376. Cellular signalling mechanisms in the airways

P3402

Effect of TGF-β on FoxO activity in airway smooth muscle cells

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Background: TGF-β is a mediator of abnormal airway smooth muscle (ASM) function in asthma and COPD. TGF-β triggers ASM cell (ASMC) hyperplasia and increases intracellular oxidants whilst reducing antioxidant enzyme expression. The O subfamily of forkhead box transcription factors (FoxO1, -3 and -4) activate antioxidant enzyme, cell cycle inhibitor and pro-apoptotic genes.
Aims & objectives: Determine whether TGF-β reduces FoxO activity in ASMCs leading to attenuated antioxidant enzyme expression and resistance to apoptosis.
Methods: mRNA and protein expression were determined by qRT-PCR and Western blotting, respectively. FoxO transcriptional activity was determined by a luciferase reporter assay. Smad activity was inhibited by infection with adenoviral vectors expressing dominant-negative Smad3 (DN-Smad3) and Smad7 genes, and histone deacetylase (HDAC) activity by treatment with trichostatin A (TSA).
Results: TGF-β (1 ng/ml) reduced the mRNA levels of the FoxO target genes BimEL (∼75%; $p<0.01$), PGC-1a (∼90%; $p<0.01$), Mn-superoxide dismutase (MnSOD) and catalase (∼50%; $p<0.01$) after 24hrs. TGF-β also reduced FoxO transcriptional activity (∼25%; $p<0.05$) 24hrs post-treatment. The inhibition of MnSOD, catalase and BimEL by TGF-β was reversed by DN-Smad3, Smad7 and TSA. TGF-β reduced FoxO3 (∼40%; $p<0.05$) and FoxO4 (∼70%; $p<0.001$) nuclear protein expression after 24hrs. TGF-β strongly increased FoxO1 mRNA and cytoplasmic protein levels (∼12-fold; $p<0.05$), however, nuclear levels were only weakly increased (∼3-fold; $p<0.05$) whilst DNA binding activity was unaffected, suggesting nuclear exclusion of FoxO1.
Conclusion: TGF-β decreases FoxO activity in ASMCs possibly by reducing FoxO nuclear expression.

P3403

Mast cells influence the physiological functions of fibroblasts

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Rationale: Mast cells (MC) play a role in allergic disease and anaphylaxis, by releasing their granules containing pro-inflammatory and pro-fibrotic mediators. Recent studies, suggest that mast cells may have a role in non-allergic chronic lung diseases. Since tissue remodelling and fibrosis is a key feature in these diseases our hypothesis is that the mast cell-fibroblast interplay is altered.
Methods: MC isolated from human peripheral blood and fibroblasts (HFL-1 fibroblasts and primary fibroblasts from lungs) were used in the experiments. The effects of the MC proteases; chymase and tryptase on fibroblasts were analyzed with western blot and q-PCR. To investigate the impact of cell-cell interaction, co-cultures between MC and fibroblasts was established and differentiation and production of extracellular matrix proteins were investigated.
Results: Mast cell tryptase significantly increased HFL-1 cell migration while chymase had no effect. This effect was not due to increased cell proliferation as neither tryptase nor chymase influenced the cell number. Tryptase triggered increased expression of alpha-smooth muscle actin, a marker for myofibroblasts. This was accompanied by a change in the production of collagens and proteoglycans. MC adhered directly to HFL-1 and primary fibroblasts when using co-cultures. The cell-cell contact caused alterations in the fibroblast phenotype and in the extra cellular matrix production.
Conclusions: These data indicate that MC and their mediators influence fibroblast phenotype and the production of extra cellular matrix proteins. Our results suggest that mast cells may play an important role in tissue remodelling and fibrosis by modulating the physiological function of fibroblasts.

