 expression.

Hence, MKP-1 is not only an endogenous suppressor of inflammatory response, but it also seems to mediate therapeutic effects of certain anti-inflammatory drugs. Even though β_2-receptor agonists are primarily used as bronchodilators, they may also have anti-inflammatory effects [19]. Increased MKP-1 expression could be a significant mechanism mediating the anti-inflammatory effects of β_2-receptor agonists. The present findings further emphasize the potential of MKP-1 as a novel anti-inflammatory drug target, and its significance in the pathophysiology and treatment of airway inflammation.

In conclusion, we found that β_2-receptor agonists increased MKP-1 expression and suppressed p38 MAPK phosphorylation (i.e. activity) as well as TNF production in macrophages. Importantly, MKP-1 mediated the anti-inflammatory effects of salbutamol *in vivo*, and this is a

novel finding. The results presented here emphasize the importance of MKP-1 as a novel anti-inflammatory drug target.

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Author Contributions

Conceived and designed the experiments: TK EM RK. Performed the experiments: TK TH MH RK. Analyzed the data: TK EM RK. Contributed reagents/materials/analysis tools: EM RK. Wrote the paper: TK EM RK.

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Suppression of cytokine production by glucocorticoids is mediated by MKP-1 in human lung epithelial cells

Tiina Keränen, Eeva Moilanen, Riku Korhonen

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Suppression of cytokine production by glucocorticoids is mediated by MKP-1 in human lung epithelial cells

Tiina Keränen, Eeva Moilanen, and Riku Korhonen

The Immunopharmacology Research Group, University of Tampere School of Medicine, and

Tampere University Hospital, Tampere, Finland

Corresponding author:
Riku Korhonen
Immunopharmacology Research Group, University of Tampere School of Medicine,
Medisiinarinkatu 3, FIN-33014 University of Tampere, Finland
Phone: +358 50 396 9644
Fax: +358 3 364 0558
Email: riku.korhonen@staff.uta.fi

Abstract

Mitogen-activated protein kinase phosphatase 1 (MKP-1) expression is induced by inflammatory factors and serves as an endogenous p38 MAPK suppressor to limit inflammatory response. Glucocorticoids are most effective anti-inflammatory drugs and they are used for the treatment of various inflammatory disease, such as asthma and COPD. We investigated the role of MKP-1 in the inhibition of cytokine production by dexamethasone in human A549 bronchial epithelial cells. We found that dexamethasone increased MKP-1 expression, inhibited p38 MAPK phosphorylation and suppressed TNF and MIP-3α production in A549 cells. Interestingly, the suppression of p38 MAPK phosphorylation and the inhibition of TNF expression by dexamethasone were attenuated in cells where MKP-1 expression was silenced by siRNA. In conclusion, these data suggest that dexamethasone increases MKP-1 expression and this results in the suppression of p38 MAPK signaling leading to the inhibition of cytokine production in human bronchial epithelial cells. These results point to the role of MKP-1 is an important factor in the therapeutic effects of glucocorticoids in the treatment of inflammatory lung diseases.

Key words

MKP-1; DUSP1; cytokine; inflammation

Nonstandard abbreviations: AP-1, Activator protein-1; COPD, chronic obstructive pulmonary disease; COX2, cyclooxygenase 2; DUSP, dual-specificity phosphatase; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MIP, macrophage inflammatory protein; MKP, mitogen-activated protein kinase phosphatase; NF- κB, nuclear factor κB ; siRNA, small interfering RNA; TNF, tumor necrosis factor

1. Introduction

Mitogen-activated protein kinases (MAPKs) are an evolutionary conserved family of intracellular signaling proteins that regulate many physiological cellular processes, such as cell growth, proliferation, differentiation, motility, stress response, survival and apoptosis [1,2]. The three main MAPK groups are p38 MAP kinases, JNK (Jun N-terminal kinase) and ERK (extracellular signal-regulated kinase) [1,2]. MAPK pathways are three-tier kinase cascades that are activated in response to several extracellular signals, such as cytokines, growth factors and bacterial substances through G-protein-coupled and/or kinase-linked receptors. MAPK are activated by phosphorylation of threonine and tyrosine residues, and their targets include transcription factors and other cellular regulatory proteins [3,4]. p38 MAPK and JNK have also a marked role in the regulation of inflammation and immune response. They regulate the production of inflammatory cytokines (for example TNF and IL-6) and other mediators, such as prostaglandins and nitric oxide, during inflammatory response, and they augment Th1 type immune response and support the activation and functions of Th1 cells. [1,2,5,6].

Mitogen-activated protein kinase phosphatases (MKPs) belong to dual specificity phosphatases (DUSPs) that dephosphorylate tyrosine and threonine residues of their target proteins. MKPs specifically dephosphorylate MAPKs, which makes them endogenous suppressors of activated MAPK pathways. MKPs include eleven members, and they display differences in substrate specificity among MAPKs, tissue distribution, cellular location and expressional pattern [7,8]. MKP-1 is an interesting member of MKPs. It is a nuclear phosphatase and it regulates p38 MAPK, and in some cells, JNK activity [6,9]. By inhibiting p38 MAPK, MKP-1 suppresses inflammatory gene expression (such as TNF and IL-6) and attenuates acute inflammatory response by inhibition

of p38 MAPK [10,11]. MKP-1 has been reported to mediate certain anti-inflammatory drug effects. For instance, anti-rheumatic gold-compounds and phosphodiesterase 4 inhibitors increase MKP-1 expression, and MKP-1 mediates, at least partly, the anti-inflammatory effects of these drugs [12,13]. Other members of MKP family (such as MKP-5 and DUSP2) have also been found to regulate inflammatory response [14,15], but their role in anti-inflammatory drug effects is not known.

Dexamethasone is a long-acting glucocorticoid. Glucocorticoids are used in the treatment of various inflammatory disease, such as asthma, COPD, osteoarthritis, autoimmune diseases and allergies [16]. Glucocorticoids are highly effective anti-inflammatory drugs, at least in the short-term treatment. Glucocorticoids prevent extensive inflammatory cell activation and function as well as production of a large number of inflammatory mediators [17]. They inhibit activity of important transcription factors nuclear factor κB (NF-κB) and activator protein-1 (AP-1) [16]. Glucocorticoids reduce the expression of various cytokines such as TNF, interleukins and hematopoietic growth factors including GM-CSF as well as inflammatory enzymes, e.g. COX-2 and iNOS which produce prostanoids and nitric oxide, respectively [17]. Glucocorticoid receptor (GR) dimerizes and binds to glucocorticoid response elements (GREs) within the promoter sequence in a number of genes and upregulates transcription. MKP-1 promotor contains a glucocorticoid response element GRE [18]. It has been shown that dexamethasone increases MKP-1 expression and the anti-inflammatory effect of dexamethasone is at least partly mediated by MKP-1 [19-22]. Ovalbumin-sensitized mice were found to have increased activation of p38 MAPK pathway and downregulation of MKP-1 expression in the lung tissue. In these mice, treatment with dexamethasone and p38 MAPK inhibitor effectively reduced lung inflammation

and inactivated p38 MAPK and upregulated MKP-1 in the lung tissue [23]. In the present study we investigated the effects of dexamethasone on MKP-1 expression and further, whether MKP-1 is involved in the anti-inflammatory effects of dexamethasone.

2. Methods

2.1 Materials

Recombinant human IL-1β was purchased from R&D systems Inc. (Minneapolis, Mass, USA) and BIRB 769 [1-(5-*tert*-butyl-2-*p*-tolyl-2H-pyrazol-3-yl)-3(4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl)urea] from Axon Medchem BV (Groningen, the Netherlands). All other reagents were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless otherwise stated below.

2.2 Cell culture

A549 human lung epithelial cells (ATCC, Manassas, VA, USA) were cultured at +37 °C in 5 % CO_2 atmosphere in Ham´s F-12K (Kaighn´s modification) medium supplemented with 5% (v/v) heat-inactivated FBS (fetal bovine serum), 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (all from Invitrogen, Paisley, UK). For experiments, cells (4 x 10^5 cells/ well) were seeded on 24-well plates and the cell monolayers were grown for 48 h before the experiments were started.

For siRNA experiments, cells were seeded at the density of 5 x 10^4 cells/well on a 24-well plate in 500 µl of medium with 5% (v/v) heat-inactivated FBS without antibiotics followed by transfection with non-targeting control siRNA (siNEG) or MKP-1 siRNA pool (siMKP-1). Cells were incubated for 24 h before transfection and for additional 24 h before the experiments were started.

Dexamethasone and IL-1β were dissolved in phosphate buffered saline (PBS). Other compounds of interest at concentration indicated or the solvent (DMSO, 0.1 % v/v) were added to the cells in

fresh culture medium containing 5 % FBS and the supplements. Cells were further incubated for the time indicated.

2.3 Preparation of cell lysates and Western Blot analysis

At the indicated time points, culture medium was removed. Cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer containing 10 mM Tris-HCL, 5 mM EDTA, 50 mM NaCl, 1 % Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 µM *n*-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged (12 000 g, 10 min, +4 °C) and supernatants were collected and mixed in a ratio 1:4 with SDS loading buffer (62.5 mM Tris-HCL, pH 6.8, 10 % glycerol, 2 % SDS, 0.025 % bromophenol blue and 5 % β-mercaptoethanol) and stored at -20 °C until analyzed. Protein concentrations in the samples were measures by the Coomassie Brilliant Blue method (Coomassie Protein Assay Reagent Kit; Pierce, Rockford, IL, USA).

