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NICOTINE AND ENDOTOXIN IN AIRWAY HYPERREACTIVITY AND INFLAMMATION

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NICOTINE AND ENDOTOXIN IN AIRWAY HYPERREACTIVITY AND INFLAMMATION

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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This thesis is dedicated to my parents

ABSTRACT

Active, as well as passive cigarette smoke exposure is detrimental to the human health, negatively affecting the cardiovascular, respiratory and immune systems. In addition, it is also carcinogenic.

The present thesis focused on the effects of cigarette smoke and two of its major components nicotine and endotoxin (lipopolysaccharide, LPS) on airway hyperreactivity (AHR) and inflammation, in both the lower and upper airways. The roles of nicotine and LPS were analyzed in murine *in vitro* and *in vivo* models. Further, the effects of smoking on the expressions of activin receptor-like kinases (ALK) were analyzed in tonsils from heavy smokers and matching non-smokers.

The first four papers of this thesis illustrated the highly complex interactions induced by nicotine and LPS involving direct airway smooth muscle contractions, indirect nerve-mediated airway contractions and epithelium-dependent relaxations. Short-term exposure (1 day) to nicotine in an *in vitro* organ culture model resulted in decreased epithelium-dependent airway relaxations via cyclooxygenase (COX) and microsomal prostaglandin E synthase 1 (mPGES-1) pathways. Long-term exposure (4 days) in the same model caused increased smooth-muscle-mediated airway contractions via transcriptional upregulation of kinin-receptors, involving mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinases (JNK) and phosphodiesterase (PDE) 4 pathways. In parallel, JNK-mediated transcriptional upregulation of toll-like receptors (TLR) was also seen. This resulted in enhanced contractile responses to the prototypical TLR4 agonist LPS, but at the same time decreased LPS-mediated AHR to electric field stimulation (EFS). In analogy with the latter, *in vivo* experiments using subcutaneously implanted pumps that delivered nicotine during 24 consecutive days, followed by 3 days of intranasally instilled LPS, demonstrated that the AHR induced by LPS was markedly decreased in the nicotine-treated mice. This highlighted the role of neuronal mechanisms in the development of lower airway AHR.

The fifth paper used human tonsils to investigate the effect of chronic cigarette smoke exposure on the expression of ALK. A novel cotinine assay was developed to quantify the level of cigarette smoke exposure in tonsil homogenates. Results showed a positive correlation between increased levels of smoke exposure and increased tonsillar mRNA expressions of IL-8, ALK1 and 2.

In conclusion, both nicotine and LPS contribute to the development of AHR. Nicotine modulates the response to LPS in murine models both *in vitro* and *in vivo*. Altered ALK expressions in smokers may contribute to the poor prognosis that characterizes tonsillar cancer among smokers. These results may contribute to the development of specific and personalized treatment strategies for patients that are actively or passively exposed to nicotine or cigarette smoke.

LIST OF SCIENTIFIC PAPERS

I. XU Y, Zhang Y and Cardell L-O.
Nicotine enhances murine airway contractile responses to kinin receptor agonists via activation of JNK- and PDE4-related intracellular pathways.
Respiratory research 2010;11(1):13.

II. XU Y and Cardell L-O.
Nicotine impairs cyclooxygenase-2-dependent kinin-receptor-mediated murine airway relaxations.
Toxicology and applied pharmacology 2014;275(1):12-21.

III. XU Y, Zhang Y and Cardell L-O.
Nicotine exaggerates LPS-induced airway hyperreactivity via JNK-mediated up-regulation of Toll-like receptor 4.
American journal of respiratory cell and molecular biology 2014;51(3):370-9.

IV. XU Y and Cardell L-O.
Long-term nicotine exposure dampens LPS-induced nerve mediated airway hyperreactivity in murine airways.
Manuscript

V. XU Y and Cardell L-O.
Increased tonsillar expression of ALK1 and 2 among smokers.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AHR	Airway hyperreactivity
ALK	Activin receptor-like kinases
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
ERK	Extracellular signal-regulated kinases
ET	Endothelin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
HPV	Human papillomavirus
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
Mch	Methacholine
mPGES-1	Microsomal prostaglandin E synthase-1
NF-κB	Nuclear factor kappa-B
PDE	Phosphodiesterase
PGE_2	Prostaglandin E_2
PRR	Pattern recognition receptors
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TRPV1	Transient receptor potential vanilloid type 1

1 BACKGROUND

1.1 AIRWAY HYPERREACTIVITY

Airway hyperreactivity (AHR) is a major and defining feature of asthma and to a lesser extent chronic obstructive lung diseases (COPD). It is defined as an exaggerated bronchoconstrictor response to direct pharmacological stimuli, such as histamine and methacholine, or indirect stimuli, such as exercise, cold air and hyperventilation (Cockcroft *et al.*, 2006).

The hyperactive phenotype of airway smooth muscle (ASM) is believed to be the primary cause of AHR. Bronchodilators, such as β-agonists, have therefore always played a central role in the treatment of AHR, both as rescue and maintenance therapy (Cazzola *et al.*, 2012). However, the use of long-acting β-agonists alone has been controversial and it is recommended that long-acting β-agonists should not be taken without simultaneous treatment with inhaled corticosteroids (Beasley *et al.*, 2010).

The relationship between airway inflammation and AHR is complex. AHR is believed to be a consequence of airway inflammation. Numerous studies have shown a positive correlation between inflammatory cell counts in bronchoalveolar lavage (BAL) and AHR (Cockcroft *et al.*, 2006). However, a number of other studies in both patients (Crimi *et al.*, 1998; Singapuri *et al.*, 2010) and animal models (Starkhammar *et al.*, 2012; Swedin *et al.*, 2009) have also suggested that inflammation and development of AHR can be two dissociated events.

ASM function can be regulated by the airway epithelium. The airway epithelium not only acts as a simple mechanical barrier, but also expresses a range of pattern-recognition receptors (PRR) and produces a number of important inflammatory mediators (Lambrecht *et al.*, 2012), as well as bronchoactive substances including acetylcholine (Proskocil *et al.*, 2004), prostaglandin E_2 (PGE_2) (Balzary *et al.*, 2006) and nitric oxide (NO) (Gourgoulianis *et al.*, 1998). A recent study has shown that bradykinin can also be an important mediator in human ASM and epithelium interaction (Deacon *et al.*, 2015).

Neuronal regulation of AHR has long been recognized. Parasympathetic fibers innervating the ASM, vascular smooth muscle and glands regulate smooth muscle tone and mucus secretion. Hypersensitive nerves can therefore lead to AHR with symptoms such as cough and dyspnea (Undem *et al.*, 2009). In the 1950s, surgical denervation and vagotomy were performed to treat severe asthma (Levine *et al.*, 1950) and COPD (Abbott *et al.*, 1953). This procedure indeed gave temporary relief of symptoms, but has been abandoned due to high complication risks and the introduction of more effective pharmacological treatment options targeting inflammation or smooth muscle. Interest in neuronal mechanisms has been revitalized during the recent years due the increasing recognition of the roll of neuronal transient receptor potential channels in airway diseases (Grace *et al.*, 2014).

1.1.1 Cigarette smoking

Tobacco smoke is a composite of irritant molecules, including nicotine, acetaldehyde, formaldehyde, nitrogen oxides and heavy metals. It is well-known that both active and passive cigarette smoke exposure cause increased AHR (Janson *et al.*, 2001; Menon *et al.*, 1992). In addition, smoke-addicted asthmatics constitute a subgroup with a poor response to local corticosteroid treatment (Comhair *et al.*, 2011; Stapleton *et al.*, 2011). Maternal cigarette smoking (Strachan *et al.*, 1998) and passive smoke exposure during childhood (Tinuoye *et al.*, 2013) both increase the risk for wheezing in early life and the development of childhood asthma.