P3404

Ageing lung shows reduced expression and activation of the *cyclic AMP response element binding protein*

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Background: Lung ageing is associated with morphological and, therefore, physiological changes. While much attention has been paid to changes of the extracellular matrix, less is known about the effect of ageing on intracellular compounds including transcription factors. As the transcription factor *cyclic AMP response element binding protein* (CREB) is involved in various cellular mechanisms, including glucose metabolism and growth factor-dependent cell survival, our study aimed at effect of lung ageing on CREB.
Methods: Lung tissues of young, adult and old mice were studied by PCR, immunoblot and immunohistochemisty. Moreover, we studied pre-senescent and senescent primary human lung fibroblasts, and the effect of known age-related factors.
Results: Lung tissue was characterized by an age-dependent reduction of activated (phosphorylated) and total CREB protein, whereas ageing did not influence the mRNA level of CREB. The mRNA of the phosphoenolpyruvate carboxykinase 1, which is a target of active CREB, was also reduced in old lung tissue. In this regard, the protein but not mRNA level of CREB was lower in senescent than pre-senescent lung fibroblasts. Down-regulation of CREB by siRNA transfection caused a lower maximal population doubling and higher cell death of human fibroblasts *in vitro*. Among several age-related factors we identified the increased non-enzymatic modification of the extracellular matrix with advanced glycation end-products as one reason for the reduced level of CREB protein in old lung tissues.
Conclusion: The reduction of active CREB might contribute to morphological and physiological changes in the ageing lung by a lower transcription of CREB target genes.

P3405

Notch signaling negatively regulate the expression of MUC5AC in airway goblet cells

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Introduction and background: Goblet cell metaplasia and airway mucus hypersecretion contributed to the pathogenesis of asthma and COPD. Evidence has shown that Notch signaling modulates the development of lung epithelial and the differentiation of intestinal goblet cells. Whether Notch signaling can regulate the expression of MUC5AC, a major component of airway mucus, is still unknown.
Aim and objective: To investigate whether Notch signaling can regulate MUC5AC expression in airway goblet cells.
Methods: Expression of Notch receptors and downstream molecules in airway goblet cells was examined. Then, after transfected with pEFBOS-NIC (upregulating Notch signaling) or treated with γ-secretase inhibitor (downregulating Notch signaling), the MUC5AC expression in airway goblet cells was examined. Finally, the transcriptional regulatory mechanisms were analyzed.
Results: Airway goblet cells express Notch1, Notch3 and downstream transcription factor hes1. Notch signaling can modulate the MUC5AC expression both in vivo and in vitro. Two N-box sites (hes1 binding site), -1439 and -893, were identified within mouse *MUC5AC* promoter. One N-box site (-293) was identified within human *MUC5AC* promoter. The Notch intracellular domain NIC, the active form of Notch receptors, activates the reporters driven by mouse or human *MUC5AC* promoters. Site-directed mutagenesis reporter assays confirmed that mouse N-box (-893) and human N-box (-293) negatively regulated *MUC5AC* promoter activity. ChIP assays showed that Hes1 bound to the *MUC5AC* promoter in both mouse and human airway goblet cells.
Conclusions: Notch signaling negatively regulates the MUC5AC expression through Hes1-dependent mechanism in airway goblet cells.

P3406

25-hydroxide-vitamin D induces TSLP expression in human bronchial epithelial cells via the VDUP1 pathway

