385. Understanding disease and drug mechanisms

3446

The long-acting β2-agonist formoterol re-establishes the anti-proliferative effect of glucocorticoids in asthmatic airway smooth muscle cells (ASMC)

Michael Roth, Jun Zhong, Chong S'ng, Michael Tamm. *Pulmonary Cell Research & Pneumology, University Hospital Basel, Basel, Switzerland*

Background: Asthma is characterized by an increased mass of ASMC which show increased proliferation in vitro. As previously reported, glucocorticoids do not have a significant anti-proliferative effect on ASMC of asthmatics while although their anti-inflammatory efficacy is maintained.
Methods: We assessed whether the addition of the long-acting β2-agonist formoterol modifies the anti-proliferative efficacy of glucocorticoids (beclomethasone, budenoside, fluticasone). Primary human ASMC lines from asthmatics and controls were set up from bronchoscopic lung biopsies.
Results: Serum deprived sub-confuent cells were stimulated by 5% serum with or without a single drug or combination for a further 48 and 72 hrs. Serum alone increased cell proliferation in healthy control cells by 65% (48 hrs) and 81% (72 hrs); a significantly higher proliferation response was seen in asthmatic ASMC (82% at 48 hrs; 123% at 72 hrs). In the presence of different glucocorticoids alone serum-induced proliferation was significantly reduced in healthy cells (max. 48%) but not in asthmatic ASMC. Treatment with formoterol alone reduced serum-induced proliferation by max. 24% at any time point, with no differences between asthmatic and healthy ASMC. When formoterol was combined with fluticasone a 78% reduction of ASMC proliferation was achieved.
Conclusions: Our results show that the β2-agonist formoterol re-establishes the anti-proliferative effect of fluticasone in asthmatic airway smooth muscle cells. This synergistic action may exert an anti-remodeling effect on the asthmatic airway supporting the clinical benefit of combination therapy.

3447

A novel target of formoterol, a dual-specificity phosphatase DUSP4 on regulation of corticosteroid budesonide sensitivity

Yoshiki Kobayashi[1], Nicolas Mercado[1], Anna Miller-Larsson[2], Peter Barnes[1], Kazuhiro Ito[1]. *[1]Airway Disease, NHLI, Imperial College, London, United Kingdom; [2]R&D Lund, AstraZeneca, Lund, Sweden*

Introduction: We previously reported that formoterol, a long-acting β_2-adrenoceptor agonist, activated a serine/threonine protein phosphatase PP2A which is involved in the restoration of corticosteroid (CS) sensitivity by formoterol (Kobayashi et al ERJ 2009;34:583s). We explored other phosphatases and identified dual-specificity phosphatase DUSP4 as a possible novel target of formoterol.
Aims: To confirm that DUSP4 is activated by formoterol and is involved in regulation of CS sensitivity.
Methods: U937 monocytic cells were incubated with IL-2 and IL-4 for 48 h to induce CS insensitivity. CS sensitivity was determined by the CS budesonide ability to inhibit TNFα-induced IL-8 production and to translocate glucocorticoid receptor (GR) from cell cytoplasm to nucleus. Phosphatase activity of immunopurified DUSP4 was measured by fluorescence-based assay. The nuclear/cytoplasmic GR ratio and phosphorylation levels of GR-Ser^{226} and JNK1 kinase were analysed by western-blotting. In some experiments DUSP4 was knocked down by siRNA.
Results: Formoterol increased DUSP4 activity, which was reduced under IL-2/IL-4 exposure. Knock-down of DUSP4 reduced GR nuclear translocation and CS sensitivity. Knock-down of DUSP4 also abrogated the dephosphorylation of GR-Ser^{226} and JNK1 by formoterol - the effects involved in GR nuclear translocation and restoration of CS sensitivity.
Conclusions: Formoterol regulates sensitivity to budesonide through activation of DUSP4 which dephosphorylates JNK1 and which in turn leads to dephosphorylation of GR-Ser^{226}. This novel mechanism by formoterol may contribute to the clinical efficacy of combination of formoterol and budesonide.

3448

Tiotropium reduces established pulmonary inflammation in a 12 weeks cigarette smoke mouse model of COPD

Lutz Wollin, Florian Gantner, Michael Pieper. *Respiratory Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany*

Rationale: Tiotropium bromide (Spiriva®) is the only marketed long acting anticholinergic for the treatment of chronic obstructive pulmonary disease (COPD).

