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Systems Biology based studies on anti-inflammatory compounds

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Chapter 6

Beta-adrenergic receptor agonists induce the release of Granulocyte Chemotactic Protein-2, Oncostatin M, and Vascular Endothelial Growth Factor from macrophages.

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Beta-adrenergic receptor agonists induce the release of Granulocyte Chemotactic Protein-2, Oncostatin M, and Vascular Endothelial Growth Factor from macrophages.

Vascular endothelial growth factor (VEGF), oncostatin M (OSM), and granulocyte chemotactic protein-2 (GCP-2/CXCL6) are up-regulated in U937 macrophages and peripheral blood macrophages exposed to LPS, beta₂-adrenergic receptor (β_2 -AR) agonists (e.g. zilpaterol, and clenbuterol) and some other agents that induce intracellular cAMP (prostaglandin E₂, forskolin, and dibutyryl cAMP). LPS in combination with β_2 -agonists and cAMP elevating agents had an additional effect on the release of VEGF, OSM, and CXCL6. These proteins are up-regulated after 16-24 h of exposure and this is mediated by the β_2 AR, as determined by time course experiments and the use of a selective β_2 -AR antagonist (ICI 118551). Beta₂-AR agonists are used as bronchodilators in the treatment of asthma, but appear to have no effect on the chronic inflammation of the disease. However, the up-regulation of VEGF, OSM, and CXCL6 may have adverse effects on the inflammatory process of asthma. These mediators are involved in the recruitment of neutrophils, airway remodelling and angiogenesis, known features of chronic inflammatory diseases. We propose that the up-regulation of these proteins could play a role in the adverse effects of prolonged excessive usage of β_2 -AR agonists on the airways besides the desensitization of the β_2 -AR.

Introduction

Beta₂-adrenergic agonists are used as bronchodilators in the treatment of pulmonary diseases¹⁻⁵. Although the major action of β_2 -agonists on the airways is relaxation of airway smooth muscle, these drugs have several other effects mediated through β_2 -adrenergic receptors (β_2 -AR) expressed on other cells as well. These additional actions of β_2 -agonists may contribute to the efficacy of β_2 -agonists in relieving asthma symptoms. Beta₂-agonists inhibit plasma exudation in the airways by acting on β_2 -AR on capillary venule cells. They inhibit the secretion of bronchoconstrictor mediators from eosinophils, macrophages, T-lymphocytes, and neutrophils. Despite these inhibitory effects on inflammatory cells *in vitro*, β_2 -agonists do not appear to reduce the chronic inflammation of asthma. Desensitization is more readily seen in inflammatory cells than in airway smooth muscle cells and may account for this discrepancy^{6,7}. In a previous paper⁸ we described a genomics-based screening methodology to categorize anti-inflammatory drugs. For this purpose, we used U937 macrophages which

were stimulated with LPS in the absence or presence of different classes of anti-inflammatory compounds (e.g. MAP kinase inhibitor, corticosteroid, proteasome inhibitor, and β_2 -agonists). Using microarrays the effects on the transcriptome of the exposed cells were investigated. The expression patterns were subsequently analyzed using pattern recognition tools, which revealed that different classes of anti-inflammatory drugs show distinct and characteristic mRNA expression patterns that can be used to categorize known compounds and to discover and classify new leads. We were able to show that zilpaterol, a poorly characterized β_2 -agonist, gives rise to an almost identical expression pattern as the β_2 -agonists clenbuterol and salbutamol. Furthermore, we identified specific biomarkers, vascular endothelial growth factor (VEGF), oncostatin M (OSM), and granulocyte chemotactic protein-2 (GCP-2/CXCL6) which were exclusively up-regulated by U937 macrophages exposed to LPS in combination with β_2 -agonists, compared to the treatment with LPS alone or LPS in combination with other anti-inflammatory compounds (e.g. dexamethasone, SB203580 and proteasome inhibitor). In the present study we investigated if these mRNA markers were also up-regulated at the protein level by peripheral blood macrophages and if these markers are up-regulated by U937 macrophages in a dose-dependent manner. Furthermore, we established if this up-regulation was mediated by the β_2 -AR and we followed the course of this regulation in time. In our experiments we used two β_2 -AR agonists of a different chemical structure: clenbuterol and zilpaterol.