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Background: Airway epithelial cells (AEC) express 1α-hydroxylase and are able to covert 25-hydroxide–vitamin D (25[OH]D) to an active form that plays a role in mucosal immunity. Thymic stromal lymphopoietin (TSLP), a cytokine mainly produced by AECs during allergic asthma reactions, plays a critical role in the activation of Th2 inflammatory responses. Therefore, we hypothesized that 25(OH)D would enhance the expression of TSLP in airway epithelia (16HBE cell line) and that vitamin D3 upregulated protein 1 (VDUP1) could be involved in this process.
Methods: 16HBE cells were cultured with 25(OH)D, and TSLP and VDUP1 mRNA and protein expression were then determined by means of quantitative PCR, ELISA, and Western blot analysis, as appropriate. The role of VDUP1 on TSLP expression was assessed in untreated and 25(OH)D-treated 16HBE cells where VDUP1 levels were manipulated via overexpression or siRNA-mediated silencing. The effect of 10^{-6} M itraconazole, a chemical inhibitor of 1α-hydroxylase, on the expression of TSLP was also determined.
Results: 25(OH)D significantly induced TSLP and VDUP1 mRNA expression in 16HBE cells. Silencing of VDUP1 dramatically inhibited 25(OH)D-mediated induction of TSLP, and overexpression of VDUP1 upregulated baseline TSLP expression and 25(OH)D-induced TSLP expression in 16HBE cells. Inhibition of 1α-hydroxylase reduced 25(OH)D-induced TSLP expression in 16HBE cells.
Conclusion: These data demonstrated that vitamin D increased TSLP expression in 16HBE cells via upregulation of VDUP1.

P3407

Acetylcholine leads to STAT-1 mediated oxidative/nitrosative stress in human bronchial epithelial cells

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The induction of nitric oxide synthase (iNOS) expression via the signal transducer and activator of transcription 1 (STAT-1) is involved in the mechanism of oxidative/nitrosative stress. Oxidative/nitrosative stress and Acetylcholine (ACh) are implicated in the activation of the bronchial epithelial cells in COPD. We aimed to investigate whether ACh generates oxidative/nitrosative stress in bronchial epithelial cells during airway inflammation of COPD and to evaluate the effects of anticholinergic drugs and long-acting β2-agonists in this mechanism.
Human bronchial epithelial cells (16HBE) were stimulated (4 hrs, 37°C) with induced sputum supernatants (ISSs) from healthy controls (HC) (n=6), healthy smokers (HS) (n=6) or COPD patients (n=6), respectively, as well as with ACh (10 μM). STAT-1 pathway activation (Ser727 and Tyr701) and iNOS were evaluated in the cell lysates using Western blot analysis, while reactive oxygen species (ROS) in the cells and nitrotyrosine in the supernatants were evaluated by flowcytometry and by ELISA, respectively. The effect of Tiotropium (Spiriva®) (20nM), alone or in combination with Olodaterol (1nM), was tested.
ISSs from COPD patients and ACh significantly increased the phosphorylation of STAT-1Ser727 and STAT-1Tyr701, the expression of iNOS and the production of ROS/nitrotyrosine in stimulated 16HBE when compared with ISSs from HC or HS subjects. The use of Tiotropium and Olodaterol alone well controlled these events. These results support the use of Tiotropium and Olodaterol to reduce the oxidative/nitrosative stress generated by ACh during airway inflammation of COPD via the STAT-1 pathway activation in human bronchial epithelial cells.

P3408

TLR2 and TLR4 induced tolerance in alveolar macrophages; differential effect on TNFα and IL-8 release

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Alveolar macrophages (AM) play a key role in the pathogenesis of COPD. Bacterial stimulation of toll-like receptors (TLR) on AM causes secretion of inflammatory mediators such as IL-8, promoting neutrophil migration to the lung. Repeat stimulation of TLR results in a reduced inflammatory response, known as tolerance.
To emulate events during bacterial colonisation of the lung, we investigated the effects of repeated TLR stimulation in AM from COPD subjects.
AM from healthy (n=7) and COPD (n=9) patients were isolated. For tolerance induction, treatment with media, LPS (1μg/ml) or Pam3CSK4 (0.1μg/ml) was performed over a 24 hour period. Cells were then washed before a repeat stimulation. Levels of IL-8 and TNFα were measured post-treatment.
Repeat LPS exposure reduced TNFα production but had no effect on IL-8 release from AM (table 1). Differences between healthy and COPD AM responses were negligible. Pre-incubation with LPS before Pam3CSK4 stimulation appeared to prime cells for further IL-8 production compared to levels secreted after two consecutive Pam3CSK stimulations.