Similar observations have been made in laboratory animals. Mice repeatedly exposed to side-stream cigarette smoke exhibit increased airway contractile responses to carbachol, endothelin-1 (ET-1) and potassium (Lei et al., 2008), while chronically exposed guinea pigs exhibit increased airway reactivity to bradykinin and capsaicin, without altering their responses to methacholine or histamine (Bergren, 2001). This suggests that bradykinin plays an important role in tobacco smoke-induced AHR.

Nicotine is the main addictive compound in cigarette smoke and also freely available over-the-counter as a smoke cessation aid. Once inhaled, nicotine is quickly taken up by the bloodstream and distributed throughout the body, to act primarily on nicotinic acetylcholine receptors, leading to the classical symptoms of heart palpitation and increased blood pressure. Acute nicotine exposure causes a transient mild airway relaxation (Streck *et al.*, 2010), whereas the effects of long-term exposure on the respiratory system are much more complex. Many of the detrimental health effects of cigarette-smoke are believed to be due to the ability of nicotine to affect the immune system, leading to both inflammatory (Vassallo *et al.*, 2008) and anti-inflammatory effects (Wang *et al.*, 2003). The effect of nicotine on AHR is however less studied.

1.1.2 Toll-like receptors

Toll-like receptors (TLR) are the most well-known family of PRR. They are essential components of the innate immune system, and are found on a variety of airway cells including epithelial, smooth muscle and infiltrating inflammatory cells. Thirteen TLRs have been identified in mice and four sets of them can be found on the cell surface. They recognize conserved microbial patterns such as lipopolysaccharide (LPS) of Gram-negative bacteria (TLR4), lipoteichoic acids of Gram-positive bacteria and bacterial lipoproteins (TLR1/TLR2 and TLR2/TLR6), flagellin (TLR5) and Toxoplasma gondii profilin-like proteins (TLR11) (Akira *et al.*, 2006). Our group has previously shown that TLR is widely expressed and functional on human airway smooth muscle (Mansson Kvarnhammar *et al.*, 2013).

Dysregulation in airway TLR expression can lead to both acute and chronic inflammatory lung diseases including asthma and COPD (Lafferty *et al.*, 2010). The "hygiene hypothesis" suggests that microbial infections, especially during childhood, can protect us from developing allergic diseases such as asthma (Tantisira *et al.*, 2001). However, there are also studies suggesting that certain infections, such as respiratory syncytial viruses and rhinoviruses in childhood increase the risk of asthma later in life (Feldman *et al.*, 2015). The role of TLRs in our innate immune defense against microbial infections has led to the speculation that TLR signaling pathways might be involved in the regulation of the extremely delicate balance in Th1/Th2 responses, and thus depending on the activation pattern, either prevents or promotes asthmatic diseases (Schroder *et al.*, 2007).

LPS is a prototypical TLR agonist primarily activating TLR4. It is an important component of the Gram-negative bacterial cell wall and often utilized to model the effect of bacterial infections under experimental conditions. LPS has long been known to be an active component of cigarette smoke (Hasday *et al.*, 1999). In fact, cigarette smoke is one of the major sources of LPS in indoor environments (Larsson *et al.*, 2004). Exposure to LPS in both occupational environments (Larsson *et al.*, 1994; Vogelzang *et al.*, 1998) and at home (Michel *et al.*, 1996) has been shown to negatively affect respiratory health and lung function. Our group has shown that activation of TLR4 by LPS can cause AHR both in isolated airway segments (Bachar *et al.*, 2004) and in an *in vivo* mouse model (Starkhammar *et al.*, 2012).

1.1.3 Bradykinin and related kinins

Bradykinin and related kinins are peptides that act as pro-inflammatory mediators. They are produced from both blood (plasma kallikrein-kinin system) and local tissue (tissue kallikrein-kinin system) in response to inflammatory stimuli. Bradykinin is formed from the kininogen precursor after proteolytic cleavage by the enzyme kallikrein. It can be further converted by carboxypeptidase N (kinase I) to des-Arg^9-bradykinin. Both bradykinin and des-Arg^9-bradykinin are degraded by kininase II, an enzyme identical to angiotensin-converting enzyme and neutral endopeptidase (Moreau *et al.*, 2005; Zhang *et al.*, 2013). It has been proposed that accumulation of bradykinin might be the mechanism behind angiotensin converting enzyme inhibitor induced cough and angioedema (Fox *et al.*, 1996; Mahmoudpour *et al.*, 2013).

Two classes of kinin receptor ligands are recognized by the corresponding receptor subtypes: B_1 and B_2 receptors, both belonging to the family of G-protein coupled receptors (GPCR). Kinin B_1 receptors are expressed at low levels in healthy tissue but induced following inflammatory stimuli, whereas the kinin B_2 receptors are constitutively expressed. The actions of bradykinin are mainly mediated by the B_2 receptors, whereas the actions of des-Arg^9-bradykinin are mainly mediated by the B_1 receptors (Leeb-Lundberg *et al.*, 2005; Zhang *et al.*, 2004). Icatibant, the selective antagonist for the kinin B_2 receptor has been approved

for the treatment of hereditary angioedema within the European Union since 2008 (Wu *et al.*, 2015).

Increased levels of bradykinin are found in BAL of asthmatics following allergen challenge (Christiansen *et al.*, 1992). Directly inhaled bradykinin causes a potent bronchoconstriction in asthmatic patients, but even in high concentrations has no effect in healthy individuals (Barnes, 1992). Thus, bradykinin has occasionally been suggested as a marker, more sensitive than methacholine, for demonstrating AHR (Berman *et al.*, 1995; Suguikawa *et al.*, 2009).

In murine models, the kinin receptors mediate both bronchoconstriction and epithelium-dependent airway relaxations, as well as mucus secretion, edema and cough. Effects are dependent on the localization of the receptor and the pre-contractile state. Relaxations have been shown to be caused by the release of the cyclooxygenase (COX) product and bronchodilator PGE_2 (Li *et al.*, 1998). Kinin B_1 receptors have also been suggested to be essential for LPS-induced AHR and acute lung injury in mice (Campanholle *et al.*, 2010). In guinea pig, chronic exposure to tobacco smoke increases the airway reactivity to bradykinin without altering the response to methacholine or histamine (Bergren, 2001), suggesting a special role for bradykinin in smoke-induced AHR.

1.1.4 Mitogen-activated protein kinases

The mitogen-activated protein kinases (MAPK) signaling cascade is composed of a family of protein kinases whose functions and regulations have been conserved during evolution. There are at least three main groups of MAPK in mammalian cells including extracellular signal-regulated protein kinase 1/2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK). They are activated by a three-tiered sequential phosphorylation starting from MAPK kinase kinase, MAPK kinase to MAPK, and are inactivated by MAPK phosphatases.

Once activated, MAPKs can induce translocation and activation of their downstream transcriptional factors to regulate gene expression (Duan *et al.*, 2006). In airways, MAPK activation can lead to ASM proliferation (Hirst *et al.*, 2000), cytokine production (Hayashi *et al.*, 2000) and mucus hypersecretion (Chen *et al.*, 2001; Zhang *et al.*, 2013).

Studies in animal models have revealed that the activities of ERK1/2 (Kumar *et al.*, 2003), p38 (Taube *et al.*, 2004) and JNK (Nath *et al.*, 2005) in the lungs are enhanced in allergic airway inflammation and AHR. Endobronchial biopsies of asthmatic patients show significant phosphorylation of ERK1/2 and p38 compared to controls. The degree of phosphorylation also correlates positively with the severity of the disease (Liu *et al.*, 2008). Our group has shown that cytokines increase AHR to bradykinin *in vitro* via activation of JNK and downstream transcription factor nuclear factor-kappaB (NF-κB) that leads to increased expression of kinin receptors (Zhang *et al.*, 2005; Zhang *et al.*, 2004). SP600125, a JNK MAPK inhibitor, reduces ozone- (Williams *et al.*, 2007) and allergen- (Nath *et al.*,

2005) induced airway inflammation and AHR. MAPK inhibitors have been under clinical development for many years for the treatment of corticosteroid-resistant asthma and COPD in both oral and inhaled form, showing promising results (Singh *et al.*, 2015).