Clenbuterol was originally designed as a potent bronchodilator but only reached the clinic in a few countries. Nowadays it is mostly known for its anabolic action in athletes and cattle and approved as bronchodilator in horses ^{1,9}. Zilpaterol is a relatively new and rather unknown β_2 -AR agonist which was originally designed as an growth promoter in cattle ¹⁰.

β_2 -agonists signal through β_2 -AR receptors which belong to the family of GTP-binding protein-coupled receptors (GPCRs). Beta₂-AR activates adenylate cyclase via G_s regulatory proteins. Binding to the β_2 -AR results in the production of the second messenger cyclic adenosine-3',5'-cyclic monophosphate (cAMP) ¹¹⁻¹⁶, which is involved in many metabolic pathways ^{13,16-20}. We investigated if cAMP is involved in the up-regulation of VEGF, OSM, and CXCL6. Therefore we incubated U937 macrophages with cAMP elevating agents; prostaglandin E₂ (PGE₂) which induces cyclic AMP by binding to its own receptor, forskolin, a direct activator of adenylate cyclase and the membrane permeable stable derivate of cAMP, dibutyryl cAMP, in the absence or presence of LPS.

Materials and Methods

Chemicals

Lipopolysaccharide (LPS, *E. coli* 0111:B4), ICI 118551, clenbuterol, dibutyryl cAMP, prostaglandin E₂, forskolin were obtained from Sigma Aldrich (St. Louis, MO, USA) and zilpaterol from Intervet Inc. (Millsboro, US).

Cell cultures

Human monocyte-like histiocytic lymphoma cells U937²¹ obtained from the ATCC (CRL-1593.2) were grown in RPMI-1640 medium, supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine (Life technologies, Breda, The Netherlands) at 37 °C and 5% CO₂ in a humidified atmosphere. U937 monocytic cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA, 10 ng/ml, overnight, Omnilabo, Breda, The Netherlands) as described previously²². The PMA-differentiated macrophages were allowed to recover from the PMA treatment for 48 h, during which the culture medium was replaced daily.

Peripheral blood monocytes (PB-MO) were isolated from human EDTA-blood with Rosette SepTM human monocyte enrichment cocktail (Stemcell Technologies Inc, Meylan, France) as described previously²². The monocytes (0.5x10⁶ cells per well) were cultured in 24 well cell culture plates containing RPMI-1640 medium supplemented with 10% (v/v) human serum and 2 mM L-glutamine and were allowed to differentiate into peripheral blood macrophages (PB-MØ) for eight days. Following this procedure, the macrophage maturation has been described to give rise to the characteristically morphology and phenotype of primary macrophages²³.

Macrophage activation

U937 macrophages were cultured at a concentration of 1x10⁶ cells per well. Cells were incubated for 16 h with LPS (1 µg/ml), LPS in the presence of a dilution series of zilpaterol (1x10⁻⁸ - 1x10⁻⁴ M), or LPS in the presence of a dilution series of clenbuterol (1x10⁻¹⁰ - 1x10⁻⁶ M) respectively.

Human PB-MØ were cultured in 12 well plates at a concentration of 1x10⁶ cells per well and pre-incubated with 1x10⁻⁶ M zilpaterol or 1x10⁻⁷ M clenbuterol at 37 °C. After 30 minutes LPS (1 µg/ml) was added and the cells were incubated for a further 16 h at 37 °C. For the time course experiments U937 cells (1x10⁶ cells per well) were incubated with LPS (1 µg/ml), LPS in the presence of β_2 -AR agonists (1x10⁻⁶ M zilpaterol or 1x10⁻⁷ M clenbuterol), or LPS in the

presence of β_2 -AR agonists in combination with ICI 118551 (equal concentration as β_2 -AR agonist). Culture medium was collected at 8, 16, and 24 h respectively. Furthermore, U937 macrophages were incubated with cAMP elevating compounds, dibutyryl cAMP (1×10^{-4} M), forskolin (1×10^{-5} M), prostaglandin E₂ (1×10^{-4} M), zilpaterol (1×10^{-6} M), and clenbuterol (1×10^{-7} M) in the presence or absence of 1 μ g/ml LPS. The concentrations of the various compounds were chosen as such to achieve similar levels of TNF- α inhibition. All incubations were performed in triplicate. The culture medium was collected and stored at -20 °C until analysis. The cells were lysed in 0.1 M NaOH and used for protein determination by the modified method of Bradford (Bio-Rad, Veenendaal, The Netherlands).