Table 1. TNFα and IL-8 Release Following LPS-induced Tolerance in COPD AM

1st 24 hr 2nd 24 hr	Media Media	Media LPS	LPS LPS
TNFα ng/ml (s.e.m)	0.01 (0.007)	11.2 (2.9)	3.0 (1.8)*
IL-8 ng/ml (s.e.m)	6.6 (2.0)	299.4 (42.7)	352.7 (64.5)

Media+LPS v LPS+LPS *$p<0.05$.

Chronic LPS exposure in COPD lungs results in diminished TNFα production but continual IL-8 release. This may cause neutrophil recruitment and inflammation, further contributing to disease pathogenesis.

P3409

TLR4 signaling potentiates airway secretion from the swine submucosal gland via NO/cGMP/cGK pathway

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Rationale: Airway secretion plays an important role in the airway defense as a part of innate immunity. A major fraction of the airway fluids appears to be derived from submucosal gland in response to ACh. Toll-like receptor 4 (TLR4) recognizes gram-negative bacteria and activates the innate immune systems. However, the biological role of TLR4 in the airway secretion is not well understood.
Methods: Freshly isolated swine tracheal submucosal gland cells were investigated their secretory activities as ionic currents by applying a patch-clamp technique. LPS was used as a potent TLR4 ligand. The expression of TLR4 was estimated by both the immunofluorescent staining and RT-PCR. The involvement of NO/cGMP/cGMP-dependent protein kinase (cGK) pathway was investigated by applying both the NOS and cGK inhibitors. The synthesis of endogenous NO was estimated by an intracellular NO indicator, DAF-2DA.
Results: LPS significantly potentiated the ACh-evoked ionic currents. This potentiating effect was completely abolished by the pretreatment of anti-TLR4 antibody or TLR4 antagonist. The immunofluorescent staining and RT-PCR revealed the abundant expression of TLR4 on tracheal submucosal glands. Two different inhibitors of each NOS and cGK completely abolished the LPS-induced potentiating effect, respectively. LPS further increased the ACh-induced synthesis of NO.
Conclusions: Our studies revealed that TLR4 signaling could potentiate the electrolyte and water secretions from tracheal submucosal glands via the activation of NO/cGMP/cGK pathway. These findings suggest that TLR4 takes part in the airway mucosal innate immune systems as one of important pathogen-specific secretagogue.

P3410

Flagellin potentiates the Ca2+-dependent electrolytes secretion from airway submucosal gland in a TLR4-independent, TLR5-dependent and cGK-dependent manner

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Background: Airway surface fluids are mainly secreted from submucosal glands and play important roles in the airway defense via the upregulation of mucociliary transport. Although Toll-like receptors (TLRs) recognize distinct pathogen-associated molecular patterns, it is not well known whether TLRs directly upregulates the airway secretions. Recently, we have reported that TLR4 works as one of important secretagogue in the airways (AJRCMB 2011). In the present study, we focused on the TLR5 signaling as other candidate for a potentiator of airway secretion.
Methods: Freshly isolated swine tracheal submucosal gland cells were prepared and measured their secretory activities as ionic currents by applying a patch-clamp technique. The expression of TLR5 was estimated by both the immunofluorescent staining and RT-PCR. The intracellular mechanisms were investigated by applying some inhibitors of NOS or cGMP-dependent protein kinase (cGK).
Results: Flagellin, a TLR5 ligand, showed significant potentiating effects on ACh-evoked ionic currents. These effects were abolished by the pretreatment with anti-TLR5 antibody, but not by specific TLR4 antagonist. Two different inhibitors of each NOS and cGK abolished these effects. We also revealed that the expression of TLR5 on the tracheal submucosal gland cells.
Conclusion: Our study showed that flagellin caused the potentiating effects on airway secretions in a TLR5-dependent, TLR4-independent manner and that NO/cGMP/cGK pathway was involved in these intracellular mechanisms. These finding suggest that TLR5 also works as a potentiator of airway secretion in the airway mucosal immune systems.