1.2 OROPHARYNGEAL CANCER

Oral and pharyngeal cancer, grouped together, is the sixth most common cancer in the world. About 90% of all oropharyngeal cancers, including tonsillar cancers are squamous cell carcinomas. There is wide geographical variation in the incidence of oropharyngeal cancers in the world. While the majority of oropharyngeal cancer incidences in Western countries are attributed to the rise of local infections with human papillomavirus (HPV), specifically HPV16. It should not be forgotten that the majority of the world's oropharyngeal cancer cases are found in developing countries, where smoking and alcohol rates are still high (Warnakulasuriya, 2009). Patients with HPV-positive tonsillar cancer, in particular those who are also never-smokers, are reported to have significantly better clinical outcome than patients with HPV-negative tonsillar cancer. The latter are almost always heavy smokers (Ang *et al.*, 2010; Dalianis, 2014; Hong *et al.*, 2013; Maxwell *et al.*, 2010).

1.2.1 Cigarette smoking

Smoking and alcohol are considered traditional risk factor for tonsillar squamous cell carcinoma. Smoking is also found to be an important negative prognostic factor for the overall survival (Dalianis, 2014; Hong *et al.*, 2013) and disease recurrence (Maxwell *et al.*, 2010), regardless of HPV status. Patients who smoke during radiotherapy also have a lower rate of response (Browman *et al.*, 1993). This suggests that HPV-positive and smoke-induced tonsillar squamous cell carcinomas have distinctly different pathogenesis. Hence, tobacco smoke exposure is damaging to oral health, stimulating tonsillar squamous cell carcinoma development regardless of the pathogenesis. The mechanisms behind this are not fully known.

1.2.2 Activin receptor-like kinases

Activin-receptor like kinases (ALK) are receptors for the transforming growth factor-beta (TGF-β) superfamily. They are involved in several cancer-related cellular processes including proliferation, differentiation, adhesion, migration and apoptosis (Graham *et al.*, 2006). Among the seven different ALKs found in humans, ALK1 and 5 are possibly the most studied in the context of cancer. ALK1 and ALK5 (also termed TGF-βI receptor) have antagonizing effects in endothelial cells. While ALK1 promotes growth and proliferation and

thereby carcinogenesis, ALK5 inhibits the process (Jonker, 2014). ALK5 conditional knockout mice are found to develop spontaneous squamous cell carcinoma (Honjo *et al.*, 2007). ALK5 expression was found to be decreased in human head and neck squamous cell carcinoma tumor cells (Eisma *et al.*, 1996). At the same time, high levels of ALK1 in tumor tissue from patients with head and neck squamous cell carcinoma is correlated with advanced T classification (greater tumor size), positive N classification (metastasis to nearby lymph nodes), advanced TNM stage (faster growing and spreading cancer), and poor prognosis (Chien *et al.*, 2013). Inhibitors of ALK1 are currently under clinical trials for the treatment of solid tumors (Cunha *et al.*, 2011; Jonker, 2014) . Blockage of ALK1 signaling using an ALK ligand trap ALK1-Fc in combination with cisplatin was recently found to inhibit tumor growth in murine head and neck cancer models more efficiently than chemotherapy alone (Hawinkels *et al.*, 2015).

2 AIMS OF THE THESIS

The overall aim of this thesis is to increase the understanding of the mechanisms behind cigarette-smoke induced airway hyperreactivity and inflammation.

Nicotine and LPS are two of the most important components of cigarette smoke. Understanding their functions and interactions in *in vitro* and *in vivo* murine models, as well as in human samples can reveal mechanisms that will allow the development of new therapeutic options for cigarette-smoke induced airway diseases.

The specific aims of the thesis are to:

1. Investigate the effects of nicotine exposure on GPCR agonist mediated airway contractions in isolated murine tracheal segments and explore the underlying mechanisms;

2. Investigate the effects of nicotine exposure on kinin receptor agonist induced airway relaxations in isolated murine tracheal segments and explore the underlying mechanisms;

3. Investigate the effects of long-term nicotine exposure on cell-surface TLR expression in isolated murine tracheal segments and its subsequent effect on LPS-induced AHR;

4. Investigate the effects of long-term nicotine exposure in mouse *in vivo* on LPS-induced AHR and explore the underlying mechanisms, with emphasis on neuronal mechanisms;

5. Investigate the effect of cigarette smoke exposure on tonsil ALK expression using a novel cotinine assay to quantify the level of cigarette smoke exposure in tonsil tissue.

3 MATERIAL AND METHODS

3.1 *IN VITRO* MURINE MODEL

3.1.1 Tissue preparation and organ culture

Organ culture was performed according to previous protocols (Zhang *et al.*, 2004). Tracheae from 10-week-old male BALB/c mice were dissected and cut into 4 segments (or 2 segments for EFS experiments) and placed individually in wells of a 48-well plate (or 24-well plate for EFS experiments; Ultra-low attachment, Sigma, St Louis, MO) with 1 ml serum-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml).

The tracheal rings were incubated for 1, 2 or 4 days at 37°C in humidified 5% CO_2 in air in the presence of vehicle or nicotine, with/without different concentrations of LPS (0.01, 0.1 and 1 μg/ml), with/without specific MAPK inhibitors (SP600125, U0126 or SB203580). Tracheal rings were moved into new wells containing fresh medium with supplements of nicotine and/or LPS and/or inhibitors every day.

The experimental protocol was approved by the Stockholm north animal ethics review board (Dnr N153/11) and Malmö-Lund's animal ethics review board (Dnr M15-09).

3.1.2 Epithelial removal

For the epithelium removal experiments, the epithelium from the whole trachea were mechanically scraped away gently by moving a coarse metallic rod in and out of the trachea, prior to cutting into segments and organ culture.

3.1.3 *In vitro* pharmacology

Contractile or relaxant responses of the tracheal segments were measured in temperature-controlled (37°C) myographs (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark), containing Krebs–Henseleit buffer solution and continuously equilibrated with 5% CO_2 and 95% O_2. The tracheal segments were mounted on two L-shaped metal prongs, and gradually stretched over the course of one hour to reach a resting tension of 0.8 mN (or 1.6 mN for EFS experiments). One prong was connected to a force–displacement transducer for continuous recording of isometric tension by the Chart software (ADInstruments Ltd, Hastings, UK). The other prong was connected to a displacement device, allowing adjustment of the distance between the two parallel prongs.

In vitro pharmacology was performed according to previous protocols (Adner *et al.*, 2002; Bachar *et al.*, 2005b). The viability of the segments was first tested using 60 mM KCl. KCl was later washed out with Kreb-Henseleit buffer solution three times until the segments reached basal tension. For contraction studies, each segment was incubated with 3 μM of the non-selective COX inhibitor indomethacin for 30 min before administration of agonists to inhibit epithelium-dependent relaxations. GPCR agonists such as ET-1, 5-hydroxytryptamine (5-HT) and bradykinin were then administered cumulatively to produce their concentration-effect curves.

To test relaxant properties, segments were pre-constricted with 1 μM carbachol, and after reaching stable plateaus, the concentration-effect curves for bradykinin-, des-Arg^9-bradykinin-, isoprenaline- and PGE_2-induced relaxations were produced in the absence of indomethacin. To further dissect the mechanisms behind the relaxation responses, the selective COX-1 inhibitor piroxicam (5 nM), the selective COX-2 inhibitor DuP-697 (5 nM), the non-selective COX inhibitor indomethacin (2 μM) or the selective EP_2 inhibitor PF04418948 were added 30 min prior to carbachol preconstriction in attempts to inhibit the relaxation responses.