Before Western blot analysis, the samples were boiled for 5 min. Equal aliquots of protein (20 µg) were loaded on 12 % SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred to membrane by iBlot® Dry Blotting System (Life Technologies, Carlsbad, California) following manufacturer's instructions. After transfer the membrane was blocked in TBS/T (20 mM Tris-base (pH 7.6), 150 mM NaCl, 0.1 % Tween-20) containing 5 % non-fat milk for 1 h at room temperature. For detection of phosphorylated proteins, membranes were blocked in TBS/T containing 5 % bovine serum albumin (BSA). Membranes were incubated overnight at +4 °C with the primary antibody and at room temperature for 1 h with the secondary antibody, and the

chemiluminescent signal was detected by ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The chemiluminescent signal was quantified with FluoChem program (version 3.1) and Image Quant TL 7.0 Image Analysis software.

Following antibodies were using in the Western blot analysis: MKP-1 (sc-1102; Santa Cruz Biotechnology, CA, USA), p38 MAPK (ab27986; Abcam, Cambridge, UK) and phosphor-p38 MAPK (#9211; Cell Signaling Technology Inc., MA, USA), as well as actin (sc-1616), polyclonal anti-goat (sc-2020) and polyclonal anti-rabbit (sc-2004) antibodies (the three latter from Santa Cruz Biotechnology, CA, USA).

2.4 RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

At the indicated time points, the culture medium was removed, and cell homogenization and total RNA extraction was carried out by using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Inc., MO, USA) according to the manufacturer´s instructions. Reverse transcription of RNA to cDNA was performed by TaqMan® Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA, USA), according to the supplier´s instructions. The primer and probe sequences and concentrations were optimized according to the manufacturer´s guidelines in TagMan® Universal PCR Master Mix Protocol part number 4304449 revision C (Applied Biosystems, Branchburg, NJ, USA). The following primer and probe sequences were used: for human GAPDH 5'-AAGGTCGGAGTCAACGGATTT-3' (forward), 5'-GCAACAATATCCACTTTACCAGAGTTAA-3' (reverse) and 5'-CGCCTGGTCACCAGGGCTGC-3' (probe containing 6-FAM as the 5´-reporter dye and

TAMRA as the 3´-quencher). Primers and probes were obtained from Metabion (Martinsried, Germany). Expression of human MKP-1, TNF and MIP-3α mRNA were measured using TagMan gene expression assays (Applied Biosystems).

PCR reaction parameters were as follows: incubation at +50 °C for 2 min and at +95 °C for 10 min, and thereafter 40 cycles of denaturation at +95 °C for 15 s and annealing and extension at +60 °C for 1 min. A standard curve method was used to estimate the relative mRNA levels. When calculating the results, MKP-1, TNF and MIP-3α mRNA levels were first normalized against GAPDH.

2.5 Down-regulation of MKP-1 by siRNA

MKP-1 siRNA SMARTpool (5´-AGGAGGAUACGAAGCGUUU-3´, 5´-UUUGUGAAGCAGAGGCGAA-3´, 5´-GCUUACCUUAUGAGGACUA-3´, 5´-CCAACCAUUUUGAGGGUCA-3´) was purchased from Dharmacon (Lafayette, CO, USA). Nontargeting control siRNA (5´-AATTCTCCGAACGTGTCACGT-3´) was obtained from Qiagen (Valencia, CA, USA).

A549 cells were transfected with siRNA using Dharmafect transfection reagent (Dharmacon) according to the manufacturer´s instruction. Fresh cultured medium was changed after 24 h transfection and cytokines and dexamethasone were added into the culture medium. Cells were further incubates for the time indicated and gene expression was analyzed. Transfection efficacy was monitored with green fluorescent siRNA oligos (siGLO green transfection indicator;

Dharmacon RNA Technologies). Approximately 90 % of the cells emitted green fluorescence signal when transfected with siGLO and Dharmafect.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium samples were kept at -20 °C until assayed. The concentrations of human TNF (Duoset® ELISA Development System human TNF kit; R&D Systems Europe Ltd., Abindgon, UK) and MIP-3α (Duoset® ELISA Development System human MIP-3α kit; R&D Systems Europe Ltd.) were determined by ELISA according to the manufacturer´s instructions.

2.7 Statistics

Results are expressed as mean ± standard error of mean (S.E.M.). When appropriate, statistical analyses were performed with GraphPad Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA). P values less than 0.05 were considered significant.

3. Results

3.1 Dexamethasone enhanced MKP-1 expression in human lung epithelia cells.

In this study, we investigated the effects of dexamethasone on MKP-1 expression and inflammatory gene expression in A549 human lung epithelial cells. First, the expression of MKP-1 was investigated in A549 cells in response to IL-1β and the combination of IL-1β and dexamethasone over time. MKP-1 mRNA expression was increased by IL-1β at 1 h (Fig. 1). MKP-1 expression was then returned near the basal level at 2 h and then remained slightly elevated up to 24 h. In cells treated with a combination of IL-1β and dexamethasone, MKP-1 expression was further enhanced compared to IL-1β -stimulated cells at 1 h, and in the follow-up, MKP-1 mRNA levels remained moderately elevated up to 24 h.

MKP-1 is an endogenous suppressor of p38 MAPK activity. Therefore, we investigated the effect of dexamethasone on p38 MAPK phosphorylation. The phosphorylation of p38 MAPK was increased in response to IL-1β and it was inhibited by dexamethasone in A549 human lung epithelial cells at time point 2 h (Fig. 2).

3.2 Dexamethasone inhibited TNF and MIP-3α expression in activated human lung epithelial cell.

TNF is a key inflammatory mediator and MIP-3α a potent chemokine present in inflamed pulmonary tissue, and their expression is increased by inflammatory signals [24-26]. Therefore, we continued by investigating the effects of dexamethasone on TNF and MIP-3α production. Dexamethasone inhibited IL-1β-induced TNF and MIP-3α mRNA and protein levels in A549 lung

epithelial cells (Fig. 3 and 4). Since dexamethasone inhibited p38 MAPK phosphorylation and TNF and MIP-3α expression, we tested the effect of p38 MAPK inhibitor on TNF and MIP-3α levels. p38 MAPK inhibitor BIRB 796 inhibited IL-1β-induced TNF and MIP-3α release in human lung epithelia cells (Fig. 3B and 4B).

3.3 MKP-1 regulated TNF production in human lung epithelial cells

We then proceeded to investigate the role of MKP-1 in the inhibition of TNF expression by dexamethasone. In these experiments, we silenced MKP-1 by siRNA. MKP-1 protein levels in cells stimulated with IL-1β or combination of IL-1β and dexamethasone were effectively reduced in cells transfected with MKP-1 siRNA (siMKP-1) compared to the cells transfected by control siRNA (siNeg) (Fig. 5A). This shows that MKP-1 expression was effectively down-regulated by siRNA. Further, p38 MAPK phosphorylation was inhibited by dexamethasone in cells treated with control siRNA, but the inhibition of p38 MAPK phosphorylation was attenuated in cells treated with MKP-1 siRNA (Fig. 5B). In cells transfected with control siRNA, dexamethasone inhibited TNF mRNA levels, while in MKP-1 siRNA transfected cells, TNF mRNA expression was not reduced by dexamethasone (Fig. 6). These results suggest that MKP-1 is present and functional in A549 cells and it mediates the inhibition of p38 MAPK phosphorylation and TNF expression by dexamethasone.

4. Discussion

The aim of this study was to investigate the effect of dexamethasone on MKP-1 expression and on TNF and MIP-3α production in A549 human lung epithelial cell. We found that dexamethasone increased MKP-1 expression, inhibited the phosphorylation of p38 MAPK and suppressed the production of TNF and MIP-3α. Importantly, the effect of dexamethasone on p38 MAPK phosphorylation and cytokine expression was attenuated in cells transfected with MKP-1 siRNA. This suggests that anti-inflammatory effects of dexamethasone are partly mediated by MKP-1.

Chronic obstructive pulmonary disease (COPD) and asthma are chronic inflammatory lung diseases characterized by persistent airway inflammation. Inflammation results in progressive airway obstruction and dyspnea, and, in advanced stage, fibrotic changes in the lung, reduced quality of life and increased mortality. Inflammatory and immune cells, including macrophages, activated T lymphocytes, dendritic cells and granulocytes, are often present in the inflamed lung. Those cells together with lung epithelial cells produce excessive amounts of inflammatory factors, such as cytokines (e.g. TNF, IL-1 and IL-6,), chemokines (e.g. MCP-1, IL-8 and MIP-3α), inflammatory mediators (prostaglandins, nitric oxide) and growth factors (GM-CSF) that sustain and amplify the airway inflammation [27-29]. Inhaled glucocorticoids are used in the treatment of COPD and asthma to dampen the chronic inflammation [30].

Mitogen-activated protein kinase phosphatases (MKPs) belong to a larger family of DUSPs, and they are endogenous suppressors of MAPK activity [31,32]. MKPs controls MAPK activity by dephosphorylating the activation domain (Thr-X-Tyr, threonine and tyrosine residues) of MAPK,

which inactivates the kinase. MKPs regulate the inflammatory response as well as other cellular functions including apoptosis, cell cycle, proliferation and differentiation [7,9,33].

MKP-1 is a well characterized member of the MKP phosphatase family. MKP-1 is expressed in many cell types and tissues. It is a nuclear tyrosine/threonine phosphatase that regulates primarily the activity of p38 MAPK, and in some cells, JNK [5,34]. p38 MAPK pathway is an important regulator of inflammation and immune response [2]. p38 MAPK inhibitors attenuate the production of inflammatory mediators [10,34-36], and they have potent anti-inflammatory effects in experimental models of inflammatory diseases [37-39]. Studies with MKP-1(-/-) mice have shown that MKP-1 suppresses inflammatory gene expression (such as TNF and IL-6), and attenuates acute and chronic inflammatory response by inhibiting p38 MAPK [10,11,34,40]. Despite excessive inflammatory response, MKP-1(-/-) mice display defective anti-microbial responses due to reduced IL-12 production, impaired Th1 response and excessive IL-10 release [41-43].