We recently demonstrated that tiotropium reduces established cigarette smoke (CS)-induced lung inflammation in a short term mouse model.
Aim: To investigate the therapeutic anti-inflammatory activity of tiotropium on CS-induced lung inflammation in a chronic 12 weeks mouse model.
Methods: All mice except controls were exposed to CS for 3 weeks (W) to elicit pronounced lung inflammation. Thereafter, 4 groups of mice were either further exposed to CS or room air (RA) with or without tiotropium treatment up to W12. Tiotropium was administered by inhalation (0.1 mg/mL for 5 min) 1 h prior to CS or RA exposure. Cell counts in the bronchoalveolar lavage (BAL) were determined at W3, W8, and W12.
Results: Lung inflammation induced by 3 W of CS exposure was resolved 5 W after termination of smoke exposure regardless of Tiotropium treatment. Continued CS exposure increases lung inflammation to a maximum at W8. Tiotropium treatment of existing inflammation for 5 W completely inhibited the increase in neutrophil number and reduced monocytic inflammation. After 9 W of Tiotropium treatment the number of monocytes was reduced to baseline levels of healthy animals. Neutrophil number was significantly reduced by 58% after 9 W of Tiotropium treatment compared to sham treated CS exposed mice.
Conclusion: The therapeutic anti-inflammatory properties of tiotropium bromide may contribute to the beneficial influence of tiotropium in chronic inflammatory airway diseases like COPD.

3449
Effects of aclidinium on human lung fibroblast activation *in vitro*
Javier Milara[1], Adela Serrano[2], Teresa Peiró[2], Ricardo Guijarro[3], Amadeu Gavaldà[4], Montserrat Miralpeix[4], Esteban Morcillo[2], Julio Cortijo[5].
[1]CIBERES, Health Institute Carlos III, Valencia, Spain; [2]Department of Pharmacology, Faculty of Medicine, University of Valencia, Valencia, Spain; [3]Thoracic Surgery Unit, University General Hospital Consortium, Valencia, Spain; [4]R&D Centre, Almirall, Barcelona, Spain; [5]Research Unit, University General Hospital Consortium, Valencia, Spain

Introduction: Muscarinic activation of human lung fibroblasts is associated with pathological remodelling in the airways of patients with asthma and chronic obstructive pulmonary disease (COPD).
Aims: To investigate the *in vitro* effects of aclidinium bromide, a novel, long-acting muscarinic antagonist, on human lung fibroblast activation.
Methods: Lung fibroblasts, isolated from human bronchus, were pre-incubated with aclidinium (10^{-9}M-10^{-7}M), the ERK 1/2 inhibitor PD98059 (10μM) or the cAMP analogue dbcAMP (1mM) for 30 min and then exposed to carbachol (10μM) for 48 h. Collagen type I and α-smooth muscle actin (αSMA) expression were measured by RT-PCR, Western blot (WB) and immunofluorescence. ERK 1/2 phosphorylation was measured by WB and intracellular cAMP levels by cAMP Biotrak enzyme immunoassay. Fibroblast proliferation was assessed using a BrdU kit, and fibroblast migration by wound closure assay.
Results: Aclidinium, PD98059 and dbcAMP attenuated carbachol-induced increases in αSMA and collagen type I mRNA and protein levels. Aclidinium and dbcAMP prevented carbachol-induced increases in phospho-ERK 1/2. Carbachol (10μM) prevented isoprenaline (1μM)-induced cAMP upregulation, which was completely reversed by aclidinium 10^{-7}M. Carbachol-dependent increases in lung fibroblast proliferation (2-fold) were reduced by aclidinium 10^{-7}M (1.1-fold), PD98059 (1.3-fold) and dbcAMP (1.2-fold). Aclidinium 10^{-7}M, PD98059 and dbcAMP reduced fibroblast wound closure by 30%, 28% and 40%, respectively.
Conclusions: Aclidinium blocks carbachol-induced lung fibroblast proliferation probably by a direct effect at muscarinic receptors. Aclidinium may alleviate lung fibroblast activation in patients with asthma and COPD.