3.1.4 Electric field stimulation

Electric field stimulation (EFS) was used to access nerve-induced contractions, and carried out according to previous protocols (Bachar *et al.*, 2005a). Tracheal segments were stimulated with EFS by two electrodes placed at opposite sides of the tracheal segments (Current Stimulator Model CS200, J.P. Trading, Aarhus, Denmark). Each segment was first contracted with 60 mM KCl to test its viability. After washing out KCl, and returning to resting state, each segment was given five training impulses of 4 Hz, 55 mA (≈10 V). Later, segments were given a 2log EFS series of 0.2 – 12.8 Hz. Each impulse was 0.8 ms long with duration of 1 min, followed by 1.5 min rest. Segments were washed and then incubated for 30 min with 3 μM of the non-selective COX inhibitor indomethacin before EFS, followed by the same procedure after incubation with 3 μM indomethacin and 10 μg/ml LPS for 1 hr. The experiment ended with a control contraction to 0.1 μM carbachol.

3.2 *IN VIVO* MURINE MODEL

3.2.1 Treatment protocol

10-week old female BALB/c mice were implanted with Alzet® osmotic pumps (model 2004, Durect Corporation, USA) containing either sterile phosphate-buffered saline (PBS) or PBS-buffered nicotine tartate salt solution (24 mg/kg/day, Sigma-Aldrich, USA). The mice were

then housed for 24 days in groups of four in temperature- and humidity-controlled rooms with 12:12 hour light-dark cycle. Standard food and water were provided *ad libitum*. At day 25, the mice started to receive daily intranasal instillations of LPS for 3 consecutive days. At day 28, lung mechanics were measured with a flexiVent^{TM} small animal ventilator. Bronchoalveolar lavage fluid was obtained. The right lung lobes were fixated in formalin for histological analysis and the left lung lobes were snap-frozen in liquid nitrogen for protein analysis (Fig 1).

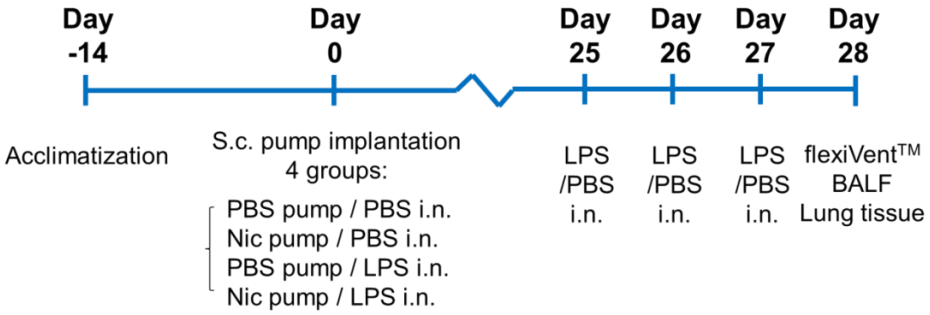

Fig 1. *In vivo* treatment protocol.

Animal procedures were reviewed and approved by the Stockholm north animal ethics review board (Dnr N153/11 and N258/13).

3.2.2 Subcutaneous osmotic pump implantation

Mice were anesthetized by continuous inhalation of isoflurane and a small incision was made behind the neck using aseptical techniques. A pre-conditioned Alzet® osmotic pump (model 2004, Durect Corporation, USA) with an infusion rate of 0.25 µl/h , containing either sterile phosphate-buffered saline (PBS) or PBS-buffered nicotine tartate salt solution (24 mg/kg/day, Sigma-Aldrich, USA) was implanted in a subcutaneous pouch. The incision was closed using 2-3 sterile Reflex clips (Alzet®, USA). Mice were allowed to recover for 30 min before returning to home cage. Clips were removed 7-10 days post-operation.

3.2.3 Intranasal LPS instillations

Mice were anesthetized with isoflurane and 20 µl of LPS from Escherichia coli 0127:B8 (0.1 mg/ml, Sigma-Aldrich, USA) or PBS was placed as a big droplet in their nostrils while the mice were held in an upright posture. The droplet was sniffed in by the mice reaching their lungs.

3.2.4 Airway mechanics

Mice were anesthetized with pentobartial sodium (90 mg/kg body weight) and then placed on a heating pad (37°C). A tracheotomy was performed and a 18-gauge cannula was inserted. The mouse was then connected to a flexiVent™ small animal ventilator (Scireq, Montreal, Canada). After ventilation was started, bilateral thoracotomy was performed, in order to equalize pleural pressure to atmospheric and to exclude chest wall contribution to the mechanics. Animals were ventilated at a tidal volume of 12 ml/kg body weight and the positive end-expiratory pressure (PEEP) was kept at 3 cm H_2O. An i.v. catheter was inserted into the tail vein. Acetyl-ß-methylcholine (Mch, Sigma-Aldrich, St Louis, MO, USA) was injected through the tail vein in increasing doses (0.01, 0.03, 0.1, 0.3, 1 and 3 mg/kg body weight) to induce airway contractions. Nerve blockage was achieved by pretreatment with tetrodotoxin (6 µg/kg, Tocris Bioscience, United Kingdom) prior to Mch challenge. A broadband forced oscillation manoeuvre was carried out and measurements fitted with a constant phase model to compute Newtonian resistance (Rn), tissue damping (G) and tissue elastance (H) (Bates et al., 2011).

3.2.5 Bronchoalveolar lavage

BAL was collected after airway mechanical measurements by carefully filling the lungs with 0.7 ml of ice-cold PBS which was withdrawn 3 times with a syringe. The fluid was centrifuged at 4°C, 1200 rpm, for 10 minutes and the supernatant was stored at −80°C until use.

Total cell number was counted on Fast Read 102® plastic counting slides immediately after mixing 10 µl cell suspensions with 90 µl Gentiana blue. Differential cell counts were performed on May-Grünwald/Giemsa stained cytospin preparations, counting a minimum of 300 cells, in a blinded manner.

MMP-9 and TIMP-1 in BAL were measured with ELISA kits (mouse total MMP-9 Quantikine ELISA kit and mouse TIMP-1 kit Quantikine Immunoassay, R&D systems, USA), according to the manufacturers' instructions.

3.2.6 Histology

Right lung lobes were fixated in formaldehyde, dehydrated, embedded in paraffin and sectioned with a microtome. Sections were immersed in UltraClear™ followed by decreasing concentrations of ethanol for deparaffinzation and then immersed in deionized water for rehydration. The slides were then stained with hematoxylin and eosin (H&E) for morphology and semi-quantification of inflammation, or picrosirius red for visualization of collagen.

Peribronchial, perivascular and parenchymal inflammatory cell infiltration were semi-quantitatively graded in a blinded manner as 0 to 3 (0=no cell infiltration, 3=abundant) under a light microscope (Olympus BX50 microscope with Nikon Digital Sight DS-5M camera) under 400x magnification.

3.2.7 Lung homogenate

Left lung lobes were snap-frozen in liquid nitrogen and stored in -80°C after lung physiological measurements and BAL collection. Upon use, the samples were defrosted and washed free of visible blood using ice-cold PBS. Lung homogenate was obtained by adding 10 µl of PBS to every 1 mg lung tissue and homogenized at 5000 rpm in two 20s cycles (Precellys®24, Bertin technologies, USA) with 2.8mm ceramic beads.

Every 20 µl of lung homogenate were then digested with 80 µl of 0.5M acetic acid and pepsin mixture and the amounts of acid- and pepsin soluble collagen were measured with Sircol™ collagen assay (Biocolor, UK) according to the manufacturer's instructions.

3.3 HUMAN TONSILS

Twenty seven pairs of tonsils were collected from patients between 15 and 40 years old undergoing bilateral tonsillectomy at the Ear- nose- and throat clinic at Malmö University Hospital, Sweden for chronic tonsillitis. Apart from the tonsillar symptoms, all subjects were healthy and did not receive any medications. The allergy statuses of the patients were determined by blood Phadiatop® testing for allergen-specific IgE.

Directly after surgery, tonsils were cut into small pieces. Some pieces (20-40 mg) were placed in RNAlater (QIAGEN, Germany) for 24 hrs at room temperature and then kept at −80°C until use, while others (50-150 mg) were snap-frozen in liquid nitrogen and then kept at −80°C until use.