P3411

Sodium transport mediated by concentration-sensitive sodium channel in mouse alveolar epithelium

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Introduction: The concentration-sensitive Na^+ channel (Na_C), a member of the family of voltage-dependent Na^+ channels, opens in response to increased Na^+ concentration in the extracellular fluid ($[Na^+]_o$). Although the expression of Na_C in alveolar epithelial type II (AT II) cells has been reported, the physiological role of Na_C in lung tissue has not yet been established. Various ion channels in the alveolar epithelium are involved in maintaining the alveolar fluid balance; therefore, we hypothesized that Na_C-mediated Na^+ transport contributes to the clearance of Na^+ from the alveolar fluid. In this study, we examined Na_C distribution in mouse lung tissue and the $[Na^+]_o$-dependent influx of Na^+ into mouse alveolar epithelium.
Methods: We used immunohistochemistry and immunofluorescence to study mouse lung tissue using antibodies against Na_C and other ion transport proteins. In situ hybridization was performed using a digoxigenin-labeled antisense probe for Na_C mRNA. Na^+ dynamics in mouse alveolar epithelium were analyzed using sodium-binding benzofuran isophthalate and an image analyzer Argus-50.
Results: The alveolar epithelial type I (AT I) cells showed positive staining with anti-Na_C antibodies. Similarly, Na_C mRNA signals were detected in the AT I cells by in situ hybridization. Confocal laser scanning microscopy showed the presence of Na_C in the cell membrane of the AT I cells. Na_C was partially colocalized with γ epithelial sodium channels (ENaC). Results of image analysis showed that Na^+ influx into alveolar epithelium was dependent on elevation of $[Na^+]_o$.
Conclusions: These findings suggest that Na_C, expressed in AT I cells, is involved in Na^+ transport in the alveolar epithelium.

P3412

Characterisation of endogenous IL-33 using a model of *Alternaria*-induced pulmonary inflammation

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Background: IL-33 is a potential mediator of chronic inflammatory diseases including asthma. However, endogenous forms of IL-33 are poorly understood. Here we have used a model of *Alternaria alternata* (ALT) challenge to investigate *in vivo* mechanisms of IL-33 processing and release.
Methods: Cytokines were quantified in bronchoalveolar lavage (BAL) from BALB/c mice by MSD assay. Mast cells were differentiated from bone marrow over 5 weeks and stimulated with BAL or recombinant IL-33 for 24 hours.
Results: Intranasal challenge with 50μg ALT, but not 25μg house dust mite, extract in 50μL to anaesthetised BALBɱice caused a spike (∼2000pg at 15-30min) of IL-33 in BAL followed by rapid clearance (below detection limit of 2pg within 4-5 hrs). Key downstream effects were an increase in 5 and 24 hours BAL IL-5 (∼400pg and ∼600pg, respectively) and eosinophilia at 24 hours. Dependence on the IL-33-ST2 axis was confirmed in ST2 and IL-33 KO mice which showed no IL-5 or eosinophilia after challenge. IL-33 is considered an alarmin, released on cell damage, yet histological analysis of ALT challenged lungs showed no overt damage to the airway epithelium. The presence of active IL-33 in WT but not IL-33KO BAL was confirmed *ex vivo* by stimulation of mouse mast cells. Western blot analysis of BAL demonstrated the presence of full length IL-33 (∼31kDa) and a ∼20kDa processed form in WT, but not IL-33KO, ALT-treated mice.
Conclusion: IL-33 is released rapidly and in large quantities following ALT exposure. This occurs in the absence of overt epithelial injury and results in generation of a ∼20kDa processed form of IL-33 *in vivo*. These data support the current view of IL-33 as an alarmin-type molecule.