Glucocorticoids are very effective and widely used anti-inflammatory drugs in the treatment of various inflammatory conditions, such as asthma and COPD, rheumatic and other autoimmune diseases, as well as allergic and hypersensitivity reactions [16]. At cellular level, glucocorticoids prevent inflammatory cell migration and accumulation into the inflamed tissues as well as leukocyte activation and function, such as release of inflammatory mediators and cytotoxic functions [17]. Glucocorticoids inhibit the activity of key inflammatory transcription factors NF-κB and AP-1 [16], and thereby reduce the production of cytokines, such as TNF and

interleukins, and chemotactic and hematopoietic growth factors as well as the production of prostaglandins and nitric oxide through COX-2 and iNOS pathways, respectively [17].

In this study, we found that dexamethasone increased MKP-1 levels and inhibited the production of cytokine and chemokine production in human lung epithelial cell. Importantly, the phosphorylation of p38 MAP kinase and the inhibition of TNF production by dexamethasone was attenuated, when MKP-1 was silenced with siRNA. These results strongly suggest that anti-inflammatory effects of glucocorticoids are mediated by MKP-1 in human lung epithelial cells. MKP-1 expression has been shown to be increased by glucocorticoids [44]. In knockout mice model, MKP-1 has been shown to mediate the suppression of macrophage functions and systemic inflammation by glucocorticoids [20,45]. Also, attenuation of ozone-induced airway constriction by glucocorticoids has been shown to be mediated by MKP-1 [46]. Our results suggest that anti-inflammatory therapeutic effects of glucocorticoids in human lung epithelial cells are also mediated by MKP-1. This likely contributes to the therapeutic effects of glucocorticoids used in the treatment of chronic inflammatory lung diseases asthma and COPD. Airway epithelium has been shown to produce many inflammatory factors and play an important role in airway inflammation [47].

Besides MKP-1 is an important endogenous anti-inflammatory factor [6,9], MKP-1 has been found to mediate certain anti-inflammatory drug effects other than those of glucocorticoids. Our group has previously reported, that disease-modifying anti-rheumatic gold compounds reduce the expression of IL-6, cyclooxygenase-2 and matrix metalloproteinase 3, and those effects were mediated by MKP-1 [12]. Recently, we have demonstrated that phosphodiesterase (PDE) 4

inhibitor rolipram and β_2-receptor agonists salbutamol and terbutaline increased MKP-1 levels and suppressed inflammatory response in wild-type mice, but the response was impaired in MKP-1(-/-) mice [13,48]. Hence, MKP-1 is not only an endogenous suppressor of inflammatory response, but it is also an important mediator of anti-inflammatory drug effects.

5. Conclusions

In this study, we found that dexamethasone increased MKP-1 expression and suppressed p38 MAPK phosphorylation (i.e. activity) as well as cytokine and chemokine production in human lung epithelia cells. Importantly, the effect of dexamethasone was decreased in cells where MKP-1 was silenced by siRNA. This suggests that anti-inflammatory effects of dexamethasone are partly mediated by MKP-1 in human lung epithelial cells. The results presented here emphasize the importance of MKP-1 as a mediator of therapeutic effects of glucocorticoids in inflammatory lung diseases as well as its potential as a novel anti-inflammatory drug target.

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7. Author Contributions

Participated in research design: Tiina Keränen, Riku Korhonen, Eeva Moilanen

Conducted experiments: Tiina Keränen

Performed data analysis: Tiina Keränen, Riku Korhonen, Eeva Moilanen

Wrote or contributed to the writing of the manuscript: Tiina Keränen, Riku Korhonen, Eeva Moilanen

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9. Footnotes

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Figure Legends

Fig 1. Dexamethasone enhanced MKP-1 expression in A549 cells

A549 cells were stimulated with IL-1β in the presence or in the absence of dexamethasone for the time indicated. MKP-1 mRNA was measured by quantitative RT-PCR, and MKP-1 mRNA expression levels were normalized against GAPDH mRNA levels. Results are expressed as mean ± S.E.M., n=4. One-way ANOVA with Bonferroni´s post test was performed, and statistical significance is indicated with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between IL-1β and control or IL-1β+ Dexa and IL-1β at each time point.

Fig 2. Dexamethasone reduced the phosphorylation of p38 MAPK in A549

A549 cells were stimulated with IL-1β in the absence or in the presence of dexamethasone for the time indicated, and the phosphorylation of p38 MAPK was detected by Western blot. The chemiluminescent signal was quantified, and phosphorylated p38 MAPK was normalized against total p38 MAPK. Results are expressed as mean ± S.E.M, n=4. One-way ANOVA with Bonferroni´s post test was performed, and statistical significance is indicated with *** $p < 0.001$.

Fig 3. Dexamethasone and the p38 MAPK inhibitor BIRB 769 inhibited TNF production in A549 cells

A549 cells were stimulated with IL-1β in the absence or in the presence of dexamethasone (A and B) or the p38 MAPK inhibitor BIRB 769 (B) for the time indicated (A) or for 24 h (B). In A, TNF mRNA was measured by quantitative RT-PCR and TNF mRNA expression levels were

normalized against GAPDH mRNA levels. In B, TNF protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M., n=4. One-way ANOVA with Bonferroni´s post test was performed and statistical significance is indicated with ** $p < 0.01$ and *** $p < 0.001$.

Fig 4. Dexamethasone and the p38 MAPK inhibitor BIRB 769 inhibited MIP-3α production in A549 cells

A549 cells were stimulated with IL-1β in the absence or in the presence of dexamethasone (A and B) or the p38 MAPK inhibitor BIRB 769 (B) for the time indicated (A) or for 24 h (B). In A, MIP-3α mRNA was measured by quantitative RT-PCR and MIP-3α mRNA expression levels were normalized against GAPDH mRNA levels. In B, MIP-3α protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M**.,** n=4. One-way ANOVA with Bonferroni´s post test was performed and statistical significance is indicated with *** $p < 0.001$.

Fig 5. MKP-1 siRNA effectively down-regulated MKP-1 expression

A549 cells were transfected with MKP-1 siRNA (siMKP-1) or control siRNA (siNeg) for 24 h. Cells were stimulated with IL-1β in the absence or in the presence of dexamethasone for 1 h (A) or preincubated 1 h and stimulated for 30 min (B). In A, MKP-1 protein production was detected by Western blot. The chemiluminescent signal was quantified, and MKP-1 was normalized against actin. In B, phosphorylated p38 MAPK was detected by Western blot. The chemiluminescent signal was quantified, and pp38 was normalized against total p38. Results are expressed as

mean ± S.E.M, n=4. One-way ANOVA with Bonferroni´s post test was performed and statistical significance is indicated with n.s = not significant, ** $p < 0.01$ and *** $p < 0.001$.

Fig 6. Dexamethasone inhibited TNF production more in transfected A549 cells transfected with control siRNA but not in cells transfected with MKP-1 siRNA

A549 cells were transfected with MKP-1 siRNA (siMKP-1) or control siRNA (siNeg) for 24 h. Thereafter the cells were preincubated with dexamethasone for 1 h and stimulated with IL-1β for 4 h. TNF mRNA was measured by quantitative RT-PCR and TNF mRNA expression levels were normalized against GAPDH mRNA levels. Results are expressed as mean ± S.E.M, n=6. Two-way ANOVA with Bonferroni´s post test was performed and statistical significance is indicated with n.s = not significant, ** $p < 0.01$.

Figure 1.

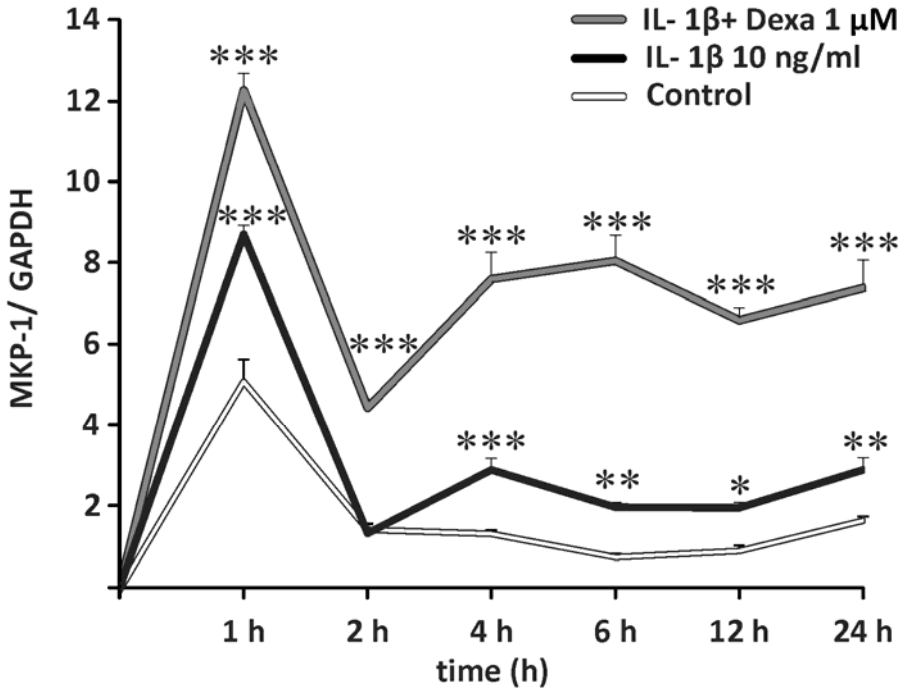


Figure 2.