3450
The pre-clinical pharmacology of the inhaled muscarinic antagonist GSK573719 predicts once-daily clinical dosing
Dramane I. Lainé, Mark A. Luttmann, James J. Foley, Chris J. Dehaas, Charles J. Kotzer, Michael Salmon, William L. Rumsey. *Respiratory CEDD, GlaxoSmithKline, King of Prussia, United States*

Introduction: GSK573719 is a new, long-acting muscarinic antagonist offering sustained 24-h bronchodilation currently in phase III clinical trials for COPD.
Objectives: To assess the pharmacology of GSK573719 in pre-clinical studies.
Methods/Results: In CHO cells transfected with recombinant human M_3 receptors, GSK573719 demonstrated pM potency (–log pA_2=23.9pM) in an acetylcholine (ACh)-mediated Ca^{2+} mobilisation assay. Concentration-response curves indicated competitive antagonism with partial reversibility after drug wash out. Using isolated human bronchial strips in static tissue baths, GSK573719 was a potent, competitive antagonist (–log pA_2=316pM) vs carbachol. Superfusion of bronchial strips with carbachol containing buffer showed that GSK573719 was slowly reversible in a concentration-dependent manner (1–100nM). Time to 50% restoration of contraction at 10nM was >600 min (vs 413 min with tiotropium bromide, 10nM). In conscious guinea pigs, GSK573719 dose-dependently blocked ACh-induced bronchoconstriction with long duration of action; a 2.5μg intratracheal dose elicited >50% bronchoprotection for >24h.
Conclusions: GSK573719 is a potent muscarinic antagonist that demonstrates slow functional reversibility at cloned human M_3 receptors and at endogenous mAChR in isolated human bronchus. This profile translates into 24h duration of bronchodilation in the clinic, suitable as a once-daily treatment for COPD.
Funded by GSK.
*Formerly at GSK.

3451
Synergistic bronchoprotective activity of the long-acting beta 2-agonist olodaterol with tiotropium (long-acting M_3 antagonist) and ciclesonide (inhaled steroid) on the ovalbumin-induced bronchoconstriction in anaesthetized guinea pigs
Thierry Bouyssou, Paola Casarosa, Michael Pieper, Andreas Schnapp, Florian Gantner. *Respiratory Diseases Research, Boehringer-Ingelheim Pharma GmbH & Co. KG, Biberach an der Riß, Baden Würtenberg, Germany*

Inhaled corticosteroids (ICS) and long-acting β_2-agonists (LABAs) fixed dose combinations (FDCs) are widely used for the treatment of asthma while the long-acting muscarinic antagonist (LAMA) tiotropium is a standard therapy of chronic obstructive pulmonary disease (COPD). Additional improvement of lung function might be expected with the use of the triple therapy "ICS + LABA + LAMA".
The aim of this study was to assess potential synergistic bronchoprotection activity of olodaterol (LABA) combined with tiotropium (LAMA) and ciclesonide (steroid), against ovalbumin-induced bronchoconstriction in the anaesthetized guinea pig.
Bronchoconstriction was induced by inhalation of a single dose of ovalbumin (50 μg/kg) and lung resistance was recorded for 10 minutes in anaesthetized guinea pigs. Compounds were given intra-tracheally 2 h before OVA challenge. Olodaterol dose-dependently inhibited the OVA-induced bronchoconstriction with an ED_{50} of 0.2 μg/kg. Combination of olodaterol with either tiotropium or ciclesonide displayed bronchoprotection in a dose-dependent manner with ED_{50} values 10–fold lower (0.02-0.03 μg/kg) than the ED_{50} of olodaterol alone ($p<0.05$). The triple combination led to even better bronchoprotection with an ED_{50} of 0.003 μg/kg ($p<0.05$).
This study shows that olodaterol in combination with tiotropium and ciclesonide elicits potent synergistic bronchoprotective activity in the guinea pig. This effect is significantly higher than the summarized values of the respective monotherapies and supports the concept of the triple therapy.