Enzyme-Linked Immunosorbent Assays (ELISA)

The concentration of VEGF, OSM and CXCL6 in the culture supernatants were determined by ELISA. Immunoassays for CXCL6 and oncostatin M were purchased from R&D systems (Oxon, UK). The VEGF ELISA was obtained from Prepotech (London, UK). The immunoassays were performed according to the manufactures instructions. The enzyme activity of lactate dehydrogenase (LDH) was quantitatively determined using the *in vitro* assay for LDH activity from Roche Diagnostics (Mannheim, Germany) for automated clinical chemistry analyzer Hitachi 911 (Hitachi, Japan).

Results

Beta₂-AR agonists, zilpaterol and clenbuterol induced the release of VEGF, OSM and CXCL6 by U937 macrophages exposed to LPS in a dose-dependent manner (Fig. 1). The concentrations used were not toxic, as was determined by measuring LDH activity in the culture medium of exposed cells (data not shown). Clenbuterol induced the release of the three proteins more potently than zilpaterol, as was demonstrated by the lower concentration needed, but a lower maximum was obtained.

To investigate if the release of these compounds was not restricted to tumor cells like the human monocyte-like histiocytic lymphoma U937 cells, we used macrophages derived from freshly isolated peripheral blood monocytes from healthy donors, in order to confirm the inducing effect of β_2 -AR agonists on the VEGF, OSM and CXCL6 release. The concentrations of zilpaterol and clenbuterol used showed maximum up-regulation of the three compounds and equally inhibited the TNF- α release (data not shown). From Figure 2 it can be concluded that the release of VEGF, OSM as well as CXCL6 were significantly augmented by zilpaterol as well as clenbuterol, both in combination with LPS.

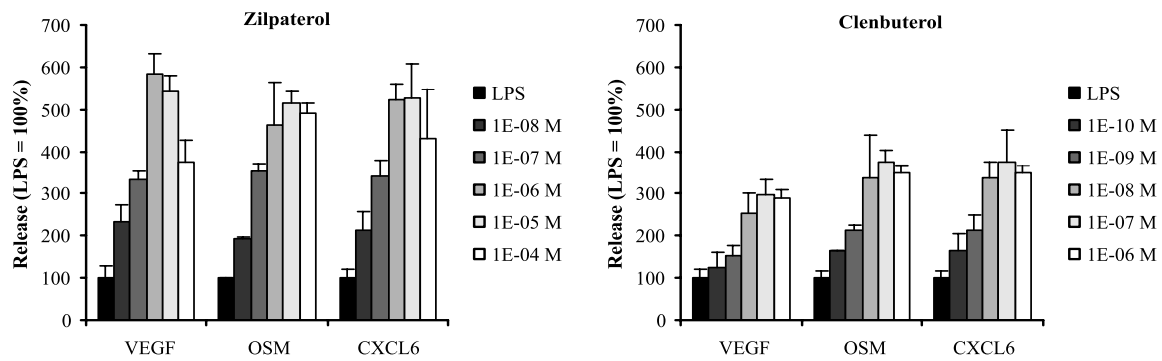


Figure 1 Induction of VEGF, OSM, and CXCL6 release from U937 macrophages incubated for 16 h with 1 μ g/ml LPS in the absence (black bars) or presence of a dilution series of zilpaterol (1×10^{-8} , 1×10^{-7} , 1×10^{-6} , 1×10^{-5} , and 1×10^{-4} M respectively) or a dilution series of clenbuterol (1×10^{-10} , 1×10^{-9} , 1×10^{-8} , 1×10^{-7} , and 1×10^{-6} M respectively). The release of VEGF, OSM, and CXCL6 was determined by ELISA, corrected for protein content and calculated with respect to the release from U937 cells exposed to LPS alone. The results are presented as means \pm SD of a triplicate measurement.

