## 90. Airway smooth muscle cells and fibroblasts: cell biology

**P742**

**TGFβ1-induced extracellular matrix production enhances airway smooth muscle cell proliferation**

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The fibrogenic cytokine transforming growth factor-β1 (TGFβ1) is an important mediator in airway remodelling. TGFβ1 overexpression in mice induces airway smooth muscle (ASM) hyperplasia, one of the characteristics of airway remodelling in asthma. The mechanisms underlying this response are unclear. As studies of ASM cell proliferation showed that TGFβ1 alone has very weak mitogenic properties, combined effects with other factors such as Gq- or Gi-protein coupled receptor agonists may be involved. Here, we hypothesized that the mitogenic effects of TGFβ1 on ASM are indirect and require prolonged exposure to allow deposition of extracellular matrix (ECM) proteins. To address this hypothesis, we investigated the effects of acute and prolonged treatment with TGFβ1 (2 ng/mL), alone and in combination with the muscarinic receptor agonist methacholine (MCh, 10μM) on human ASM cell (hASMc) proliferation. TGFβ1 had no acute effect on hASMc

proliferation. However, pretreatment with TGFβ1 for 7 days increased hASMc proliferation, and potentiated the mitogenic response to PDGF. The presence of MCh during TGFβ1 pretreatment considerably enhanced this effect of TGFβ1. Interestingly, the TGFβ1-induced effects on cell proliferation as well as the potentiating effects of MCh were inhibited by the integrin-blocking peptide RGDS (Arg-Gly-Asp-Ser), whereas RGDS had no direct effect on hASMc proliferation. Accordingly, pretreatment with TGFβ1 induced an increase in fibronectin protein expression, which was enhanced by MCh stimulation. In conclusion, our results indicate that pretreatment with TGFβ1 enhances hASMc proliferation, which is mediated by ECM proteins and enhanced by muscarinic receptor agonists.

**P743**

**Omalizumab inhibits IgE-induced extracellular matrix deposition by asthmatic airway smooth muscle cells**

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Increased serum IgE levels in allergic asthma contribute to inflammation. Neutralizing humanized anti-IgE antibodies, such as Omalizumab, effectively reduce inflammation. In this study we determined the effect of human activated IgE in the presence and absence of Omalizumab on extracellular matrix deposition by human airway smooth muscle cells (8 asthma patients; 8 healthy controls). Confluent cells were stimulated by 5% human pooled serum or IgE (0.1 – 10 ng/ml) in the presence or absence of Omalizumab (1 – 100 ng/ml) for 72 hours in the presence of [3H]-proline (0.5 microCi) and extracellular matrix deposition was monitored. Compared to non-stimulated cells, stimulation with 5% serum increased matrix deposition by +42% and IgE dose-dependently increased matrix deposition by max. +38%. Interestingly we observed the stimulatory effect of IgE in airway smooth muscle cells of both healthy controls and asthma patients with no significant difference. When the cells were pre-incubated with Omalizumab for 30 minutes the IgE-induced matrix deposition was dose-dependently reduced. The reductive effect was 100% when ratio of Omalizumab:IgE was 10:1. Furthermore, the inhibitory effect of Omalizumab was similar when applied together with IgE, or even when added 15 minutes after IgE. When added at later time points after IgE addition the inhibitory effect of Omalizumab was reduced but significant until 45 minutes. We observed no difference of either IgE induced matrix deposition or the inhibitory effect of Omalizumab comparing healthy control to asthma patient's cells. Our results indicate that IgE increases airway remodeling and that Omalizumab significantly reduces this effect.