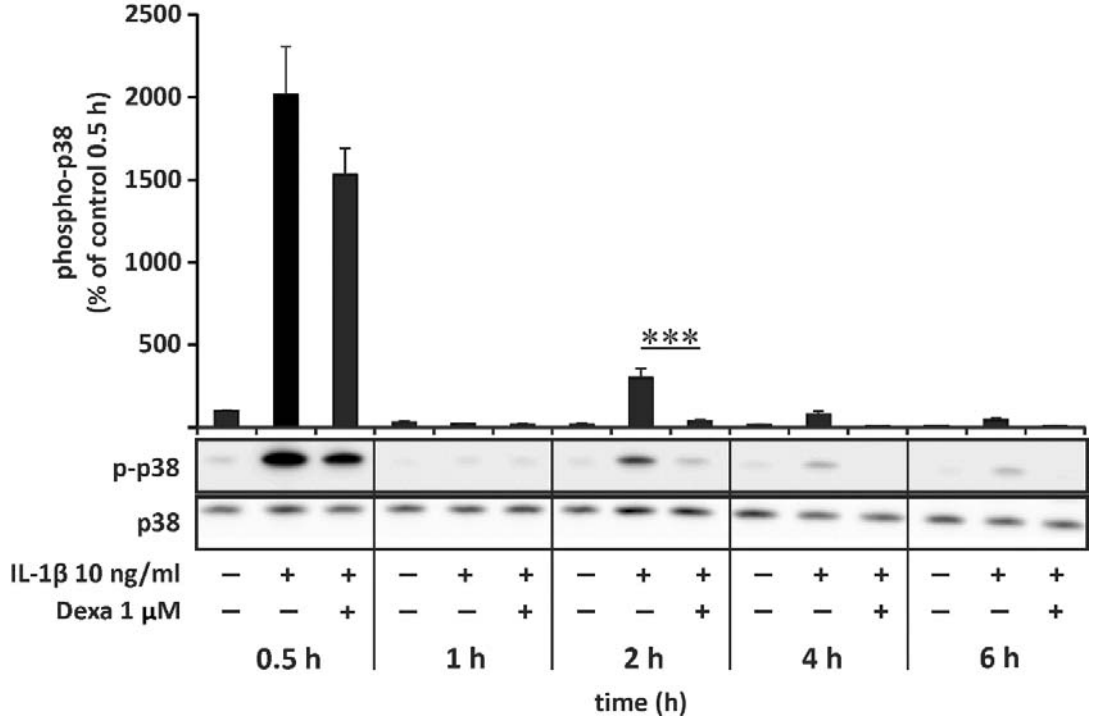
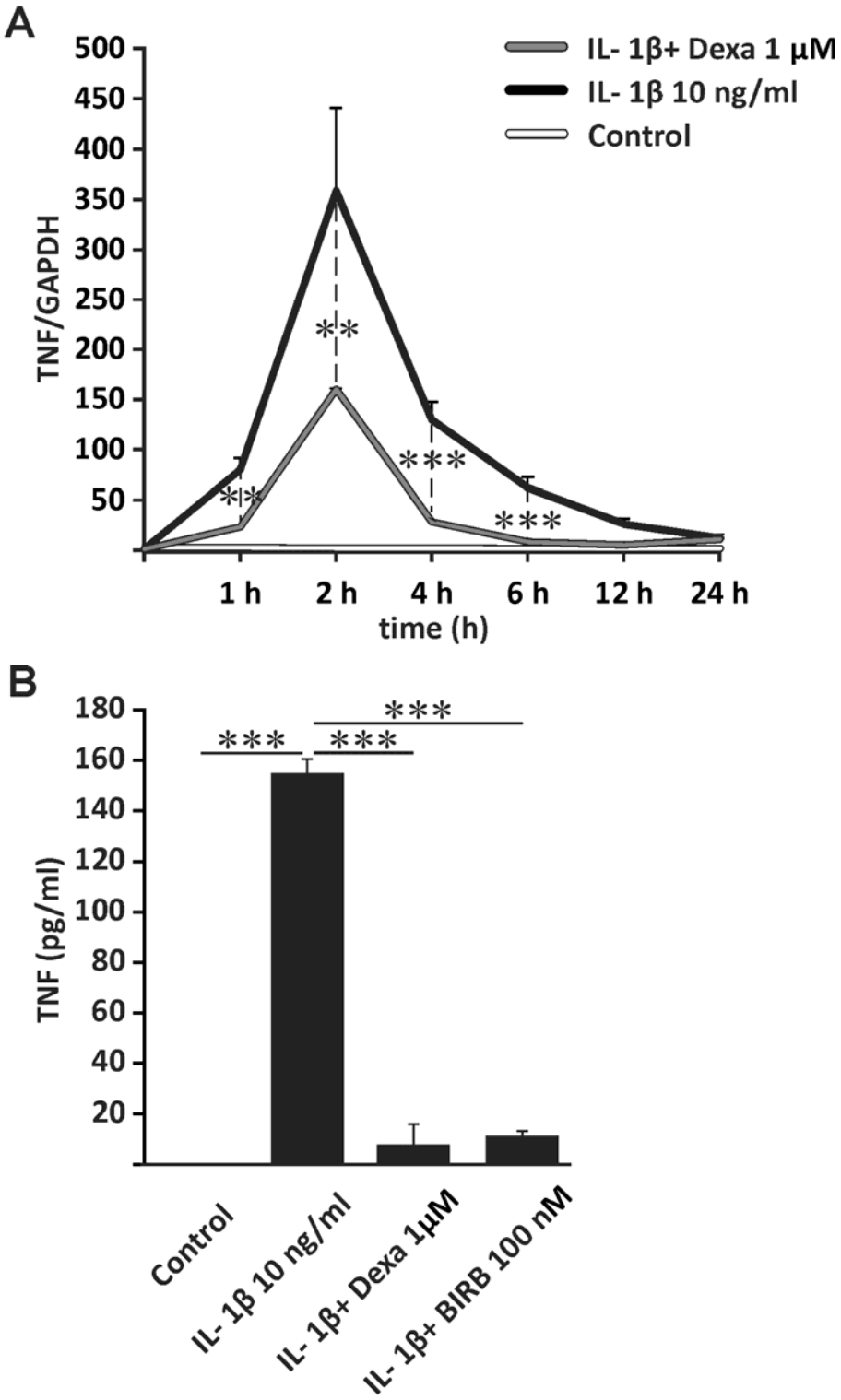


Figure 3.


A
IL- 1β+ Dexa 1 μM
IL- 1β 10 ng/ml
Control
TNF/GAPDH
500
450
400
350
300
250
200
150
100
50
**
***
***
***
1 h
2 h
4 h
6 h
12 h
24 h
time (h)
B
TNF (pg/ml)
180
160
140
120
100
80
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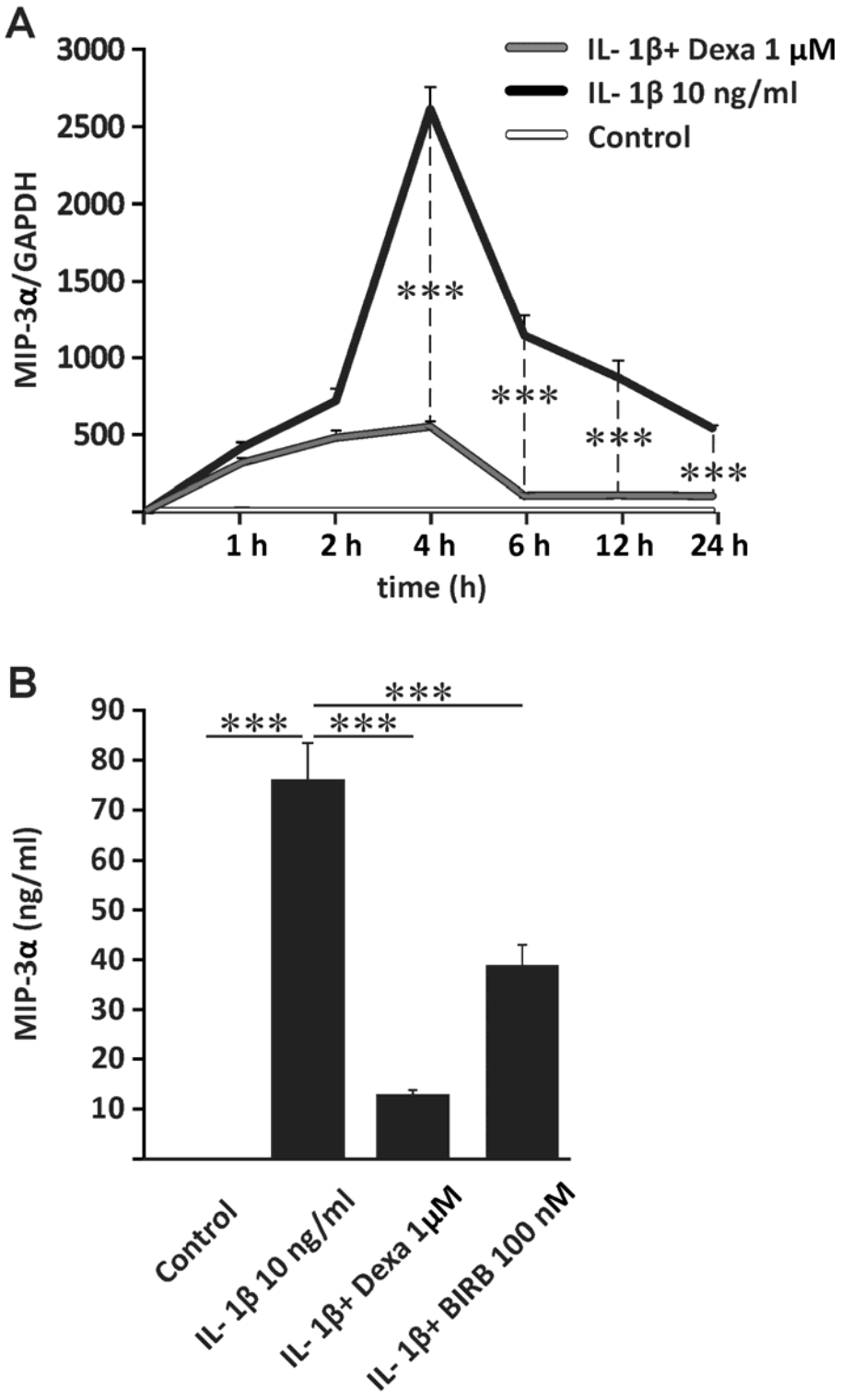
***
Control
IL- 1β 10 ng/ml
IL- 1β+ Dexa 1μM
IL- 1β+ BIRB 100 nM

Figure 4.