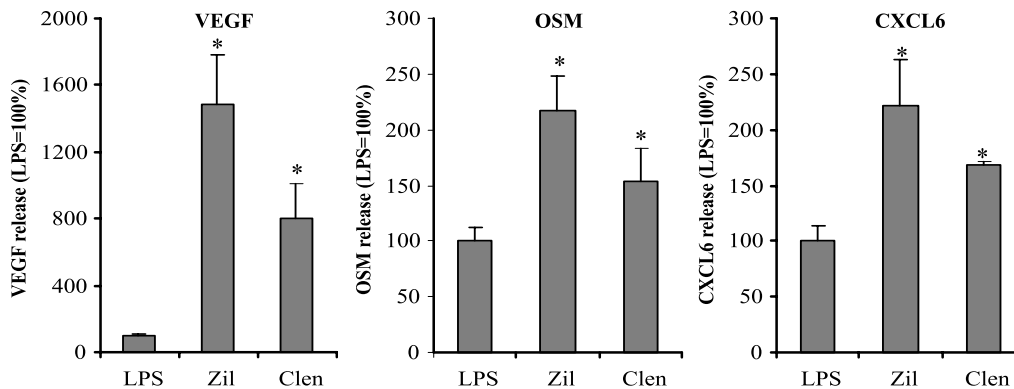


Figure 2 Release of VEGF, OSM, and CXCL6 by peripheral blood macrophages exposed to 0.1 μ g/ml LPS in the absence (LPS) or presence of 1×10^{-6} M zilpaterol (Zil) or 1×10^{-7} M clenbuterol (Clen). The release of VEGF, OSM, and CXCL6 is corrected for protein content and calculated with respect to the release from PB-M \emptyset exposed to LPS alone. The results are presented as means \pm SD of a triplicate measurement. * Different according to Student's *t*-test (assuming normal distributions and equal variance, $p < 0.05$). The *p*-values are calculated with respect to PB-M \emptyset cells treated with LPS alone.

Since β_2 -AR agonists mediate their effect via the β_2 -AR, we investigated if the β_2 -AR was involved in the inducing effect of zilpaterol and clenbuterol on the VEGF, OSM, and CXCL6 production by using a selective β_2 -AR antagonist ICI 118551. From Figure 3 it can be concluded that the induction of the three compounds was reversed by the addition of ICI 118551. Figure 3 also shows that the release of VEGF, OSM and CXCL6 was particularly induced at longer incubation times and that this induction was also achieved by U937 cells exposed to LPS in the absence of the β_2 -AR agonists, or LPS in combination with a β_2 -AR agonist and ICI 118551.

Since the main pathway by which activation of β -ARs exerts their effects is related to the elevation of intracellular cyclic AMP levels, we determined the effect of other agents which are known to elevate intracellular cyclic AMP levels. U937 macrophages were incubated in the presence or absence of LPS with or without β_2 -AR agonists or cAMP elevating compounds.

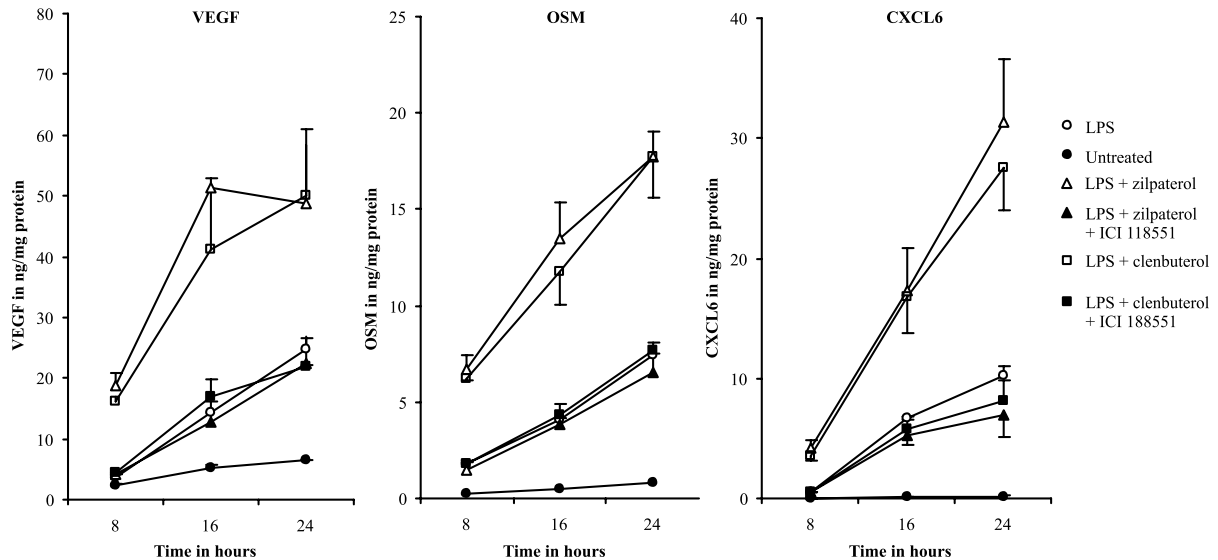


Figure 3 Release of VEGF, OSM, and CXCL6 from untreated U937 macrophages, U937 macrophages after exposure to LPS (1 μ g/ml) alone, LPS in combination with 1×10^{-6} M zilpaterol, LPS in combination with 1×10^{-7} M clenbuterol, LPS in combination with zilpaterol and 1×10^{-6} M ICI 118551, or LPS in combination with clenbuterol and 1×10^{-7} M ICI 118551. The levels of VEGF, OSM, and CXCL6 released by the macrophages in the culture medium were determined at the time points indicated and are corrected for protein content. The results are presented as means \pm SD of triplicate measurements.