**P744**

**Neo-vascularisation in asthma: Altered angiogenic potential of ASM cells from asthmatic patients**

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**Background:** Airway remodeling is a key pathology in asthma with increasing changes of the airway wall structure, including thickening of airway smooth muscle (ASM) bundles and increased vascularisation. ASM cells require oxygen and nutrition which are delivered via blood vessels. Understanding the mechanism of neo-vascularisation and the necessary changes of the airway structure may therefore lead to novel strategies to counteract remodeling.
**Methods:** The capacity of ASM cells of asthmatics and non-asthmatics to induce sprouting was determined with a sprouting assay, the release of VEGFA was measured and proliferation of HMEC-1 cells stimulated with conditioned muscle cell medium (CM) of asthmatic or non-asthmatic donors was analyzed.
**Results:** CM of ASM cells from asthmatics showed an increased angiogenic potential in the angiogenesis sprouting assay. HMEC-1 spheroids incubated with CM of cell-lines obtained from asthma patients developed more and longer sprouts compared to control CM. Proliferation of HMEC-1 cells stimulated with CM from ASM cells of asthmatics was increased and thus confirmed the sprouting assay results. Next, we analyzed VEGFA levels in CM of ASM cells from asthmatic and non-asthmatic donors. Cells from asthmatics released more VEGFA compared to controls.
**Conclusion:** Our results indicate that the angiogenic potential of ASM cells from asthmatic patients is higher compared to that of control cells and thus may directly contribute to neo-vascularisation through the increased release of VEGFA. Counteracting this process may lead to novel asthma therapies.
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**P745**

**Carrier-mediated transport of tiotropium in bronchial smooth muscle cells: The role of pH-dependent organic cation transporters**

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Organic cation transporters (OCTs) play an important role in drug absorption, tissue distribution and elimination. Cationic drug transport mechanisms in the airway tissue, however, are poorly understood. To assess the role of OCTs in airway drug transport, uptake of the inhaled bronchodilator tiotropium (a cationic, quaternary ammonium compound) by human bronchial smooth muscle cells was measured. Primary bronchial smooth muscle cells showed a carrier-mediated, concentration-, time-, and temperature-dependent uptake of tritiated tiotropium. Tiotropium uptake was reduced by various OCT inhibitors, including L-carnitine, the pH-dependent organic cation/carnitine transporter 2 (OCTN2)-carried zwitterion. OCTN2 expression was confirmed by RT-PCR and immunofluorescence analysis. Selective suppression of OCTN2 expression using lentivirus-mediated production of shRNA reduced tiotropium uptake in smooth muscle cells. Although bronchial smooth muscle cells also express corticosteroid-sensitive OCTs, tiotropium uptake was insensitive to corticosteroids in these cells. In contrast, tiotropium uptake was pH-dependent, with nearly 50% lower rates at acidic (pH 5.7) than at alkaline (pH 8.2) extracellular pH. These findings demonstrate the existence and functional role of a pH-dependent uptake machinery for tiotropium in human bronchial smooth muscle cells. We suggest that inflammation-associated airway acidification, through OCTN2 inhibition, could interfere with the disposal of tiotropium by smooth muscle in the airway and increase its concentration at muscarinic receptor sites.

**P746**

**Role of endosomal and cytosolic pattern recognition receptors in dsRNA-induced cytokine expression in human airway smooth muscle cells**

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**Background:** Rhinovirus infections are major triggers of asthma exacerbations. Double-stranded RNA (dsRNA), a viral replication intermediate, is sensed by pattern recognition receptors (PRRs): endosomal TLR3 and cytosolic RIG-I-like receptors (RLRs). dsRNA induces cytokine expression in airway smooth muscle cells (ASMCs) via TLR3, but little is known about involvement of RLRs.
**Objective:** Investigate if both TLR3 and RLRs are involved in dsRNA-induced cytokine expression in ASMCs.
**Methods:** Cultured primary human ASMCs (n=8) were stimulated with dsRNA (10μg/ml) or dsRNA in complex with LyoVec (dsRNA/LV; 0,5μg/ml) that selectively distributes to the cytosol and activates RLRs. mRNA expression (3 and 24h) and protein production (24h) were analysed by RT-qPCR and ELISA respectively.
**Results:** At 3h, dsRNA induced significantly higher mRNA expression of IL-8, TNFα and IFNβ ($p<0.001$) than dsRNA/LV ($p<0.05$). By 24h, TNFα and IFNβ expression returned towards baseline in dsRNA-treated cells while IL-8 further increased, as did all cytokines in dsRNA/LV-treated cells, particularly IFNβ ($p<0.01$). Chloroquine, an endosomal TLR3 inhibitor, reduced only dsRNA-induced effects. dsRNA produced more IL-8, but equal IFNβ protein compared to dsRNA/LV. dsRNA and dsRNA/LV also upregulated RLR (RIG-I and MDA5) mRNA expression at 24h ($p<0.01$).
**Conclusions:** TLR3 largely mediated the prompt cytokine response to dsRNA in ASMCs. RLRs also played a role, especially in 24h antiviral IFNβ production. Upregulation of RIG-I and MDA5 further suggests involvement of cytosolic PRRs in addition to endosomal TLR3 and supports the immunomodulatory function of ASMCs in virus-infected airways.