The study was approved by the Lund University research ethics committee (Dnr LU 293-03). Informed consent was obtained from all participants.

3.3.1 Tonsil cotinine assay

Small pieces of tonsils that were snap-frozen in liquid nitrogen and stored in -80°C were defrosted in room temperature. Samples were weighed. For every mg of tissue, 3 µl of sterile PBS was added. The tissue was then homogenized together with 1.4 mm ceramic beads in a Precelleys®24 homogenizer (Bertin Technologies, France) at 5000 rpm for 20 sec x 2. The

homogenate was subsequently centrifuged and the supernatant was used for cotinine measurements using a Cotinine ELISA kit (Abnova, Taiwan) following the manufacturer's instructions.

3.4 REAL-TIME PCR

3.4.1 RNA extraction

RNA was extracted using RNeasy mini kit (QIAGEN, Germany) according to the manufacturer's instructions. Pieces from tonsils or tracheae were stored in RNAlater (QIAGEN, Germany) at -20°C or -80°C, defrosted, squeezed free of excessive RNAlater, weighed and homogenized in a Precelleys®24 homogenizer. RNA was extracted from the homogenate using a spin-column based technique (RNeasy Mini Kit, QIAGEN, Germany) according to manufacturer's protocol. RNA quality was accessed using a Nanodrop® ND-3300 spectrophotometer (Thermo Scientific, USA). A260/A280 ratio and A260/A230 ratio over 1.8 were accepted as "pure" RNA.

3.4.2 Reverse transcription

Reverse transcription of RNA (100 ng/µl) into cDNA was carried out using Omniscript™ reverse transcriptase kit (QIAGEN, Germany) with oligo$(dT)_{16}$ primers (DNA Technology A/S, Denmark) in a 20 µl reaction volume with a Mastercycler personal PCR machine (Eppendorf AG, Germany) at 37 °C for 1 hr according to the manufacturer's protocol.

3.4.3 Real-time PCR

Both TaqMan® Gene Expression Mastermix (QIAGEN, Germany) and QuantiTect™ SYBR® Green PCR kit (QIAGEN, Germany) were used for real-time PCR in the present studies.

Using TaqMan® Gene Expression Mastermix and MicroAmp® fast optical 96-well reaction plates (Applied Biosystems, USA) real-time PCR was performed in 20-µl reaction volumes according to the manufacturer's instructions. Each sample was analyzed in duplicate. Commercially available Taqman® probes were used (Applied Biosystems, USA). 40 PCR thermal cycles were ran in the Applied Biosystems 7500 Real-Time PCR System. β-actin, Hprt1 and GAPDH were used as housekeeping genes.

Using QuantiTect™ SYBR® Green PCR kit (QIAGEN, Germany), real-time PCR was performed in the Smart Cycler® II system (Cepheid, Sunnyvale, CA, USA). A reaction

volume of 25 µl was used and carried out with heating 95°C for 15 min followed by touchdown PCR i.e. denature at 94°C for 30 sec and annealing at 66 °C for 1 min for the first PCR cycle, thereafter, a 2°C decrease for the annealing temperature in every cycle up to 56°C. Finally, 40 thermal cycles with 94°C for 30 sec and 55°C for 1 min were performed. The specificity of the PCR products was checked by using the dissociation curves. Primers were designed using Prime Express 2.0 software (Applied Biosystem, Forster city, CA, USA) and synthesized by DNA Technology A/S (Aarhus, Denmark). GAPDH was used as the housekeeping gene. The primer sequences are as below (Table 1):

Table 1: Primer sequences for real-time PCR

Namn	**GenBank No**	**Primer sequences (5'-3')**
mTLR1	NM_030682.1	Fwd: 5'-CAA GCA TTT GGA CCT CTC CTT TA-3' Rev: 5'-GTA CCC GAG AAC CGC TCA AC-3'
mTLR2	NM_011905.2	Fwd: 5'-TCA CCA CTG CCC GTA GAT GA-3' Rev: 5'-CAA GAT GTA ACG CAA CAG CTT CA-3'
mTLR4	NM_021297.2	Fwd: 5'-GAA CCC TCT ATC ATG GAA GGA CTA TG-3' Rev: 5'-TTC GCC AAG CAA TGG AAC TTA-3'
mTLR5	NM_016928.2	Fwd: 5'-CTG GGG ACC CAG TAT GCT AA-3' Rev: 5'-ACA GCC GAA GTT CCA AGA GA-3
mTLR6	NM_011604.2	Fwd: 5'-ACC TGG AAG TGC TCG GTT AAA C-3' Rev: 5'-TTC CCT GTC GAT TCT CTC AGT TAT C-3'
mTLR11	NM_205819.1	Fwd: 5'-TCC TTC CTC TGA TTA GCT GTC CTA A-3' Rev: 5'-TCC ACA TAA TTT CCA CCA ACA AGT-3'
mGAPDH	XM_001473623.1	Fwd: 5'-CAT GGC CTT CCG TGT TCC TA-3' Rev: 5'-TGC TTC ACC ACC TTC TTG ATG-3'
mCOX-2	NM_011198	Fwd: 5'-CTC CCT GAA GCC GTA CAC AT-3' Rev: 5'-ATG GTG CTC CAA GCT CTA CC-3'
mTNF-α	NM_013693	Fwd: 5'-GAC TCA AAT GGG CTT TCC GA-3' Rev: 5'-TCC AGC CTC ATT CTG AGA CAG AG-3'

mB1R	NM_007539	Fwd: 5'-CCA TAG CAG AAA TCT ACC TGG CTA AC-3'; Rev: 5'-GCC AGT TGA AAC GGT TCC-3'
mB2R	NM_009747	Fwd: 5'-ATG TTC AAC GTC ACC ACA CAA GTC-3'; Rev: 5'-TGG ATG GCA TTG AGC CAA C-3'

The relative amounts of mRNA were determined by subtracting the threshold cycle (C_T) values for these genes with the CT value for the most stable housekeeping gene to obtain the ΔC_T value and then expressing amount as $2^{-\Delta CT}$ relative to housekeeping gene.

3.5 IMMUNOHISTOCHEMISTRY

3.5.1 Cryosection

After organ culture, the tracheal rings were immersed in a fixative solution consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 hrs at 4°C. The specimens were dehydrated in 20% sucrose with 0.1 M phosphate buffer (pH 7.4) for 24 hrs at 4°C, and then frozen in Tissue-Tek® O.C.T. (Sakura Finetek Europe BV, Netherlands) and stored at -80°C. Ten μm-thick sections were cut using a cryostat. These were mounted on SuperFrost™ Plus slides (Menzel GMBH & COKG, Germany) and frozen in -20°C until staining.

Immunohistochemistry was performed according to standard protocols, i.e. the sections were incubated with the primary antibody overnight at 4°C and the secondary antibody for 1 hr at room temperature in the dark. In the control experiments, either the primary antibody or the secondary antibody was omitted.

3.5.2 Paraffin-embedded tissue

Tracheal segments or lung tissue were immersed in a fixative solution consisting of phosphate-buffered formaldehyde overnight at 4°C. The tissues were dehydrated in series of ethanol from 50% to 99.5%, followed by UltraClear™ and embedded in paraffin blocks. Sections were cut into 4 μm-thick slices using a microtome and mounted on SuperFrost™ Plus slides (Menzel GMBH & COKG, Germany).

Sections were then deparaffinized, rehydrated and underwent target retrieval in 98°C citrate buffer for 25 min prior to immunohistochemical staining. The sections were permeabilized with 1% Triton-X100, blocked with 10% donkey serum in 1% bovine serum albumin (BSA)

and then incubated with the primary antibody overnight in 4°C in darkness. The sections were then incubated with the secondary antibody (raised in donkey) for 1 hr in room temperature in darkness. Appropriate IgG isotype controls were used instead of the primary antibody as control for non-specific staining.