Figure 4 shows that forskolin, butyryl cAMP, PGE_2 , the two β_2 -AR agonists all significantly induced the release of VEGF, OSM and CXCL6 from U937 macrophages. Exposure to LPS also gave rise to an increase in the release of these proteins. Interestingly, exposure of U937 cells to a combination of LPS and either a β_2 -AR agonist or a c-AMP elevating compound resulted in an even higher induction of the release of the three proteins. Only the addition of PGE_2 to LPS exposed U937 macrophages had no significant additional effect on the VEGF release compared to U937 macrophages exposed to LPS alone. These results indicate that VEGF, OSM, and CXCL6 were induced via a cAMP-dependent pathway. The release of the three proteins was elevated by LPS, β_2 -AR agonists and cAMP-elevating agents. The production is induced even more when the U937 macrophages are simultaneously treated with LPS and cAMP-elevated agents or β_2 -AR agonists.

Discussion

Our results demonstrate that VEGF, OSM and GCP-2/CXCL6 release from U937 macrophages and peripheral blood macrophages exposed to LPS are up-regulated by β_2 -AR agonists via the β_2 -AR and a cAMP dependent pathway. The expression of these markers is a relatively late event, as they become apparent 16 h after addition of the β_2 -AR agonists. This contrasts the immediate effects of β_2 -AR agonists on smooth muscle. Relaxation of smooth muscle, the main action of β_2 -agonists, occurs directly after inhalation until approximately 20 hours after inhalation³⁻⁵.

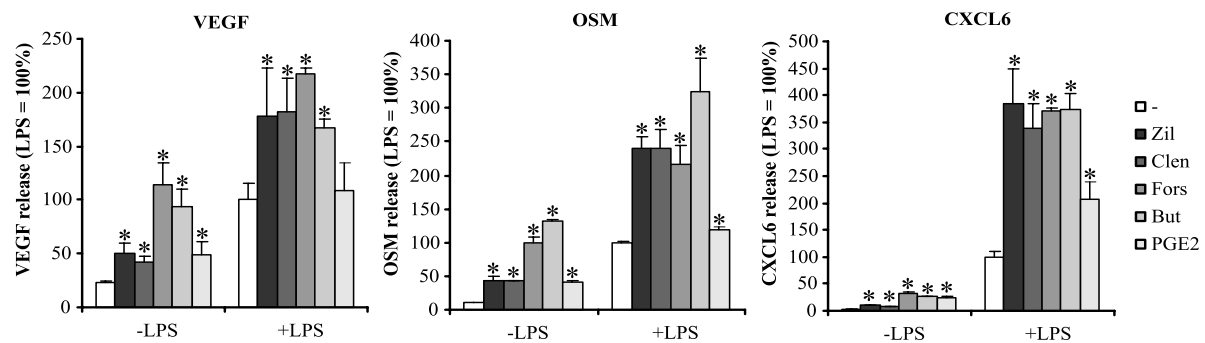


Figure 4 Release of VEGF (A), OSM (B), and CXCL6 (C) from U937 macrophages (-) or U937 macrophages incubated for 24 h with β_2 -adrenergic agonists; 1×10^{-6} M zilpaterol (Zil), 1×10^{-7} M, clenbuterol (Clen), or cAMP elevating agents; 1×10^{-5} M forskolin (Fors), 1×10^{-4} M butyryl cAMP (Butyl), or 1×10^{-4} M prostaglandin E₂ (PGE₂) respectively in the absence (-LPS) or presence of 1 μ g/ml LPS (+LPS). The concentrations of the various compounds were chosen as such to achieve similar levels of TNF- α inhibition. The release of VEGF, OSM, and CXCL6 is corrected for protein content and represented as mean percentage of LPS induction (LPS=100%) \pm SD of triplicate measurements. * Significant difference according to Student's *t*-test (assuming normal distributions and equal variance, $p < 0.05$). The *p*-values of the +LPS group were calculated with respect to U937 cells treated with LPS alone, and for -LPS group with respect to untreated U937 macrophages.