A
IL- 1β+ Dexa 1 μM
IL- 1β 10 ng/ml
Control
MIP-3α/GAPDH
3000
2500
2000
1500
1000
500
***
***
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***
1 h
2 h
4 h
6 h
12 h
24 h
time (h)
B
MIP-3α (ng/ml)
90
80
70
60
50
40
30
20
10
***
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***
Control
IL- 1β 10 ng/ml
IL- 1β+ Dexa 1μM
IL- 1β+ BIRB 100 nM

Figure 5.

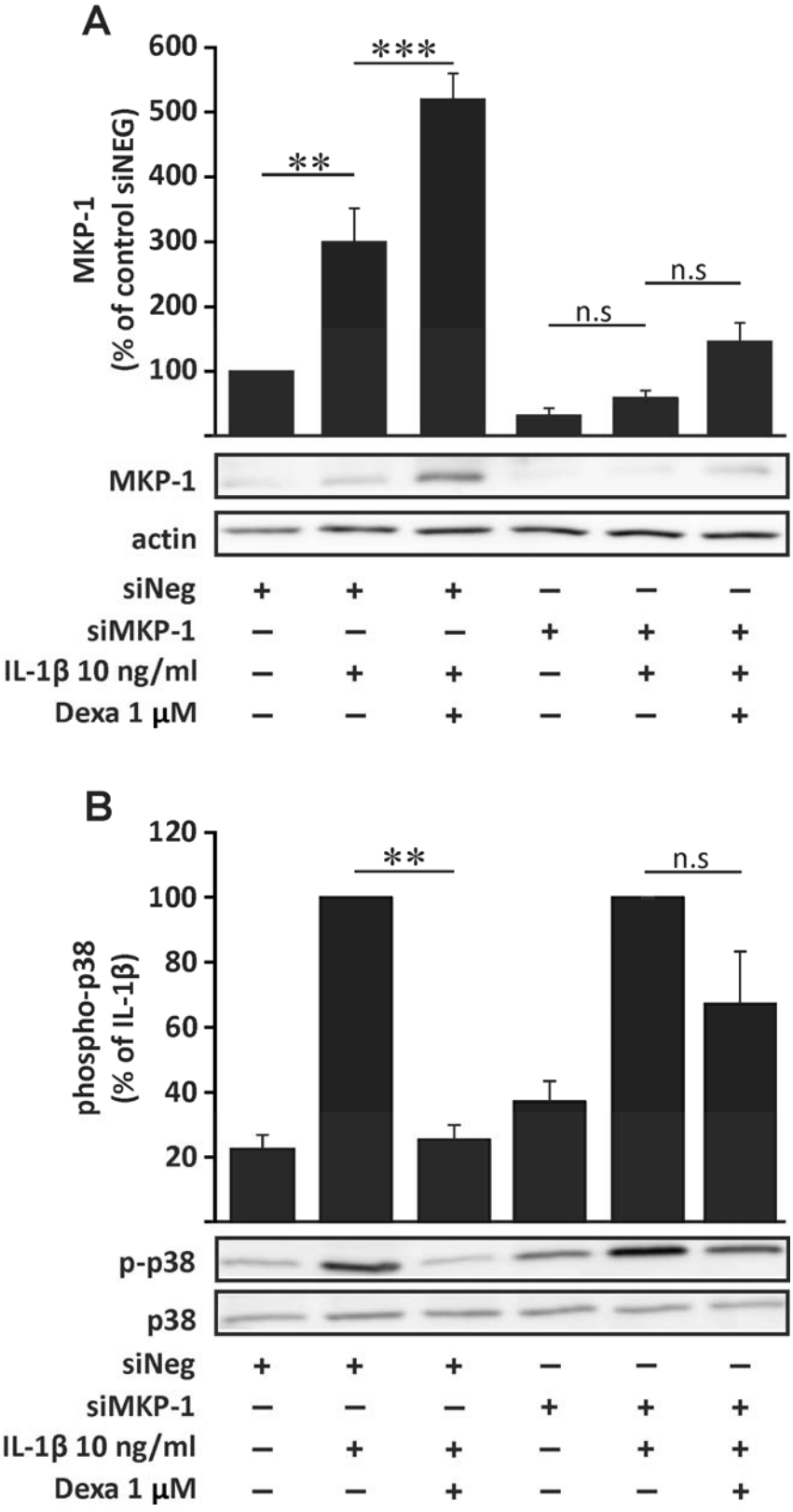
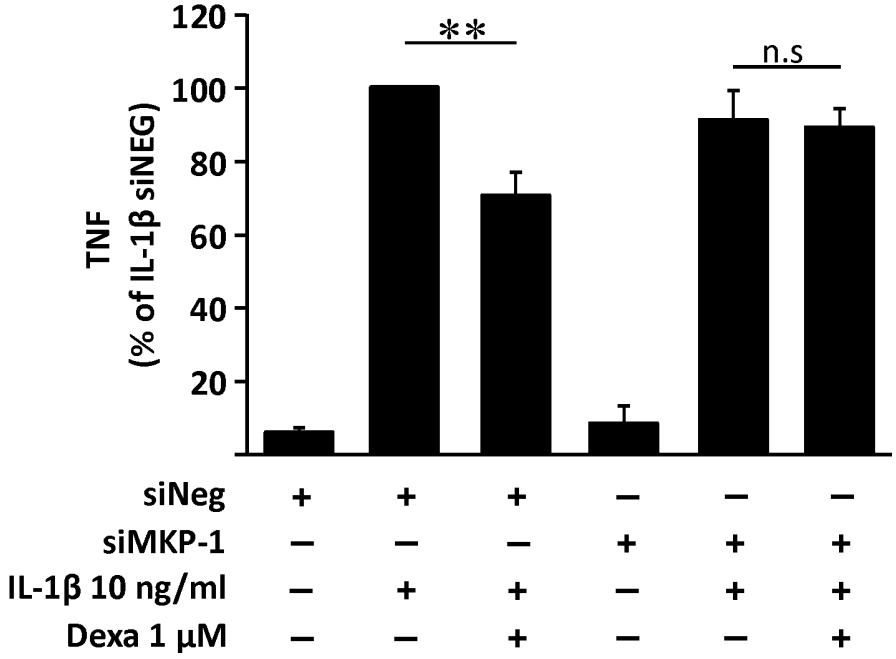

A
MKP-1 (% of control siNEG)
600
500
400
300
200
100
**
***
n.s
n.s
MKP-1
actin
siNeg + + + – – –
siMKP-1 – – – + + +
IL-1β 10 ng/ml – + + – + +
Dexa 1 µM – – + – – +
B
phospho-p38 (% of IL-1β)
120
100
80
60
40
20
**
n.s
p-p38
p38
siNeg + + + – – –
siMKP-1 – – – + + +
IL-1β 10 ng/ml – + + – + +
Dexa 1 µM – – + – – +

Figure 6.


120
100
80
60
40
20
TNF
(% of IL-1β siNEG)
**
n.s
siNeg + + + – – –
siMKP-1 – – – + + +
IL-1β 10 ng/ml – + + – + +
Dexa 1 µM – – + – – +

PUBLICATION III

β_2-receptor agonists salbutamol and terbutaline attenuated cytokine production by suppressing ERK pathway through cAMP in macrophages

Tiina Keränen, Tuija Hömmö, Eeva Moilanen, Riku Korhonen

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β_2-receptor agonists salbutamol and terbutaline attenuated cytokine production by suppressing ERK pathway through cAMP in macrophages ☆

Tiina Keränen, Tuija Hömmö, Eeva Moilanen, Riku Korhonen *

The Immunopharmacology Research Group, University of Tampere School of Medicine, and Tampere University Hospital, Tampere, Finland

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ABSTRACT

β_2-receptor agonists are used in the treatment of inflammatory obstructive lung diseases asthma and COPD as a symptomatic remedy, but they have been suggested to possess anti-inflammatory properties, also. β_2-receptor activation is considered to lead to the activation of ERK pathway through G-protein- and cAMP-independent mechanisms. In this study, we investigated the effects of β_2-receptor agonists salbutamol and terbutaline on the production of inflammatory factors in macrophages. We found that β_2-receptor agonists inhibited LPS-induced ERK phosphorylation and the production of MCP-1. A chemical cAMP analog 8-Br-cAMP also inhibited ERK phosphorylation and TNF and MCP-1 release. As expected, MAPK/ERK kinase (MEK)1/2 inhibitor PD 0325901 inhibited ERK phosphorylation and suppressed both TNF and MCP-1 production. In conclusion, we suggest that β_2-receptor agonists salbutamol and terbutaline inhibit inflammatory gene expression partly by a mechanism dependent on cAMP leading to the inhibition of ERK signaling in macrophages. Observed anti-inflammatory effects of β_2-receptor agonists may contribute to the clinical effects of these drugs.

1. Introduction

Mitogen-activated protein kinases (MAPKs) are important intracellular signaling pathways that regulate many physiological and pathophysiological events in cells. The three main MAPK cascades are extracellular signal-regulated kinase (ERK) pathway, p38 MAPK pathway and Jun N-terminal kinase pathway, which all consist of several sub-isoforms [28,32]. In general, MAPK pathways are three-tier kinase cascades that are activated in response to many extracellular signals, such as cytokines, growth factors and bacterial substances through G-protein-coupled and/or kinase-linked receptors. Upon activation, threonine and tyrosine residues in the activation motif of the given MAPK are phosphorylated by the upstream kinase in the signaling cascade [4,8]. Targets of activated MAPKs include transcription factors and other regulatory proteins, and they control many cellular processes, such as cell growth, proliferation, differentiation, motility, stress response, survival, apoptosis, and inflammation [28,32].