**P747**

WITHDRAWN

**P748**

**Airway smooth muscle cells from patients with COPD exhibit a higher degree of cellular proliferation and steroid insensitivity than that from healthy patients**

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**Rationale:** Chronic obstructive pulmonary disease (COPD) refers to chronic bronchitis and emphysema, a pair of two commonly co-existing diseases of the lungs in which the airways become narrowed. The potential contribution of airway smooth muscle to airflow obstruction, airway inflammation and airway remodeling in COPD isn't fully understood. Indeed, although it is known that the cytokine TGF-β induces the expression of such cytokines as IL-6 and IL-8, little is known regarding its role upon ASM proliferation and steroid insensitivity in COPD. Ergo, we have examined the role of both the growth factor FCS and TGF-β, upon ASM proliferation and steroid response following treatment with dexamethasone.
**Methods:** Human ASM cells were pre-treated with dexamethasone for 2 h before being stimulated with FCS ± TGF-β for 8 days. For the determination of chemokine release, supernatants were removed and IL-6 levels determined by DuoSet ELISA (R&D Systems). The degree of cell proliferation was assessed by Cell Proliferation ELISA, BrdU kit (Roche Applied Science) according to the manufacturer's instructions.
**Results:** Exposure to FCS and TGF-β induced a higher increase in cellular proliferation and IL-6 release in ASM from patients with COPD compared to healthy individuals. Furthermore, the inhibitory effect of dexamethasone in this system was almost completely ablated in the ASM cells from the COPD patients.

**P749**

**miR-221 and miR-222 target p21 and p27 in airway smooth muscle to elicit hyper proliferation in severe asthmatics**

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**Rationale:** Since microRNAs (miRNAs) were first discovered to be produced in human tissues over 10 years ago, they have emerged as important mediators in cellular physiology and pathology. Asthma is characterised by chronic airflow obstruction, chronic airway inflammation and remodeling and the airway smooth muscle (ASM) cells cultured from the biopsies of patients with asthma are of a hyper proliferative phenotype and release greater amounts of the chemokine IL-6. This ASM proliferation has been shown to be increased in response to growth factors such as FCS, and inflammatory mediators such as TGF-β. Ergo, we have examined the role of the miRNAs, miR-221 and miR-222, in both FCS and TGF-β-induced ASM proliferation and IL-6 release.
**Methods:** Human ASM cells were stimulated with FCS ± TGF-β for 8 days. For the determination of chemokine release, supernatants were removed and IL-6 levels determined by DuoSet ELISA (R&D Systems). The degree of cell proliferation was assessed by Cell Proliferation ELISA, BrdU kit (Roche Applied Science) according to the manufacturer's instructions. miRNA and mRNA expression was examined by TaqMan RT-PCR. The function of miR-221 and miR-222 were assessed through transfection with miRNA mimics and inhibitors.
**Results:** Exposure to FCS and TGF-β induced an increase in cellular proliferation, IL-6 release and miRNA-221 and miR-222 expression. This increase was greater in patients with severe asthma than those with non severe asthma. FCS and TGF-β-induced proliferation and IL-6 release were inhibited following transfection with miRNA-222 inhibitors and potentiated in the presence of miRNA-222 mimics, by the targeting of p21 and p27.