3.5.3 Confocal microscopy

The stained specimens were examined under a confocal microscope (Leica, Wetzlar, Germany or Nikon, C1plus, Nikon Instruments Inc., NY, USA) for semi-quantification of protein expressions. The fluorescence intensity was measured and analyzed by Image J software (http://rsb.info.nih.gov/ij). Either the entire preparation or 6 randomly selected sections were measured. All experiments were carried out in one single batch and measurements were done under the same microscope instrument setting on the same day to ensure comparability between the slides.

3.6 STATISTICS

All data were expressed as mean values ± S.E.M. Agonist concentration-effect curve data from individual segments were fitted to the Hill equation using an iterative, least-squares method (GraphPad Prism® 5, U.S.A.) to provide estimates of maximal contraction (E_{max}) and pEC_{50} (negative logarithm of the agonist concentration that produces half of its maximal effect). Concentration-effect curves obtained from the *in vitro* pharmacology experiments were compared using two-way analysis of variance (ANOVA). Unpaired two-tailed Student's *t*-test was used when two groups are compared to each other. One-way ANOVA were performed when three or more groups are compared with each other. Bonferroni's *post hoc* analysis was used to correct for multiple comparisons. Linear regression was used to determine correlation between data. P-values less than 0.05 were considered to be statistically significant.

4 RESULTS AND COMMENTS

4.1 NICOTINE ON AIRWAY SMOOTH MUSCLE CONTRACTION (PAPER I)

4.1.1 Time- and concentration-effect

Murine tracheal segments were cultured for one (Fig. 2A-B), two (Fig. 2C-D) or four (Fig. 2E-F) days in serum-free DMEM medium in presence of nicotine (1 and 10 μM) or vehicle (DMSO). Contractile responses monitored with myographs were induced by the following GPCR agonists: the kinin B_1 receptor agonist des-Arg^9-bradykinin (D-A-BK, Fig 2A,C,E), the B_2 receptor agonist bradykinin (BK, Fig 2B,D,F), the 5-HT receptor agonist 5-HT, the cholinergic agonist acetylcholine, the non-selective ET receptor agonist ET-1 and the selective ET_B receptor agonist sarafotoxin 6c.

The results showed that nicotine did not affect the contractile responses mediated by 5-HT, cholinergic or ET receptors (data not shown). For kinin receptor agonists, a tendency towards an increased airway contractile response to des-Arg^9-bradykinin and bradykinin was seen already after 2 days of nicotine treatment (Fig. 2C-D) and this increase reached statistical significance at day 4 (Fig. 2E-F). After 4 days of organ culture with a lower nicotine concentration (1 μM), the contractile responses to des-Arg^9-bradykinin and bradykinin were not significantly altered, while culture with 10 μM of nicotine significantly increased the E_{max} for both agonists (Fig. 2E-F). Although a tendency towards an increased pEC_{50} could be seen, it did not reach statistical significance (des-Arg^9-bradykinin: Ctrl 6.96 ± 0.17, 10 μM nicotine 7.20 ± 0.20, $p>0.05$; bradykinin: Ctrl 6.72 ± 0.38, 10 μM nicotine 7.30 ± 0.25, $p>0.05$). Two different neuronal nicotinic receptor antagonists MG624 and hexamethonium both blocked the nicotine-induced effects (data not shown).

4.1.2 Transcriptional upregulation of kinin receptors

The relative amount of mRNA for kinin B_1 and B_2 receptors was semi-quantified by real-time PCR, and their corresponding protein expression with immunohistochemistry. Four days of organ culture in the presence of nicotine (10 μM) increased the mRNA expression for both receptors, compared to controls (Fig. 3A). An increase in kinin B_1 (Fig. 4A,B,E) and B_2 (Fig. 4C,D,F) receptor protein expressions was seen in both the airway epithelial and smooth muscle cells (Fig. 4E-F). After nicotine treatment, the increase in B_1 receptor protein expression was more prominent in the smooth muscle cells than in the epithelial cells (Fig. 4E), while the increase of B_2 receptors was more prominent in the epithelial cells (Fig. 4F).

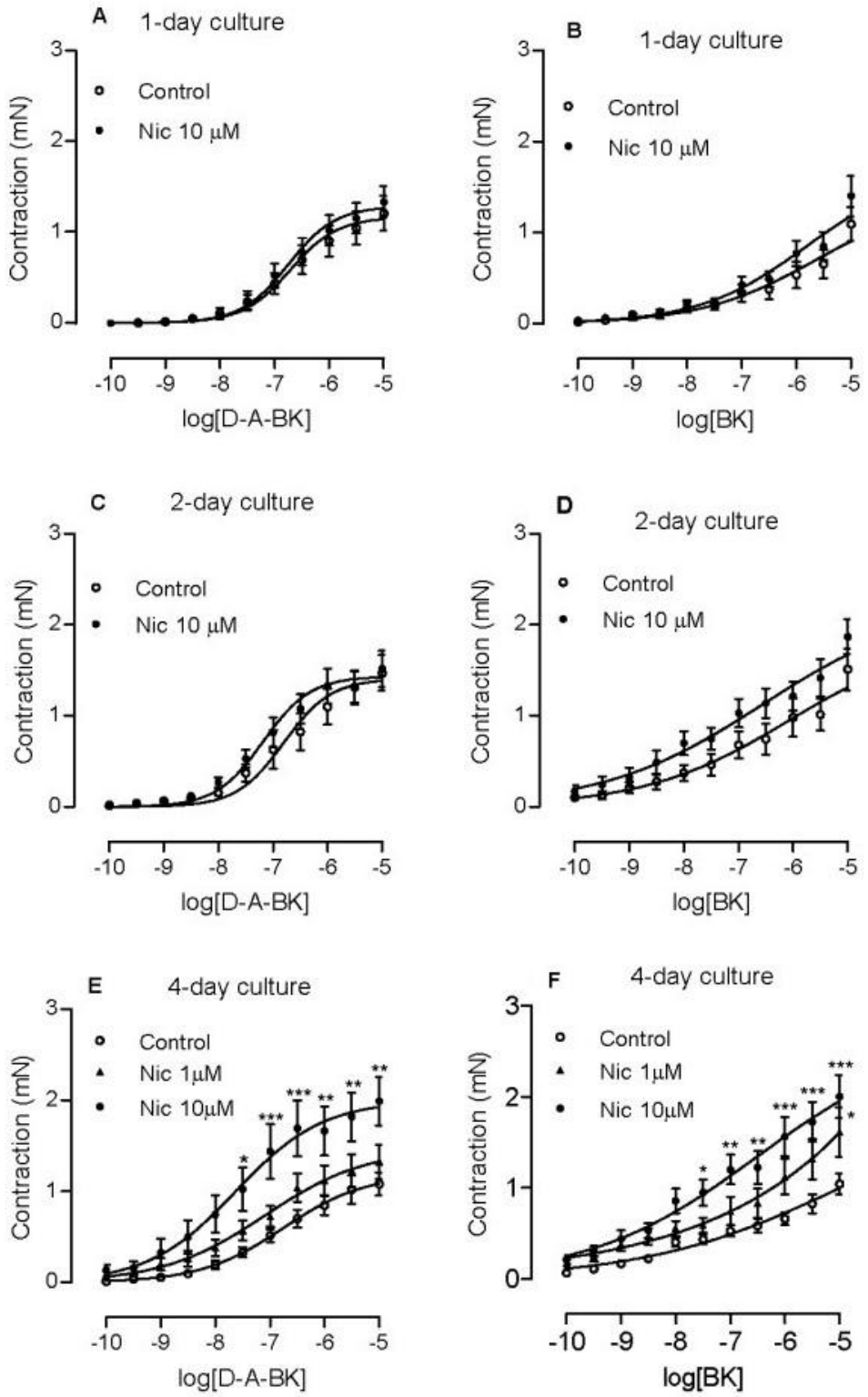

Figure 2. Time- and concentration-dependent effects of nicotine (Nic) on contractions of murine tracheal segments induced by des-Arg^9-bradykinin (D-A-BK; A, C, E) or bradykinin (BK; B, D, F). Each data point is derived from 15-22 experiments and data is presented as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA Bonferroni's *post hoc* analysis. Control vs Nic. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