ERK1/2 pathway can be activated by different extracellular signals such as growth factors, osmotic stress, mitotic signals, hormones or inflammatory cytokines. First, a ligand-receptor engagement leads to the activation of GTPase Ras, which in turn activates Raf-MEK-ERK pathway. Phosphorylated ERK translocates to the nucleus and activates there transcription factors, for example activator protein-1, and regulate gene expression to promote growth, differentiation or mitosis. In addition, phosphorylated ERK is involved in the regulation cell functions through cytosolic target proteins [35,32,28,31,43], and it has been identified as a drug target in the treatment of many cancer types [21,25,29].

ERK pathway has been found to regulate inflammation and immune response, also ERK1/2 regulates both T cell activation and antibody production in B cells [42]. Further, ERK regulates the production of inflammatory cytokines, such as tumor necrosis

Abbreviations: COPD, chronic obstructive pulmonary disease; ERK, extracellular-regulated kinase; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MEK1/2, MAPK/ERK kinase; MKP, mitogen-activated protein kinase phosphatase; PDE, phosphodiesterase; TNF, tumor necrosis factor.

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* Corresponding author at: The Immunopharmacology Research Group, University of Tampere School of Medicine, Lääkärinkatu 1, FIN-33014 University of Tampere, Finland.
E-mail address: riku.korhonen@staff.uta.fi (R. Korhonen).

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factor (TNF), interleukin-6 (IL-6) and other mediators, including prostaglandins and nitric oxide [10,46,36,45,19]. MAPK/ERK kinase (MEK)1/2 inhibitor PD 184352, which inhibits ERK1/2 activation, inhibited the development of arthritis in mouse collagen-induced arthritis model [42]. Aberrant ERK activation has been observed in chronic inflammatory diseases in man, also. For example, ERK pathway is chronically activated in synovial tissue of rheumatoid arthritis patients [37,42]. Cigarette smoke has been shown to induce prolonged ERK activation in airway cells, and chronically elevated ERK activity has been demonstrated in airway and alveolar epithelial cells of emphysema patients [23,22]. ERK inhibitor UO126 has been reported to reduce cytokine production, inflammatory cell recruitment into the lung tissue and airway hyper-reactiveness in ovalbumin-induced airway inflammation model [9].

β_2-receptor agonists are used in the treatment of obstructive lung diseases to relieve symptoms due to airway obstruction. In addition to their bronchodilating effects, β_2-receptor agonists have anti-inflammatory properties, which may contribute to their therapeutic effects [41]. For instance, β_2-receptor agonists have demonstrated beneficial effects in acute experimental lung injury model [5] and in carrageenan-induced acute paw inflammation [17]. In a small preliminary study with patients who had acute lung injury, inhaled salbutamol decreased inflammatory biomarkers in exhaled breath condensates [33]. However, the anti-inflammatory mechanisms of β_2-receptor agonists are yet not fully understood. We have shown that the inhibition of TNF release by β_2-receptor agonists is partly a result of the inhibition of p38 MAPK activity [17]. In this study, we further investigated the mechanism of the inhibitory effect of β_2-receptor agonists on the expression TNF in macrophages. We found that β_2-receptor agonists attenuated the production of TNF and also another inflammatory factor, MCP-1, most likely by inhibiting ERK phosphorylation through a mechanism dependent on cAMP.

2. Methods

2.1. Materials

Salbutamol [α-((*tert*-butylamino)methyl)-4-hydroxy-*m*-xylene-α,α′-diol], terbutaline [5-(2-(*tert*-butylamino)-1-hydroxyethyl) benzene-1,3-diol], 8-Br-cAMP (8-bromoadenosine 3′,5′-cyclic monophosphate) and LPS (lipopolysaccharide from *Escherichia coli* strain 0111:B4) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Rolipram [4-(3-cyclopentyloxy-4-methoxy-phenyl)-pyrrolidin-2-one] and PD 0325901 (N-((2R)-2,3-Dihyroxypropoxy)-3,4-difluoro-2-((2-fluoro-4-iodophenyl)amino)-benzamide) were purchased from Axon Medchem BV (Groningen, the Netherlands). Other reagents were provided by Sigma-Aldrich Inc. (St. Louis, MO, USA) unless otherwise indicated.

2.2. Cell culture

We cultured J774 mouse macrophages (ATCC, Rockville Pike, MD, USA) at +37 °C in 5% CO_2 atmosphere using Dulbecco's Modified Eagle's Medium containing glutamax-1 (DMEM; Invitrogen, Paisley, UK) supplemented with heat-inactivated fetal bovine serum [FBS, 10% (v/v)] penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) (Gibco, Wien, Austria). Cells were seeded on 24-well plates at the density of 2.5×10^5 cells/well and cells were incubated for 72 h before experiments.

Salbutamol, terbutaline, rolipram and PD 0325901 were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions, and 8-Br-cAMP and LPS were dissolved in phosphate buffered saline (PBS). LPS (10 ng/ml) and/or other compounds or the solvent (DMSO, 0.1% v/v final concentration in the experiments) were added to the cells in fresh complete culture medium. Cells were then incubated for the time indicated.

Modified XTT test (Cell Proliferation Kit II; Roche Diagnostics, Mannheim, Germany) was used to evaluate cell viability. LPS or the compounds used were not observed to affect cell viability in XTT experiments.

2.3. Animals and isolation and culture of peritoneal macrophages

C57BL/6NCrl mice were purchased from Scanbur (Copenhagen, Denmark). Mice were housed under 12:12 light-dark cycle, temperature of 22 ± 1 °C and humidity of 50–60%. Mice had free access to food and water ad libitum.

Mice were killed by CO_2 followed by cervical dislocation. Primary mouse peritoneal macrophages (PMs) were harvested by i.p. lavage using PBS supplemented with 0.2 mM EDTA. Cells were washed and resuspended in RPMI 1640 medium (Lonza, Basel, Switzerland) that was supplemented with heat-inactivated FBS (2%), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were then seeded on 24-well plates (5×10^5 cells/well). The cells were incubated overnight. Before experiments, cells were washed with PBS to remove non-adherent cells. Cells were then exposed to LPS (10 ng/ml), compounds and/or the solvent (DMSO, 0.1% v/v) in the complete culture medium for the time indicated.

2.4. Preparation of cell lysates and Western blot analysis

Culture medium was removed at the indicated time points. Cells were washed with ice-cold PBS and solubilized in cold cell lysis buffer containing Tris-HCl (10 mM), EDTA (5 mM), NaCl (50 mM), Triton X-100 (1%), sodium orthovanadate (1 mM), phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (20 μg/ml), aprotin (50 μg/ml), sodium pyrophosphate (2 mM), sodium fluoride (5 mM), and *n*-octyl-β-D-glucopyranoside (10 μM). Lysates were incubated for 20 min on ice, and then centrifuged (12,000*g*, 10 min, +4 °C). Supernatants were removed and SDS loading buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 0.025% bromophenol blue; 5% β-mercaptoethanol) was added (in a ratio of 4:1). Samples were stored at −20 °C until analyzed. Protein concentrations in the samples were determined by the Coomassie Brilliant Blue method (Coomassie Protein Assay Reagent Kit; Pierce, Rockford, IL, USA).

Samples were boiled for 5 min for Western blot analysis. Equal protein aliquots (20 μg) were loaded on SDS-polyacrylamide gel (12%) and separated by electrophoresis. iBlot® Dry Blotting System (Life Technologies, Carlsbad, California) was used to transfer proteins to membrane. Membrane was then blocked in TBS/T [150 mM NaCl, 20 mM Tris-base (pH 7.6), 0.1% Tween-20] containing non-fat milk (5%) or, if detecting phosphorylated proteins, bovine serum albumin (BSA, 5%) for 1 h at room temperature. Primary antibody incubation was done at +4 °C overnight and secondary antibody incubation at room temperature for 1 h. Chemiluminescent signal was captured by ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Captured signal was quantified with FluorChem program (version 3.1) or Image Quant TL 7.0 Image Analysis software.

Phospho-ERK1/2 (#9101) and ERK1/2 (#9102) antibodies were from Cell Signaling Technology Inc. (Beverly, MA, USA). Polyclonal anti-rabbit (sc-2004) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.5. RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

Culture medium was removed at the time points indicated. Cell homogenization and total RNA extraction were then carried out

(GenElute™ Mammalian Total RNA Miniprep Kit, Sigma-Aldrich Inc., St. Louis, MO, USA). Reverse transcription was done by TaqMan® Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA, USA). Sequences for primers and probe were designed and primer/probe concentrations were optimized using TagMan® Universal PCR Master Mix Protocol (Applied Biosystems, Branchburg, NJ, USA). Primer and probe sequences were: mouse GAPDH 5′-GCATGGCCGGCCGTGTTC-3′ (forward), 5′-GATGTCATCA-TACTTGGCAGGTTT-3′ (reverse) and 5′-TCGTGGATCTGACGTGCC-GCC-3′ (probe with 6-FAM as the 5′-reporter dye and TAMRA as the 3′-quencher). The primers and the probe were purchased from Metabion (Martinsried, Germany). Mouse TNF and MCP-1 mRNA levels were measured by TagMan gene expression assays (Applied Biosystems, Foster City, CA, USA).

Parameters in the PCR reaction were: 2 min incubation at +50 °C and 10 min at +95 °C; 15 s denaturation at +95 °C and 1 min annealing/extension at +60 °C (40 cycles). Samples were determined in duplicate. Relative mRNA levels were estimated by a standard curve method. TNF and MCP-1 mRNA levels were normalized to GAPDH.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium samples were stored at −20 °C until assayed. Mouse TNF and mouse MCP-1 protein concentrations in the culture medium were measured by Duoset® ELISA Development System (R&D Systems Europe Ltd., Abindgon, UK).