P3413

IL33 expression and release from airway epithelium

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Background: IL33 has been highlighted as a potential key player in several chronic respiratory diseases with a fibrotic aietiology. IL33 expression is thought to be predominantly in "lining cells"i.e airway epithelia, suggesting this may be a primary source of IL33; as an alarmin in response to epithelial insult.
Aim: To evaluate expression of IL33 and release in response to airway epithelial damage.
Methods: BAL was prospectively collected from post lung transplant patients (n=230) levels of IL33(ELISA) and relationship to Broncholitis Obliteran Syndrome (BOS) evaluated. Explant lung tissue from patients with IPF(n=3),COPD(n=3), Bronchiectasis(n=3) and CF(n=3) was identified and expression of IL-33 evaluated (immunohistochemistry). Epithelial damage was induced in Primary Bronchial Epithelial Cells and A549 cells by oxidative stress (H2O2) and freeze/thaw and release of IL33 evaluated(ELISA and Western Blot).
Results: IL33 conc. in post-lung transplant BAL trended towards an increase following diagnosis of BOS but did not reach statistical significance. However, elevated levels of IL-33 were associated with positive microbial culture (mean 178.197pg/ml± 42.89vs 71.31pg/ml±20.12 p=0.007). IL33 was strongly expressed in airway epithelia with a predominant nuclear location.IL33 was not detected in response to epithelial cell damage.
Conclusion: IL33 is strongly expressed in the airway epithelium of chronic respiratory conditions but does not appear to be released as an alarmin in response to airway epithelial cell damage. Elevated IL33 associated with infection in the post transplant population may contribute towards allograft dysfunction but further work is required to evaluate the cellular mechanistic pathways.

P3414

Extracellular matrix influences the inflammatory response of primary bronchial epithelial cells

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Rationale: Airway remodelling is a feature in many chronic airway diseases. This is manifested as alterations in the extracellular matrix composition. Our aim was to investigate how different extracellular matrix molecules influenced the production of inflammatory cytokines in epithelial cells.
Methods: Primary human bronchial epithelial cells were grown on plates coated with the extracellular matrix proteins collagen-1, collagen-4 and fibronectin. The cells were challenged with a cytomix consisting of TNF-alpha and IL-1-beta (both 10ng/ml) and the cytokine production was examined with proteome profiler antibody arrays (R&D) that detect the production of 40 cytokines simultaneously.
Results: Basal and induced production of IL-1-aplha was similar in cells grown on collagen-1 and fibronectin but IL-1-alpha could not be detected in cells grown on collagen-4. Low levels of G-SCF were detected in cells on collagen-1 or fibronectin stimulated with cytomix, but when cell were grown on collagen-4 the levels were dramatically higher. There were no difference in basal and induced production of GRO-alpha and Il-8 on cells grown on collagen-1, collagen-4 or fibronectin. The basal level of Il-6 was dramatically reduced on collagen-4 compared to collagen-1 and fibronectin. However, the cytomix triggered similar levels of IL-6 production regardless of matrix substrate.
Conclusions: In this study we show that the extracellular matrix actively contributes to modify cell phenotypes. Cells grown on collagen-4 that represents a "homeostatic matrix" produced a different repertoire of inflammatory mediators than cells grown on "remodeled matrix proteins" collagen-1 and fibronectin.

P3415

Inhibition of *A. fumigatus* growth by human respiratory cells

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The innate immune response to fungi mainly involves macrophages and neutrophils. By contrast, the possible participation of respiratory epithelial cells (REC) has been poorly studied. In the present study we observed that REC display the property of inhibiting mycelium development of Aspergillus fumigatus (A.f) and that this activity is linked to PI3 kinase activation.
Bronchial cells (BEAS-2B cell line) were incubated with A.f and the growth of the fungus was estimated by optical microscopy observation and measurement of galactomannan concentrations in the extracellular media. The role of PI3 kinase was evidenced by the use of the specific inhibitor LY294002 (30 μM). Spore internalization in REC was followed by epifluorescence. FITC-labeled spores (green) when outside of cells were further labelled with an anti-FITC antibody labeled with a red fluorochrome (Alexafluor 568).
In the presence of REC, most of the spores do not germinate as opposed to spores incubated in the absence of cells. The growth quantification by galactomamann concentration measurements display a 4.5 lower concentration in the presence of cells. The anti-aspergillus activity is inhibited when cells are incubated with LY294002, indicating the involvement of the PI3 kinase pathway. Of note, inhibitors of p38 MAP kinase and ERK1/2 are inactive. We evidenced that the activity is directed against the spores and not again the hyphae but is not linked to the spore internalization.
In conclusion, as macrophages and neutrophils, REC play a role in the anti-aspergillus activity. They are able to prevent the mycelium development and as such potentially prevent its dissemination.
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P3416