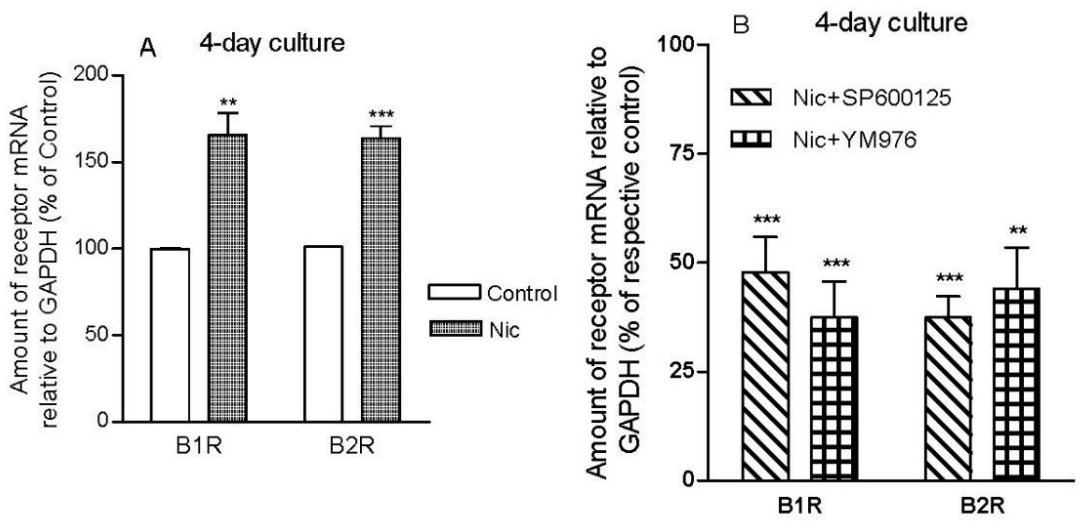

Figure 3. Kinin B_1 (B1R) and B_2 (B2R) receptor mRNA expressions**.** Each data point is derived from 3-6 experiments and data is presented as mean ± S.E.M. Statistical analysis was performed using unpaired Student's *t*-test with Welch's correction. Control vs Nic (A); Nic vs Nic+SP600125/YM976 (B). ** P < 0.01; *** P < 0.001.

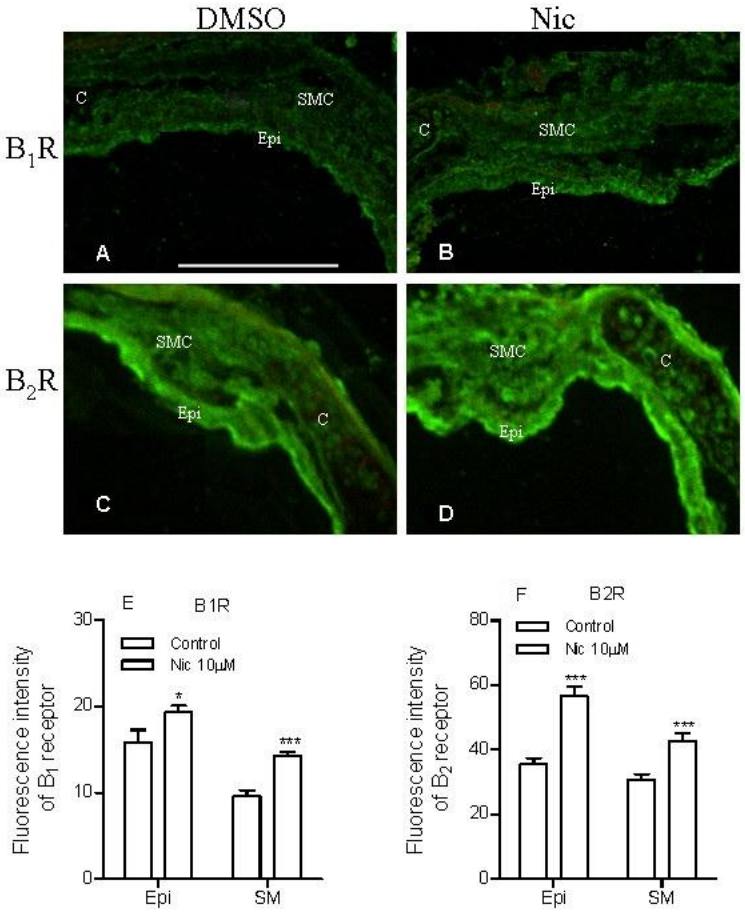

Figure 4. Nicotine (Nic) effects on kinin B_1 (B_1R) and B_2 (B_2R) receptor protein expressions. The reference bar corresponds to 25 µm. Epi = epithelium; SMC = smooth muscle cells; and C = cartilage. Each data point is derived from 6 experiments. Two-tailed unpaired Student's *t*-test with Welch's correction was performed. Control vs Nic * P < 0.05; *** P < 0.001.

4.1.3 Involvement of JNK and PDE4 pathways

Confocal-microscopy-based immunohistochemistry showed that 4 days of nicotine treatment induced activation (phosphorylation) of JNK (Fig. 5), but not ERK1/2 and p38 (data not shown). Inhibition of JNK with its specific inhibitor SP600125 abolished the nicotine-induced effects on kinin receptor-mediated contractions (Fig. 6A-B) and reversed the enhanced receptor mRNA expressions (Fig. 3B).

Similar effects were observed after administration of the specific PDE4 inhibitor YM976. YM976 decreased the nicotine-enhanced kinin receptor mRNA expressions (Fig. 3B), as well as the airway contractile responses to des-Arg^9-bradykinin and bradykinin (Fig. 6C-D).

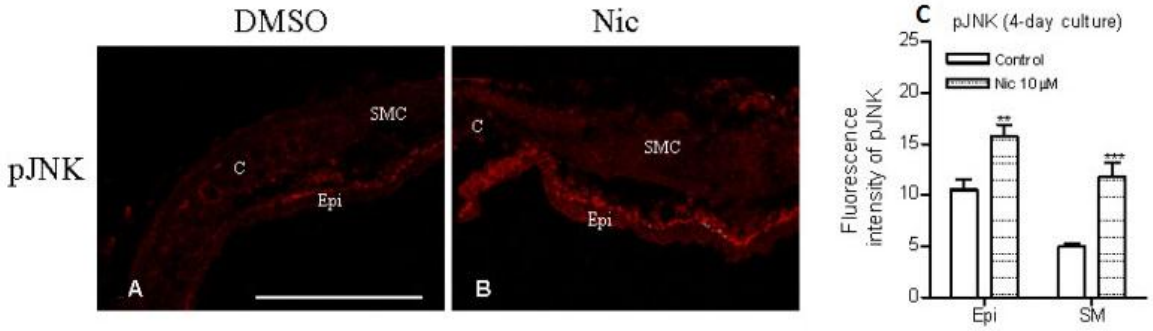

Figure 5. Nicotine (Nic) effects on phosphorylated JNK (pJNK) protein expressions. The reference bar corresponds to 25 µm. Epi = epithelium; SMC = smooth muscle cells; and C = cartilage. Each data point is derived from 6 experiments. Two-tailed unpaired Student's *t*-test with Welch's correction was performed. Control vs Nic. ** $P < 0.01$; *** $P < 0.001$.