**P750**

**Sphingosine-1-phosphate receptor expression and signalling in human airway smooth muscle cells**

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Sphingosine-1-phosphate (S1P), a bioactive lipid and ligand for five G protein coupled receptors (S1P1-S1P5), is a key regulator of cell trafficking, cell differentiation and immune responses. Recently, increased concentrations of S1P have been detected in airways of asthmatic subjects and S1P has been shown to be a potent constrictor of human airway smooth muscle (HASM) cells. The aim of this study was to investigate the expression and signalling pathways of S1P receptors in HASM cells. HASMs have been grown from bronchial biopsies of healthy individuals. Real-time PCR has been used to determine gene expression. Intracellular signalling in response to S1P was measured using MAP kinase phosphorylation, intracellular calcium and cAMP assays. Three of the five known receptors are expressed in HASM cells at mRNA level: S1P1, S1P2, and S1P3. S1P potently activated intracellular calcium flux in a concentration-dependent manner, with $EC50=4\times10^{-9}$M. Using a range of selective agonists and antagonists, S1P1 and S1P3 were found to couple to Gi and inhibit forskolin induced cAMP generation, whereas S1P2 and S1P3 signalled through intracellular calcium mobilisation. S1P also induced phosphorylation of extracellular signal-regulated kinase (Erk) and increased expression of several genes, including interleukin-6. This study demonstrates that S1P receptors may signal effectively through multiple intracellular pathways in response to S1P and activate HASM cells.

**P751**

**Heme-oxygenase (HO)-1 inhibits TNF-α induced CXCL10 secretion by airway smooth muscle cells**

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**Background and purpose:** CXCL10 induces mast cell migration towards airway smooth muscle bundles in asthma. In blood mononuclear cells, induction of heme oxygenase (HO)-1 inhibited pro-inflammatory cytokine secretion. Dimethylfumarate (DMF), which is clinically used as an anti-inflammatory medication, induced HO-1 and thereby inhibited proliferation in airway smooth muscle cells (ASMC).
**Experimental approach:** Here we assessed the anti-inflammatory effect of DMF on TNF-α induced CXCL10 secretion in human primary ASMC and the involvement of HO-1 and mitogen activated protein kinases (MAPK). ASMC were pre-incubated with DMF and/or glutathione ethylester (GSH-OEt), SB203580, or the HO-1 inducers hemin, or cobalt-protoporphyrin (CoPP) 1 hour before stimulation with TNF-α (10 ng/ml).
**Key results:** TNF-α induced the secretion of CXCL10, which was inhibited by DMF as well as by the HO-1 inducers hemin, or CoPP. Interestingly, DMF amplified the TNF-α induced phosphorylation of p38 MAPK and thereby induced the expression of HO-1. Inhibition of p38 MAPK by SB203580 reduced DMF-induced HO-1. Importantly, GSH-OEt supplementation: (i) abrogated the inhibitory effect of DMF on TNF-α induced CXCL10 secretion, (ii) counteracted DMF-induced HO-1 expression, and (iii) p38 MAPK activation.
**Conclusion and implications:** Our data indicate that DMF inhibits TNF-α induced CXCL10 by altering intracellular GSH, leading to activation of p38 MAPK and subsequent synthesis of HO-1 in ASMC. Thus, DMF might help to reduce airway inflammation in asthma.

**P752**

**Corticosteroid insensitivity and enhanced mitogen-activated protein kinase activity in airway smooth muscle cells of severe asthma**