Activation of both transcription factors STAT5 and IRF-1 is insensitive to corticosteroids in asthmatic bronchial smooth muscle cells exposed to TNFα/IFNγ

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We have previously developed a cellular model in healthy airway smooth muscle (ASM) cells where corticosteroids (CS) lose their anti-inflammatory action when cells are exposed to TNFα in combination with IFNγ. The molecular mechanisms by which TNFα/IFNγ promote CS insensitivity has not been completely investigated although an increased activation of transcription factors such as STAT5 has been implicated in other cell types. It is also not known whether TNFα/IFNγ also interfere with CS in ASM cells derived from asthmatic patients. In the present study, we found that TNFα/IFNγ-induced the expression of different steroid-resistant chemokines CX3Cl1, CCl5 and CCl11 in asthmatic ASM cells (n=3). We also found that TNFα/IFNγ (0-6hr)-increased phosphorylation of STAT5 in a time-dependent fashion reaching a maximum at 30 min followed by a lower activated state that was sustained up to 6 hr. Prior treatment with fluticasone (0.1-100 nM) did not affect cytokine-induced STAT5 activation but dose-dependently increased STAT5 phsophorylation. Similarly, the sustained activation of the transcription factor IRF-1 by TNFα/IFNγ (up to 6 hr) was also unaffected by fluticasone (01-100 nM). Together, these data show for the first time that TNFα/IFNγ induced a sustained activation of corticosteroid-resistant STAT5 and IRF-1 in asthmatic ASM cells (n=3). Because STAT5 and IRF-1 have been associated with reduced CS action in other cell types, our findings suggest the possible of involvement of these transcription factors in driving CS insensitivity in asthmatic airway smooth muscle cells.

P3417

Formoterol restores oxidative stress-induced corticosteroid insensitivity via activation of protein phosphatase PP2A

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Introduction: Oxidative stress induces corticosteroid (CS) insensitivity via the activation of phosphoinositide-3-kinase (PI3K) and reduced HDAC2 activity. We have demonstrated that the long-acting β_2-adrenoceptor agonist formoterol (FORM) restores CS sensitivity via PI3Kδ-Akt signalling inhibition (BJP 2012). However, the mechanism for this effect has not yet been elucidated. Here, we investigated whether FORM (10^{-9} M) inhibited PI3K signalling through a serine/threonine phosphatase PP2A.

Methods: CS sensitivity was determined by IC_{50} of dexamethasone on TNFα-induced IL-8 in U937 cells exposed to H_2O_2 or by budesonide ability of inhibition of LPS-induced TNFα in PBMCs transfected with PP2A. HDAC2 and PP2A were measured by fluorescence-based activity assays. Phosphorylation of Akt was used as a marker of PI3K activation determined by Western blotting and okadaic acid (OA) was used to inhibit PP2A.

Results: Dexamethasone potency, reduced 3-fold by H_2O_2, was restored by FORM in U937 cells. FORM also counteracted H_2O_2-induced increase of Akt phosphorylation, decrease of HDAC2 activation and PP2A activity. Pretreatment with OA abrogated the effects of FORM. In PBMCs from COPD patients, PP2A activity was significantly reduced by 50% vs. healthy volunteers but was restored by FORM *ex vivo* treatment. Furthermore, PP2A overexpression increased responsiveness to budesonide in PBMCs from COPD patients.