VEGF is a growth factor and is involved in angiogenesis, lymphangiogenesis, endothelial cell growth²⁴, and is mainly produced by macrophages. VEGF stimulates inflammatory cell recruitment and promotes the expression of proteases implicated in pericellular matrix degradation in angiogenesis²⁵. Angiogenesis and microvascular remodelling are known features of chronic inflammatory diseases²⁶. The newly formed vessels facilitate leukocyte influx and leak plasma proteins into the airway mucosa.²⁷ VEGF is highly expressed in asthmatic airways²⁸, and it can be envisioned that VEGF plays a role in the formation of new vessels and remodelling in asthmatic tissue²⁶. However, β_2 -agonists have been shown to decrease the plasma protein leakage and reduce the infiltration of inflammatory cells (e.g. neutrophils)²⁶. This suggests that the effects of β_2 -agonists on VEGF release are counterbalanced by other, more favourable effects of β_2 -agonists. In previously published

reports, VEGF was shown to be up-regulated by LPS, cAMP, PGE₂, and forskolin in human macrophages^{27, 29, 30} with a peak release after 24 h. This is in line with our findings that cAMP is involved in the up-regulation of VEGF and that it is a relatively late biomarker. OSM belongs to the Interleukin (IL)-6 subfamily and is involved in growth regulation, differentiation, inflammatory response, hematopoiesis, tissue remodelling, and development³¹. OSM is secreted from activated T cells, monocytes and macrophages^{31, 32}. The role of OSM is ambiguous. OSM is considered to be a late phase cytokine that inhibits the production or modulates the activities of initiators of the inflammatory response (e.g. IL-1 and TNF- α)³³. On the other hand it has been shown that OSM also possesses pro-inflammatory properties^{32, 34}. O'Hara et al³⁴ suggested that OSM may play a role in the development of airway wall remodelling by deposition of collagen in the sub-epithelial basement membrane and, as a consequence, may be a suitable target for further research in the pathogenesis of asthma and its treatment. Furthermore, OSM is able to increase VEGF release and thus indirectly may contribute to airway remodelling observed in chronic airways disease^{32, 35}. Our results suggesting that cAMP is involved in the induction of OSM is in agreement with previous findings that PGE₂, and forskolin up-regulate the release of OSM in human airway smooth muscles³⁴, and microglia, the resident macrophages of the brain³⁶. CXCL6 or GCP-2 is a CXC chemokine and is closely related to Interleukin 8. CXCL6 predominantly exert stimulatory and chemotactic activities towards neutrophils^{37, 38}. The CXCL6 expression has been reported to be regulated by a variety of inflammatory mediators, including IL-1 β and TNF- α ^{37, 39}. Recently it has been shown that Interleukin 17 levels are increased in the airways of patients with asthma. Furthermore, it was suggested that Interleukin 17 may play a role in the recruitment of airway neutrophils by releasing CXCL6 and IL-8 in human bronchial epithelial cells⁴⁰. Accumulation and activation of neutrophils can lead to a release of proteases and reactive oxygen free radicals that can contribute to mucus secretion, airway remodelling and lung tissue destruction, key characteristics of severe acute asthma⁴⁰. The induction of CXCL6 by cAMP has to our knowledge not been published to date.

In conclusion, our results revealed that VEGF, OSM and CXCL6 were up-regulated in macrophages exposed to LPS and β_2 -AR agonists via the β_2 -AR and a cAMP dependent pathway. We can only speculate about the possible adverse effects of the release of VEGF, OSM, and CXCL6 on the development of asthma. In spite of inhibiting the inflammatory response, these proteins induce the recruitment of neutrophils, cause airway remodelling and

angiogenesis, known features of chronic inflammatory diseases. The up-regulation of these proteins in a late stadium may play a role in the increased airway hyper-responsiveness to allergen after prolonged excessive usage of β_2 -agonists, besides the desensitization of the β_2 -AR³⁻⁵. In a previous study⁸ we already showed that VEGF, OSM and CXCL6 were significantly down-regulated by the corticosteroid dexamethasone. This may provide an additional explanation for the preferred usage of inhaled corticosteroids in combination with β_2 -adrenergic agonists besides the usage of the β_2 -ARs alone.

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