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**Background:** Patients with severe asthma are less sensitive to oral or inhaled corticosteroids. Relative corticosteroid insensitivity has been shown in peripheral blood mononuclear cells and alveolar macrophages in these patients.
**Aims and objectives:** Determine the response of corticosteroids in airway smooth muscle cells (ASMCs) of severe asthma, in terms of suppression of cytokine-induced chemokine release and mRNA expression, and investigate the underlying mechanisms.
**Methods:** ASMCs of non-asthmatics (NA; 12), patients with non-severe (NSA; 10) or severe asthma (SA; 10) were pretreated with dexamethasone (Dex; $10^{-10}$-$10^{-6}$ M) followed by stimulation with TNF-α at 10 ng/mL. IL-8 and eotaxin release determined by ELISA; mRNA quantified by RT-PCR. p65 NF-κB recruitment to gene promoters measured by ChIP assay; p38, JNK, and ERK expression measured by Western blot.
**Results:** Baseline and TNF-α induced eotaxin release and mRNA were higher in NSA, but not SA, compared to NA, while no differences were observed for IL-8. p65 recruitment to gene promoters did not differ. Dex ($10^{-6}$ M) suppressed induced eotaxin release by 36.7% vs 16.7% ($p<0.05$) in NSA and SA, respectively. Dex ($10^{-6}$ M) suppressed induced IL-8 release by 49.8% vs 25.7% ($p<0.01$) in NA and SA, respectively. Induced IL-8 and eotaxin mRNA was significantly inhibited by Dex ($10^{-7}$ M) in NA and NSA ($p<0.05$) but not in SA. Induced phosphorylated p38 and JNK were significantly higher in SA compared to NA, respectively ($p<0.05$).
**Conclusions:** Corticosteroid insensitivity exists in ASMCs of severe asthma, which may be associated with enhanced p38 and JNK activity.

**P753**

**MAP kinases mediate FGF-induced expression and release of VEGF in human airway smooth muscle cells: The role of azithromycin**

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**Background:** Fibroblast growth factors, FGF-1, FGF-2 and vascular endothelial growth factor (VEGF) are elevated in chronic inflamed airways. Airway smooth muscle (ASM) cells are known to synthesize VEGF. We investigated the contribution of FGF-1/-2 on the VEGF production in ASM cells, the involvement

of mitogen-activated protein kinases (MAPK) and the modulatory effects of azithromycin and dexamethasone.
**Methods:** Human ASM cells were treated with 10ng/ml of FGF-1 or FGF-2. Specific blockers for ERK1/2$^{MAPK}$ (U0126), p38$^{MAPK}$ (SB239063), JNK (curcumin), dexamethasone or azithromycin were added 30 minutes prior to stimulation. Expression of VEGF (VEGF-A, $VEGF_{121}$ and $VEGF_{165}$) was assessed by quantitative PCR, VEGF release by ELISA and MAPK phosphorylation by Western blotting.
**Results:** FGF-1/-2 upregulated mRNA expression of VEGF (VEGF-A, $VEGF_{121}$ and $VEGF_{165}$) and its release by 1.8 fold (FGF-1) and 6.1 fold (FGF-2). Transient increase in ERK1/2$^{MAPK}$ and p38$^{MAPK}$ phosphorylation and subsequent release of VEGF from FGF-1/-2 treated human ASM cells was inhibited by respective blockers. Furthermore, both dexamethasone and azithromycin reduced the VEGF secretion mediated by the p38$^{MAPK}$ pathway.
**Conclusion:** Our Results demonstrate that FGF-1 and FGF-2 upregulate VEGF production via ERK1/2$^{MAPK}$ and p38$^{MAPK}$ pathways. The anti-angiogeneic effect of dexamethasone and azithromycin may potentially contribute to tackle VEGF-mediated vascular remodelling in chronic airway diseases.

**P754**
**Effects of formoterol on TGF-β1 induced factors of extracellular matrix composition**
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**Introduction:** COPD is characterised by chronic airway inflammation resulting in extensive airway remodelling. The origin of the remodelling pathology in COPD is unknown and may result from increased EMT (epithelial-mesenchymal transition). EMT is a process which is mediated by hypoxia and several growths factors, such as transforming growth factor-beta (TGF-β1). Formoterol is a long acting beta2 agonist which can exert antiinflammatory effects by the regulation of intracellular cAMP levels. The aim of our study was to determine if formoterol can modulate down-stream mediators of EMT by cAMP induction.
**Methods:** Human bronchial smooth muscle cell culture was performed using standard protocols.Cells were stimulated with Formoterol ($10^{-10}$ to $^{-6}$ M), Dideoxyadenosin (DDA, 100μM), and/or TGF-β1 (5ng/ml); Forskolin was used as a positive control. cAMP measurement was performed by ELISA (at 1, 3 and 6 hours).CTGF, Collagen 1A1 (Col1) and Collagen4 (Col4) mRNA transcription was measured by quantitative real time PCR (18S mRNA served as reference gene). All experiments were performed as triplicates.
**Results:** Formoterol significantly increased intracellular cAMP after 1, 3 and 6 hours compared to untreated cells, whereas the maximum effect was observed after 1 hour. The cAMP increase was effectively blocked by the addition of DDA at any time point. After 24 hours, Formoterol significantly reduced TGF-β1 stimulated Col1 and CTGF but not Col4 mRNA transcription. The addition of DDA reversed this effect for Col1 but not for CTGF.
**Conclusion:** Treatment with formoterol resulted in a decrease of TGF-β1 induced mediators of extracellular matrix composition. These new findings suggest a potential role for formoterol in EMT.