3452
Mast cell generated cyclooxygenase products protect from airway hyperresponsiveness in a model of chronic asthma
Barbara Fuchs[1,2,3], Lisa Sjöberg[1,2,3], Christine Möller Westerberg[2], Maria Ekoff[2], Linda Swedin[1,3], Sven-Erik Dahlén[1,3], Mikael Adner[1,3], Gunnar Nilsson[2]. *[1]Experimental Allergy and Asthma Research, National Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden; [2]Department of Medicine, Clinical Immunology and Allergy Unit, Karolinska Institutet, Stockholm, Sweden; [3]Centre for Allergy Research, National Institute for Environmental Medicine, Karolinska Institutet, Stockholm, Sweden*

The cyclooxygenase (COX) product prostaglandin (PG) E2 is known to act bronchoprotective in asthmatics. Recent preclinical studies identified the COX-terminal microsomal PGE synthase-1 to contribute to the formation of protective PGE2. This study investigated whether the release of bronchoprotective COX products is mast cell (MC) dependent.
Chronic allergic airway inflammation in mice was induced by ovalbumin/alum injections and repeated challenges during 90 days. COX-inhibition with diclofenac (DFC) was performed. Airway hyperresponsiveness (AHR) was assessed applying forced-oscillation technique in response to methacholine. Contribution of MC-COX-products to AHR was resolved by comparing Wsh (MC-deficient) to C57BL/6 wildtype (WT) mice (MCs around central airway).
Central airway resistance, R_n was pronounced in allergen-challenged WT mice and increased in WT-DFC [cmH2O/s*mL, mean± SEM: 1.3±0.1 *vs* 1.6±0.2, p=0.04]. Wsh displayed comparable resistance [1.7±0.1] as WT-DFC. R_n in Wsh was not changed after treatment [1.9±0.2].
Both mouse strains lack MCs in peripheral lung tissue. Consequently, tissue resistance was not changed in WT and Wsh [8.5±0.5 *vs* 7.8±0.6], nor after treatment [WT-DFC 7.8±0.5, Wsh-DFC 7.6±0.6]. Although a shift in baseline tissue elastance was observed between WT and Wsh [21.9±1.4 *vs* 17.7±0.4, p=0.02], treatment did not affect baseline [WT-DFC 21.4±1.2; Wsh-DFC 17.6±0.9], nor was the reactivity changed.
The data indicates that the bronchoprotective role of PGE2 is MC dependent. It remains to be seen if this is due to PGE2 or other COX products released by MCs, or if modulating COX products are released by other cells in response to MC activation.

3453
Antitussive effect of (-) menthol mediated by nasal trigeminal TRPM8 receptors
Jana Plevkova[1], Brendan J. Canning[2], Ivan Poliacek[3], Mariana Brozmanova[1], Milos Tatar[1]. *[1]Department of Pathophysiology, Jessenius School of Medicine, Commenius University, Martin, Slovakia (Slovak Republic); [2]Asthma and Allergy Center, Johns Hopkins School of Medicine, Baltimore, United States; [3]Department of Medical Biophysics, Jessenius School of Medicine, Martin, Slovakia (Slovak Republic)*

(-) Menthol, main component of the peppermint is exploited in many over-the-

counter drugs for common cold and cough. It has been reported that (-) menthol has antitussive effect in humans and animals. However, there are conflicting evidences against that statement and also case reports of respiratory failure induced by menthol in kids. Based on the recent data about the distribution of the TRPM8 – menthol receptors expressing neurons in the upper airways, and their low proportion within the vagal nerves in lower airways we suppose, that effect of (-) menthol on cough could be mediated by TRPM8 expressed on nasal afferents. To test this hypothesis a segmental airway challenges with (-) menthol were performed in anaesthetized guinea pigs (nose, trachea and lower airways were separately treated by menthol) and under such set up citric acid cough dose response curves (DRC) were constructed according the method described by *Canning et al*., *2006*. The DRC were compared to control provocations. The (-) menthol applied topically to the trachea does not influence cough significantly. (-) menthol vapors applied directly to lower airways via cannula resulted to the tendency to facilitate cough, with visible signs of increased mucus output, loaded breathing and wheezing. The antitussive effect of menthol was observed only in the group treated by nasal (-) menthol vapors, which reduced cough response to CA significantly comparing to control response $p < 0.001$.

Our results correspond with the data describing the TRPM8 distribution in the respiratory tract, and may contribute to explanation of adverse effects induced by menthol inhalation in kids. Supported:VEGA 1/0031/11.