Conclusions: Activation of PP2A by FORM inhibits PI3K activation, thereby restoring HDAC2 activity and CS sensitivity after oxidative stress. PP2A appears to be a negative regulator of PI3K signalling and impaired PP2A in COPD may be a potential therapeutic target.

P3418

Corticosteroid insensitivity in severe asthma: Impaired nuclear translocation of glucocorticoid receptor in airway smooth muscle cells

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Background: Patients with severe asthma respond poorly to corticosteroids (CS). Airway smooth muscle cells (ASMC) of severe asthma display CS insensitivity. CS mediate their effects through activation of the glucocorticoid receptor (GR) and suppression of NF-κB activity.

Aims and objectives: Compare protein/mRNA, phosphorylation, and nuclear translocation of GR and NF-κB (p65).

Methods: ASMC of the healthy (9), non-severe (NSA; 8) and severe asthmatics (SA; 8) were obtained from endobronchial biopsies, cultured at passage 4-5. Cells were treated with TNF-α (10 ng/ml)/dexamethasone (Dex; 10^{-7} or 10^{-6} M). Whole cell protein or nuclear extracts were assessed by Western Blot, mRNA by qRT-PCR.

Results: TNF-α induced greater p65 in SA, whereas baseline and TNFα-induced nuclear abundance, and Dex suppression of p65 expression, were similar between groups. GR expressed in asthma was 49% of that in the healthy ($p<0.01$), with no difference between NSA and SA. Dex-induced nuclear translocation of GR in SA was approximately 60% of that in either the healthy or NSA at 30 min-2h, whereas baseline levels were similar. In healthy subjects, Dex maximally induced GR phosphorylation at S211 at 1 hr (5.2-fold vs baseline; $p<0.001$), which was maintained over 4 hr; phosphorylation status at 2 hr showed no difference between groups.

Conclusions: Baseline GR is decreased in ASMCs of asthma, while induced GR translocation is impaired only in SA. Although TNF-α induces greater p65 expression in SA, nuclear translocation and Dex suppression of p65 expression are similar between all groups. Impaired nuclear translocation may underlie the mechanism of CS insensitivity in SA.

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A role of mTOR in cigarette smoke-induced corticosteroid insensitivity

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Introduction: The corticosteroid insensitivity is a major barrier to the treatment of severe asthma and COPD. It has been revealed that cigarette smoke induced inactivation of HDAC2 through activation of PI3K/Akt pathway, leading to corticosteroid insensitivity. Mammalian target of rapamycin (mTOR) is one of the most important effectors of PI3K/Akt pathway. It is a serine/threonine kinase, and implicated in disease states where growth is deregulated and homeostasis is compromised.

Aims: To determine the role of mTOR on the development of corticosteroid insensitivity in COPD and under oxidative stress.

Methods: mTOR activity was calculated by the phospho-p70s6k band intensity in Western blot analysis. Corticosteroid sensitivity was determined as the 50% inhibitory concentration of dexamethason on TNF-a-induced interleukin-8 release in U937 cells.

Results: mTOR activity was increased within 5minutes after cigarette smoke stimulation in U937 cells, then decreased gradually until below the baseline. mTOR inhibitor, rapamycin, totally suppressed this increase. The EC50 value of dexamethasone was 5.0nM and the Emax value was 62.8±6.4% (n=4) in the intact cells, but after incubation with cigarette smoke extract, EC50 was increased to 47.9nM and Emax was decreased to 42.4±9.1%, suggesting cigarette smoke decreased dexamethasone sensitivity almost by one-tenth. EC50-Dex was 12.9nM and Emax was 55.9±6.9% in the presence of Rapamycin (20nM, 2hr) and rapamycin partially reversed this insensitivity.

Conclusions: Cigarrete smoke extract treatments increased mTORC1 activity in U937 cells, and rapamycin partially reversed corticosteroid insensitivity caused by cigarette smoke exposure.