**P755**
**LSC 2011 Abstract: Potential anti-inflammatory role of the cAMP effectors Epac and PKA in COPD**
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Cigarette smoke-induced release of pro-inflammatory cytokines such as interleukin-8 (IL-8) from airway smooth muscle (ASM) cells may contribute to the development of chronic obstructive pulmonary disease (COPD). Here, we investigated the role of the cAMP-effectors Epac and PKA on cigarette smoke extract (CSE)-induced IL-8 release by human ASM cells as well as the potential signalling mechanisms involved. Additionally, the impact of CSE on Epac and PKA expression was evaluated.
CSE-induced IL-8 release from ASM was reduced by the $\beta_2$-agonist fenoterol, the Epac activator 8-pCPT-2'-*O*-Me-cAMP and the PKA activator 6-Bnz-cAMP. CSE induced IκBα degradation and p65 nuclear translocation, processes that were primarily reversed by the Epac activator 8-pCPT-2'-*O*-Me-cAMP. In addition, CSE increased extracellular signal-regulated kinase (ERK) phosphorylation, which was selectively reduced by the PKA activator 6-Bnz-cAMP. Furthermore, CSE decreased Epac1 expression, but had no effects on Epac2 and PKA expression. Importantly, we observed reduced Epac1 expression in lung tissue from COPD patients.
In conclusion, our data indicate that Epac and PKA differentially decrease CSE-induced IL-8 release by ASM cells, via inhibition of NF-κB and ERK signalling, respectively.
Our findings further indicate that cigarette smoke exposure may reduce anti-inflammatory effects of cAMP in the airways via down-regulation of Epac1.

**P756**
**Caveolin-1 expression in airway smooth muscle cells is related to airway responsiveness magnitude in allergic asthma model**
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Caveolin-1 (Cav-1) is an important signalling scaffold protein involved in smooth muscle contraction; however, the role of Cav-1 in airway responsiveness has not been elucidated so far. Our aim was to determine the relation of Cav-1 expression with airway hyperresponsiveness (AHR) in a guinea pig asthma model. To evaluate this relation, three inhaled antigenic challenges were applied every 10 days to antigen (ovalbumin; OVA) sensitized guinea pigs (n=9). Antigen-induced airway obstruction, AHR to histamine, and the expression of Cav-1 in lung and airway smooth muscle cells (ASMC), was evaluated at the third OVA challenge. The control group received saline solution instead of OVA (n=6). From the first challenge on, OVA induced a transient airway obstruction and the development of AHR at the third antigenic challenge. Cav-1 mRNA levels in lung (assessed by RT-PCR) significantly decreased in our asthma model compared with the control group ($P<0.01$). Nevertheless, in the ASMC from allergic asthma guinea pigs, the expression of Cav1 mRNA, determined by PCR "in situ", was increased. This increment was confirmed by flow cytometry in ASMC and by immunohistochemistry in lung tissues. Airway obstruction and AHR was correlated with the extent of Cav-1 increment in ASMC ($P<0.05$; $r=0.69$ and 0.52, respectively). Our data suggest that Cav-1 expression in ASMC has a crucial role in airway obstruction magnitude and in the process of AHR in this asthma model.