2.7. Statistics

Results are shown as mean ± standard error of mean (S.E.M.). Statistical analyses were done using GraphPad Prism-5 software (GraphPad Software Inc., La Jolla, CA, USA). P values ⩽0.05 were considered significant.

3. Results

3.1. β_2-receptor agonists salbutamol and terbutaline inhibited TNF and MCP-1 expression in activated mouse macrophages

In this study, we investigated the effects of β_2-receptor agonist salbutamol and terbutaline on ERK phosphorylation and on inflammatory gene expression in mouse macrophages. TNF and MCP-1 are an important inflammatory mediator and chemokine, respectively, and their expression is regulated by MAPK pathway. We have previously reported that salbutamol and terbutaline alone and in combination with a phosphodiesterase (PDE) 4 inhibitor rolipram inhibited TNF mRNA and protein expression on macrophages [17]. Here, we investigated the effects of β_2-receptor agonists on MCP-1 production. β_2-receptor agonists salbutamol and terbutaline inhibited LPS-induced MCP-1 mRNA and protein expression. In addition, the inhibitory effect of the β_2-receptor agonists on MCP-1 expression was enhanced in the presence a PDE4 inhibitor rolipram. Further, cAMP chemical analog 8-Br-cAMP inhibited the expression of MCP-1 (Fig. 1).

3.2. β_2-receptor agonists salbutamol and terbutaline inhibited ERK phosphorylation in activated mouse macrophages

Second, the phosphorylation of ERK was investigated in J774 macrophages in response to LPS and the combination of LPS and β_2-receptor agonists over time. ERK phosphorylation was increased at 15 min and reached maximal level at 30 min after LPS stimulation declining near basal level at 60 min (Fig. 2). Salbutamol (Fig. 2A) and terbutaline (Fig. 2B) inhibited ERK phosphorylation

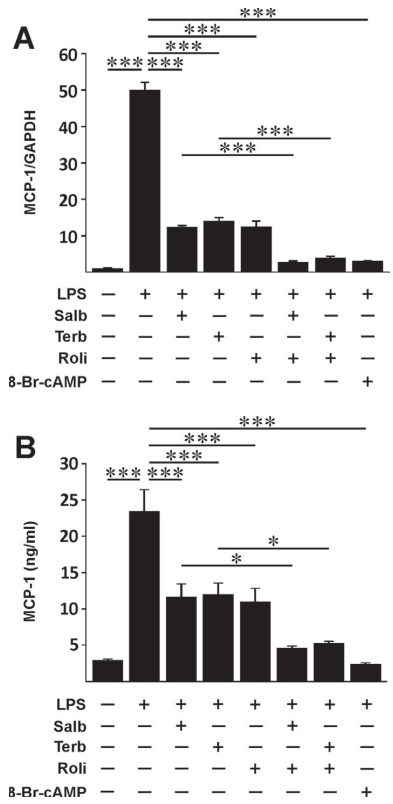

Fig. 1. β_2-receptor agonists salbutamol and terbutaline, PDE4 inhibitor rolipram and cAMP analog 8-Br-cAMP inhibited MCP-1 production in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of salbutamol (100 nM), terbutaline (100 nM), rolipram (1 μM) or 8-Br-cAMP (1000 μM) for 24 h. (A) MCP-1 mRNA was measured by quantitative RT-PCR and mRNA expression levels were normalized against GAPDH mRNA levels. (B) MCP-1 protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M., n = 4 (A), n = 8 (B). One-way ANOVA with Bonferroni's post test was performed and statistical significance is indicated with * $p < 0.05$ and *** $p < 0.001$.

at the time points of 15 min and 30 min and 8-Br-cAMP was reported to have a similar effect in a previous study [15]. Combining salbutamol and the PDE4 inhibitor rolipram further attenuated pERK levels (Fig. 3A). cAMP chemical analog 8-Br-cAMP also inhibited ERK phosphorylation in J774 macrophages (Fig. 3B).

3.3. MEK1/2 inhibitor reduced TNF and MCP-1 expression in activated mouse macrophages

We also investigated the effect of inhibition of ERK pathway on TNF and MCP-1 production. MAPK/ERK kinase (MEK)1/2 directly phosphorylates and thereby activates ERK. PD 0325901 is an inhibitor of MEK1/2 and it has been reported to prevent ERK phosphorylation and activity [3]. PD 0325901 inhibited ERK phosphorylation with maximal inhibition at 100 nM concentration in activated macrophages (Fig. 4). We then proceeded to investigate the effect of PD 0325901 at 100 nM concentration on TNF and MCP-1 expression. PD 0325901 inhibited LPS-induced TNF (Fig. 5A and C) and MCP-1 (Fig. 5B and D) mRNA and protein expression in mouse J774 macrophages. PD 0325901 inhibited

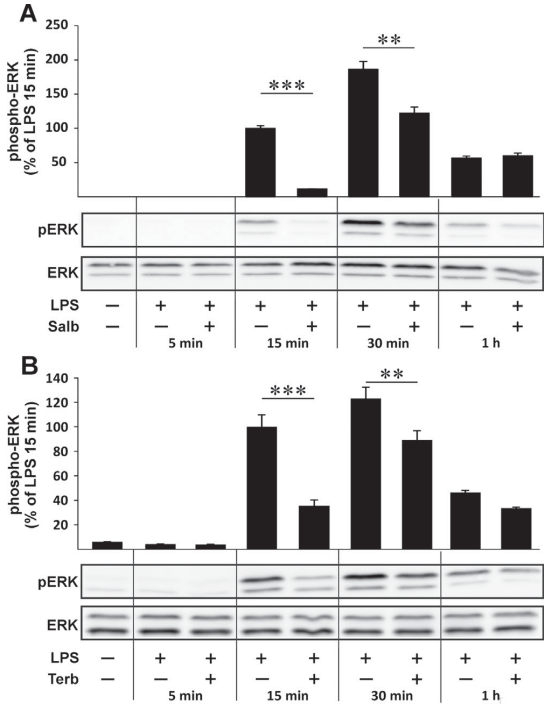

Fig. 2. Salbutamol and terbutaline reduced ERK phosphorylation in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of salbutamol (100 nM, A) or terbutaline (100 nM, B) for the time indicated, and the phosphorylation of ERK was detected by Western blot. The chemiluminescent signal was quantified, and phosphorylated ERK was normalized against total ERK. Results are expressed as mean ± S.E.M., n = 3 (A) or n = 4 (B). One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated with ** $p < 0.01$ and *** $p < 0.001$ as compared in each time point to LPS-treated cells.

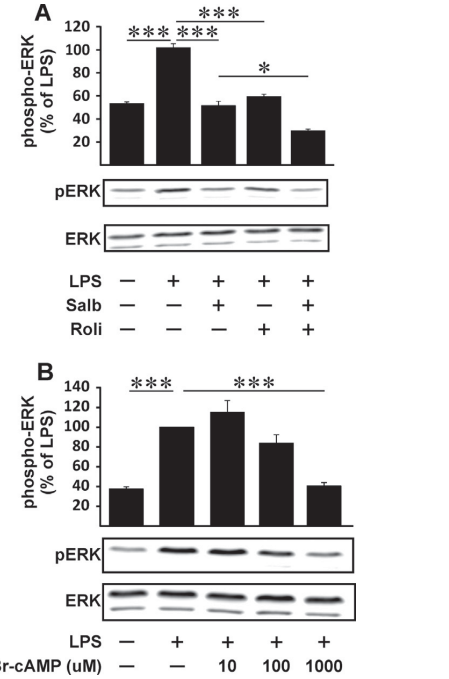

Fig. 3. Combination of salbutamol and rolipram and 8-Br-cAMP reduced ERK phosphorylation in J774 macrophages. J774 macrophages were treated with LPS (10 ng/ml) in the absence or in the presence of salbutamol (100 nM, A), rolipram (1 μM, A) or 8-Br-cAMP (10–1000 μM, B) for 30 min, and the phosphorylation of ERK was detected by Western blot. The chemiluminescent signal was quantified, and phosphorylated ERK was normalized against total ERK. Results are expressed as mean ± S.E.M., n = 4. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated with * $p < 0.05$ and *** $p < 0.001$.

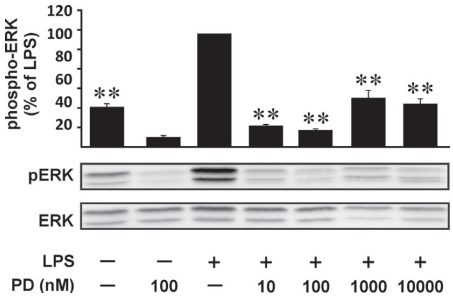

Fig. 4. MEK1/2 inhibitor PD 0325901 inhibited ERK1/2 phosphorylation in J774 macrophages. J774 macrophages were treated with LPS (10 ng/ml) in the absence or in the presence of PD 0325901 for 30 min, and the phosphorylation of ERK was detected by Western blot. The chemiluminescent signal was quantified, and phosphorylated ERK was normalized against total ERK. Results are expressed as mean ± S.E.M., n = 3. The gel is a representative of three experiments with similar results. One-way ANOVA with Dunnet's post test was performed, and statistical significance is indicated with ** $p < 0.01$.

LPS-induced TNF and MCP-1 production also in mouse peritoneal macrophages (Fig. 6).