**P757**
**Relaxant effect of *crocus sativus* (saffron) on guinea pig tracheal chains and its possible mechanism(s)**
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The relaxant effects of 4 cumulative concentrations of aqueous-ethanolic extract and safranal were examined by their relaxant effects on precontracted tracheal chains of guinea pig by 10 μM methacholine (group 1) and 60 mM KCl in two different conditions including: non-incubated tissues (group 2) and incubated tissues with 1 μM propranolol 1 μM chlorpheniramine and 1 μM atropine (group 3), (n=6). In groups 1 and 2 all concentrations of theophilline, extract and safranal showed significant relaxant effects compared to that of saline ($p<0.05$ to $p<0.001$). However, in group 3 the extracts of *Crocus sativus* showed a weak relaxant effect ($p<0.05$ only for highest concentration). The effects of last concentrations of safranal in group 1, and its all concentrations in group 2 were significantly lower than those of theophylline ($p<0.05$ to $p<0.001$). In addition to the effects of two last concentration of safranal in both groups 1 and 2 were significantly lower than that of *Crocus sativus* extract. These results showed a potent relaxant effect of *Crocus sativus* on tracheal chains of guinea pigs. The results also indicated the safranal is atleast, in part, responsible for the relaxant effect of *Crocus sativus*.

**P758**
**Cigarette smoke alters hyaluronic acid homeostasis in primary human lung fibroblasts**
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**Background:** Hyaluronic acid (HA) mediates lung tissue flexibility which is considerably reduced in asthma and COPD. The pro- or anti-inflammatory effect of HA on tissue physiology depends on the length of the HA molecules. Cigarette smoke is a major risk factor for developing COPD and also is linked to asthma severity. The aim of our study was to investigate the effect of cigarette smoke on HA homeostasis by lung fibroblasts.
**Methods:** Primary lung fibroblasts were isolated from lung tissue biopsies obtained from lung donors (controls) as well as from patients with asthma. Cells were incubated in the presence of smoke conditioned medium for 48 h. Gene expression of HA synthase 1 (HAS-1), hyaluronidase-1 (HYAL-1) and of the HA receptors CD44 and RHAMM was assessed by real time PCR.
**Results:** Cigarette smoke significantly reduced mRNA transcription of HAS-1, but induced the gene expression of HYAL-1. These results indicate an elevated HA catabolism in response to cigarette smoke. Furthermore, the expression of both HA receptors CD44 and RHAMM was significantly reduced in cells treated with cigarette smoke conditioned medium. The above described effects were more prominent in cells obtained from patients with asthma as compared to healthy controls.

**Conclusion:** Our results indicate that cigarette smoke induces HA catabolism which may lead to increased inflammation in the lung through low molecular weight HA molecules.

**P759**

**Roflumilast N-oxide, a PDE4 inhibitor, curbs bleomycin-induced lung fibroblast activation *in vitro***

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**Objective:** Activated lung fibroblasts may foster small airway thickening in COPD. Roflumilast, an oral, selective PDE4 inhibitor approved in EU for severe COPD, mitigates bleomycin (BLM)-induced lung fibrosis *in vivo*. This study addressed whether roflumilast N-oxide (RNO), the active metabolite of roflumilast, modulates effects of BLM on human lung fibroblasts (HLF) *in vitro*. RNO was used at 2nM, corresponding to therapeutic plasma levels.
**Methods:** HLF pre-incubated with RNO (2nM) were exposed to BLM for 24 or 48h. Reactive oxygen species (ROS) were quantified by 2',7'-dichlorofluorescein (DCF) accumulation from dichlorodihydrofluorescein diacetate. Total glutathione (GSH) was measured with the GSH reductase DTNB protocol. Proliferation and collagen synthesis was assessed as [$^3$H] thymidine and [$^3$H] proline incorporation (*p<0.05 vs control, $^{\#}$p<0.05 vs BLM).
**Results:** BLM (24h) at 50 and 100μg ml$^{-1}$ enhanced DCF accumulation by 1.9* and 1.4*-fold of control. RNO (2nM) reduced this increment by 50%$^{\#}$ and 85%$^{\#}$, respectively. In parallel, total GSH was reduced by BLM (100μg ml$^{-1}$) yet rescued by RNO (2nM) (nmol mg protein$^{-1}$: Control 32.2±1.1, BLM 26.6±1.3*, BLM+2nM RNO 30±1.7$^{\#}$). BLM (50μg ml$^{-1}$) increased [$^3$H] thymidine incorporation in HLF by 1.4*-fold that was abolished by RNO (2nM). Finally, RNO (2nM) reduced [$^3$H] proline incorporation that was increased to 179±35%* of control (100%) with BLM (50μg ml$^{-1}$) to 83±13%$^{\#}$ of control. RNO (2nM) did not affect basal ROS, total GSH, [$^3$H] thymidine or [$^3$H] proline incorporation.
**Conclusions:** BLM augmented ROS formation, reduced total GSH and increased proliferation and collagen synthesis of HLF *in vitro*. RNO (2nM) prevented these effects.