4. Discussion

The aim of this study was to investigate the mechanism of the inhibition of cytokine production by β_2-receptor agonists salbutamol and terbutaline in macrophages. We found that β_2-receptor agonists salbutamol and terbutaline inhibited the phosphorylation of pERK and suppressed the production of MCP-1. When combined with the PDE4 inhibitor rolipram, ERK phosphorylation and the production of MCP-1 were further suppressed, and we have obtained similar results on TNF production in our previous study [17]. 8-Br-cAMP, a chemical analog of cAMP had comparable effects on ERK phosphorylation and MCP-1 release. These results suggests that anti-inflammatory effects of β_2-receptor agonists are partly mediated by the inhibition of ERK phosphorylation by a mechanism dependent on cAMP.

β_2-receptor agonists are used in the treatment of obstructive lung diseases as symptomatic drugs due to their bronchodilating properties [6,41]. β_2-receptor agonists have been reported to have anti-inflammatory effects in addition to their effects on smooth muscle relaxation in the airways. In experimental settings, they have been shown to inhibit the expression of inflammatory mediators and to reduce capillary permeability and formation of plasma exudate and tissue edema [2,48]. Salbutamol has also been reported to reduce carrageenan-induced paw edema in rodents [47,17] by a mechanism dependent on β_2-receptors [47]. In our studies, β_2-receptor agonists salbutamol and terbutaline effectively inhibited early (15 min) ERK phosphorylation and the production of TNF and MCP-1. We have previously reported that the elevation of cAMP levels by β_2-receptor agonists or PDE4 inhibitor also lead

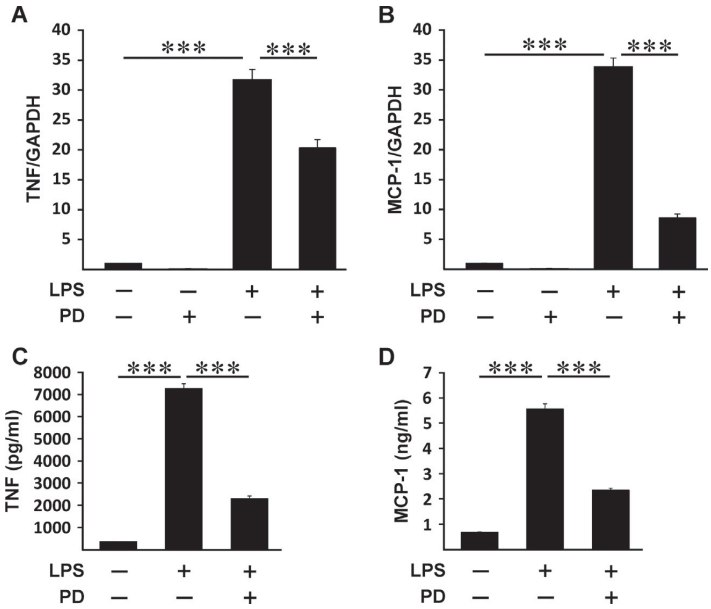

Fig. 5. MEK1/2 inhibitor PD 0325901 inhibited TNF and MCP-1 production in J774 macrophages. J774 macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of PD 0325901 (100 nM) for 4 h. In A and B, TNF or MCP-1 mRNA was measured by quantitative RT-PCR and mRNA expression levels were normalized against GAPDH mRNA levels. In C and D, TNF or MCP-1 protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M., n = 4 (A and B) or n = 6 (C and D). One-way ANOVA with Bonferroni's post test was performed and statistical significance is indicated with *** $p < 0.001$.

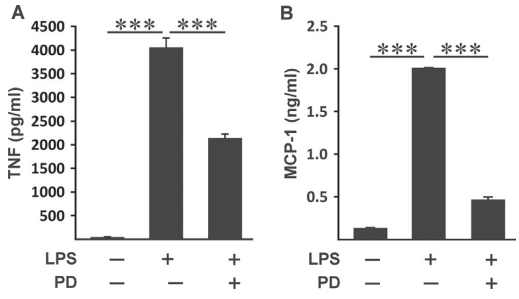

Fig. 6. MEK1/2 inhibitor PD 0325901 inhibited TNF and MCP-1 production in mouse peritoneal macrophages. Primary mouse peritoneal macrophages were stimulated with LPS (10 ng/ml) in the absence or in the presence of PD 0325901 (100 nM) for 4 h. TNF (A) and MCP-1 (B) protein accumulation in the culture medium was measured by ELISA. Results are expressed as mean ± S.E.M., n = 4. One-way ANOVA with Bonferroni's post test was performed and statistical significance is indicated with *** $p < 0.001$.

to an inhibition of p38 MAPK phosphorylation and TNF production, and also, a p38 MAPK inhibitor reduced TNF production approximately by 50% [17,18]. Further, we have shown that the elevation of cAMP levels by a PDE4 inhibitor increased the expression of MKP-1, an endogenous inhibitor of p38 MAPK activity. Importantly, in macrophages from MKP-1(-/-) mice, in which p38 MAPK activity is abnormally high, the inhibition of TNF production by a PDE4 inhibitor was clearly impaired [18]. Taken together, the data suggest that cAMP signaling suppresses TNF production in macrophages by regulating two distinct MAPK pathways. Firstly, β_2-receptor agonists inhibit early ERK phosphorylation leading to partially impaired ERK signaling and reduced TNF production. Secondly, β_2-receptor agonists increase MKP-1 expression leading to the dephosphorylation p38 MAPK and hence to the suppression of p38 MAPK pathway. This mechanism inhibits TNF production at later time points. In this study, a chemical cAMP analog 8-Br-cAMP mimicked the effect the β_2-receptor agonists suggesting that the effects of β_2-receptor agonists on ERK signaling and TNF and MCP-1 release are mediated by cAMP signaling.

It has previously been shown that β_2-receptor activation leads to the onset of ERK signaling [34,38,20,1]. Repetitive or prolonged G-protein-coupled receptor (GPCR) activation suppresses receptor signaling through activation of Gβγ- G-protein-coupled receptor kinase (GRK)-β-arrestin pathway [20,34]. β-arrestin directs β_2-receptor to inactivation and internalization, but it also serves as an activation signal for ERK pathway leading to ERK phosphorylation [34,20]. In this study, we found that β_2-receptor agonists reduced ERK phosphorylation and a chemical cAMP analog had similar effects. These results suggest that the outcome of the ERK pathway activation by β_2-receptor agonists is cell type dependent. Further, in macrophages, β_2-receptor activation results in the inhibition of ERK signaling, and this seems to be cAMP, and thereby also G-protein, dependent.

Long-acting β_2-receptor agonists are often combined with inhaled corticosteroids in the treatment of moderate/severe asthma and COPD to achieve better symptom control [13,12]. β_2-receptor agonists attenuate the expression of inflammatory factors (such as intracellular adhesion molecule, vascular cell adhesion molecule-1 granulocyte/macrophage colony-stimulating factor and IL-8) and this is further enhanced by inhaled corticosteroids [39,40,27]. Although corticosteroids are considered as the principal anti-inflammatory treatment in those diseases, potential anti-inflammatory effects of β_2-receptor agonists may contribute to the clinical effect of the combination therapy. Further, the combination of inhaled corticosteroids and β_2-agonists may improve symptom control by mechanisms not directly related to suppression of inflammation. Recently, inhaled corticosteroids

and β_2-receptor agonists have been shown to synergistically induce G-protein-coupled-receptor kinase 2 (GRK2) expression in airway smooth muscle cells. Interestingly, GRK2(-/-) mice displayed increased bronchoconstriction and they had impaired bronchodilation response to β_2-receptor agonists [14]. This suggests that β_2-receptor agonists and inhaled corticosteroids may have additive effects on both inflammation and control of airway smooth muscle tonus.

In patients with emphysema, which is a pathological organ change observed in COPD, chronically increased ERK activity has been observed [23]. In addition, cigarette smoke induces a rapid and strong phosphorylation of ERK in airway epithelial cells [23]. Also, the induction of the expression of MMP-1 by cigarette smoke is dependent on active ERK signaling [23]. MMP-1, as well as many other matrix metalloproteinases, is associated with the development of emphysema [26]. Hence, aberrant ERK activation is seen in the lungs of patients with chronic obstructive inflammatory lung diseases. Therefore, inhibition of ERK phosphorylation by β_2-receptior agonists may contribute to their observed clinical efficacy. IL-33 is an important factor in allergic airway inflammation and severe asthma by promoting Th2 differentiation, the expression of IL-4 and IL-5 as well as IgE and mucus production in the airways [24]. TNF produced by macrophages has been reported to induce IL-33 release in the airway smooth muscle cells [30]. TNF levels are increased in the airways of patients with asthma [16], and anti-TNF treatment has been reported to reduce disease severity, for example by lowering the incidence of disease exacerbations in patients with symptomatic moderate asthma [11]. Further, MCP-1 expression is increased in the lungs of COPD patients [7,44], and MCP-1 is an important chemokine for inflammatory cell migration into the inflamed lung tissue in COPD. Our previous results [17] and findings in this study together show that β_2-receptor agonists clearly reduce TNF and MCP-1 release in macrophages, and this may contribute to the therapeutic effect of the β_2-receptor agonists on the disease control in asthma.

5. Conclusions

In conclusion, we found that β_2-receptor agonists suppressed cytokine production and this was probably mediated by cAMP-dependent inhibition of ERK pathway. β_2-receptor activation appears to suppress MAPK pathway activity in macrophages, and this may contribute to their clinical efficacy in the treatment of COPD and asthma. Taken together, these results bring new information on the regulation of inflammatory gene expression by GPCR signaling and on the effects of β_2-receptor agonists in the treatment of inflammatory obstructive lung diseases.

Author contributions

Participated in research design: Tiina Keränen, Riku Korhonen, Eeva Moilanen

Conducted experiments: Tiina Keränen, Tuija Hömmö, Riku Korhonen

Performed data analysis: Tiina Keränen, Riku Korhonen, Eeva Moilanen

Wrote or contributed to the writing of the manuscript: Tiina Keränen, Riku Korhonen, Eeva Moilanen

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