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**Fibroblast cell behaviour growing into a stiffened three dimensional collagen matrix**

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**Introduction:** Idiopathic Pulmonary Fibrosis is a process that involves abnormal cell behaviour and increased tissue stiffness. The aim of this study was to elaborate a stiffened three-dimensional (3D) collagen I matrix to study cultured human cells inside.
**Methods:** 3D matrices were elaborated with native collagen I and it was non-enzymathically glycated with ribose at different conditions. Matrix stiffness was measured with Athomic Force Microscope and collagen deposition was evaluated by confocal reflexion microscope. Primary fibroblasts were obtained from normal human lungs. Cellular viability in 3D matrices was evaluated by AlamarBlue fluorescence assay and LIVE/DEAD kit at different time points.
**Results:** It was observed that fluorescence AlamarBlue assay requires more time incubation to detect viability in 3D collagen matrices than in 2D cell cultures. An increased stiffness of 3D matrices was achieved with high concentration of collagen and ribose glycation from the second week. Stiff-variability and morphological changes in collagen I fibers was dependent on the media used for the matrix elaboration and the glycation condition. Cell death was detected in higher ribose concentrations. Fibroblasts showed a better-defined morphology and viability in matrices elaborated with lower ribose concentrations and DMEM media.
**Conclusion:** The development of this 3D collagen I matrix allows the fibroblast growth inside a modified microenvironment. Cell viability depends on different variables and it dramatically decreases with higher concentrations of ribose. This innovated model could help in the study of cell behaviour and phenotype at different conditions.
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**Tiotropium reduced carbachol-induced axpressions of IL-6 and IL-8 by primary human lung fibroblasts of asthma and non-asthma subjects**

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Asthma is a reversible, obstructive airway disease of unknown etiology. Although the molecular pathology of asthma is still obscure, muscarinic receptor antagonists are currently in use to treat the disease. Human lung fibroblasts express muscarinic receptors, which may regulate and fine-tune the expression of cytokine genes. In the present study we analyzed the effect of the muscarinic receptor agonist, carbachol, on the release of IL-8 and IL-6 by primary human lung fibroblasts obtained from asthmatic and non-asthmatic subjects. Fibroblasts were grown in RPMI-1640 (+10% FCS, 1% vitamins) in the presence of increasing carbachol concentrations (10-8M to 10-6M). Carbachol dose-dependently and significantly inhibited IL-6 release in IL-1β-stimulateded fibroblasts of non-asthmatics, but not in fibroblasts of asthma patients. Furthermore, Carbachol dose-dependently increased the IL-1β-induced IL-8 release, however, with no difference comparing fibroblasts obtained from asthmatics to cells of controls. The muscarinic receptor inhibitor tiotropium alone reduced the secretion of IL-6 and IL-8 by fibroblasts. Tiotropium (10-8 M) almost completely blocked the IL1beta-induced IL-6 and IL-8 secretion. Our data indicate that tiotropium reduces an inflammatory response of lung fibroblasts elicited by muscarinic receptors. These data may provide a rationale for the beneficial effects of tiotropium in the treatment of asthma.
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