4.1.4 Comments

The present study demonstrated that long-term exposure (4 days) of mouse tracheal segments to nicotine caused a concentration-dependent increase of kinin B_1 and B_2 receptor-mediated airway contractions. Short-term nicotine exposure (1-2 days) induced no significant effects. Nicotine treatment did not affect airway contractions mediated by 5-HT, cholinergic or endothelin receptors. The increase in E_{max}, without significant change of pEC_{50}, seen after 4 days of nicotine treatment suggested an increase in kinin receptor protein expression rather than alteration of receptor sensitivity. This conclusion was further supported by the discovery of upregulated protein expressions for both B_1 and B_2 receptors using immunohistochemistry. In addition, real-time PCR revealed a parallel increase in B_1 and B_2 receptor mRNA suggesting the involvement of transcriptional mechanisms in the effects of nicotine. The

intracellular cascades involved in the kinin receptor upregulation seemed to be both JNK- and PDE4-related intracellular signal pathways.

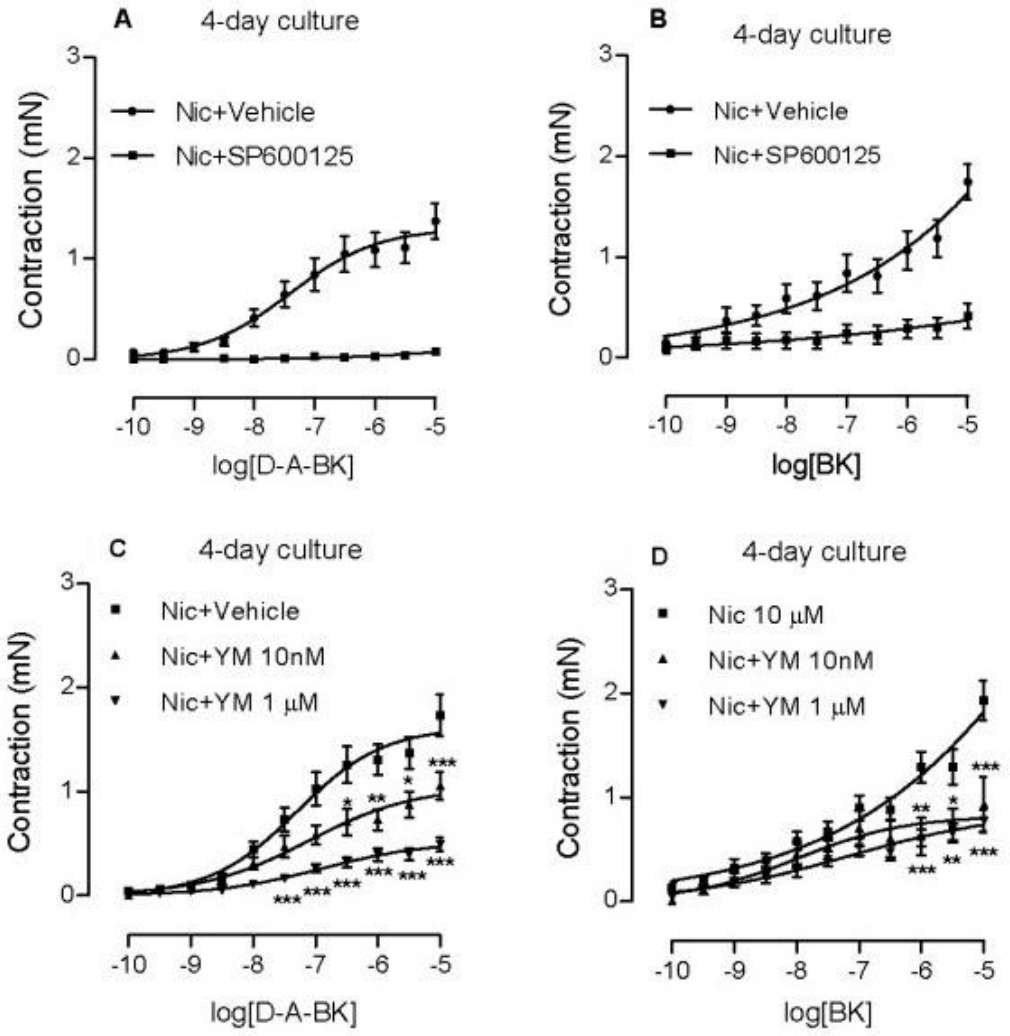

Figure 6. Effects of JNK inhibitor SP600125 (10 μM, A, B) and YM976 (YM, 1 μM, C, D) on nicotine-enhanced des-Arg^9-bradykinin- (A, C) and bradykinin- (B, D) induced contractions**.** Each data point is derived from 4-17 experiments and presented as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA with Bonferroni's *post hoc* analysis. Nic+vehicle vs Nic+inhibitor. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Many GPCRs are involved in the regulation of the contractile state of airway smooth muscle. Bradykinin, ET and M_3 muscarinic receptors are Gq-coupled while 5-HT receptors are Gi-coupled (Billington *et al.*, 2003). Our results showed that nicotine upregulated kinin B_1 and B_2 receptor-mediated airway contractions, leaving 5-HT, cholinergic and ET receptor-mediated contractions unaffected. This suggested that nicotine acted on specific targets within the airways. Thus, the effects observed were neither the result of a general hyperres-

ponsiveness nor due to alteration of downstream G-protein signaling processes. This idea was further strengthened by the finding of a simultaneous upregulation of kinin receptor function, mRNA and protein expression, revealing a special role for bradykinin in nicotine- and/or tobacco smoke-induced AHR.

JNK has long been implicated in the pathogenesis of asthma (Adcock *et al.*, 2004). In a study of human bronchial epithelial cells, ERK1/2, JNK, but not p38 was strongly activated after treatment with nicotine (Tsai *et al.*, 2006). In the present study, nicotine induced phosphorylation of JNK, but not ERK1/2 and p38. SP600125, a small molecular inhibitor for JNK, abolished the nicotine-enhanced kinin receptor-mediated contractions and the receptor mRNA expression. These results are well in line with a previous study which has demonstrated that SP600125 exhibits a powerful inhibitory effect on TNF-α induced upregulation of kinin B_1 and B_2 receptors in airways (Zhang *et al.*, 2004). SP600125 was applied at a concentration of 10 μM. At this concentration, SP600125 was found to selectively inhibit the phosphorylation of JNK, but not ERK1/2 or p38 in blood vessels (Xu *et al.*, 2008). SP600125 up to 30 μM caused no alteration in carbachol-elicited contractile responses in isolated airway segments (Zhang *et al.*, 2004).

YM976 is a selective PDE4 inhibitor shown to possess powerful anti-inflammatory and direct bronchorelaxatory effects (Aoki *et al.*, 2000). PDE4 is expressed in airway smooth muscle cells and increases intracellular concentration of the second messenger cAMP (Torphy *et al.*, 1993). A previous study has shown that inhibition of PDE4 suppresses endotoxin-induced airway inflammation and hyperreactivity (Toward *et al.*, 2001). Our results showed that YM976 attenuated the nicotine-enhanced kinin B_1 and B_2 receptor-mediated airway contractions. Inhibition of PDE4 produces a specific depression of nicotine's effects without altering control, suggesting that the nicotine-induced changes might be PDE4-specific.

The present study showed the simultaneous involvement of both JNK and PDE4/cAMP-mediated pathways in the effects of nicotine on kinin receptors. Supporting this, there have been several reports on the cross-talk between cAMP and JNK pathways. For example, cAMP has been shown to inhibit JNK activation in human airway smooth muscle cells (Kaur *et al.*, 2008) and lung cancer cells (Park *et al.*, 2016). cAMP specifically blocked activation of JNK, but not ERK 1/2 through protein kinase A (Pearson *et al.*, 2006).

4.2 NICOTINE ON AIRWAY EPITHELIAL RELAXATION (PAPERS I & II)

4.2.1 Short-term nicotine exposure

Relaxation responses to bradykinin, des-Arg^9-bradykinin, PGE_2, and isoprenaline were studied in fresh murine tracheal segments or segments after 1 day of organ culture with/without nicotine (Fig. 7). Pre-contraction was induced by 1 μM of carbachol. Pre-contraction levels were similar in all groups (data not shown).

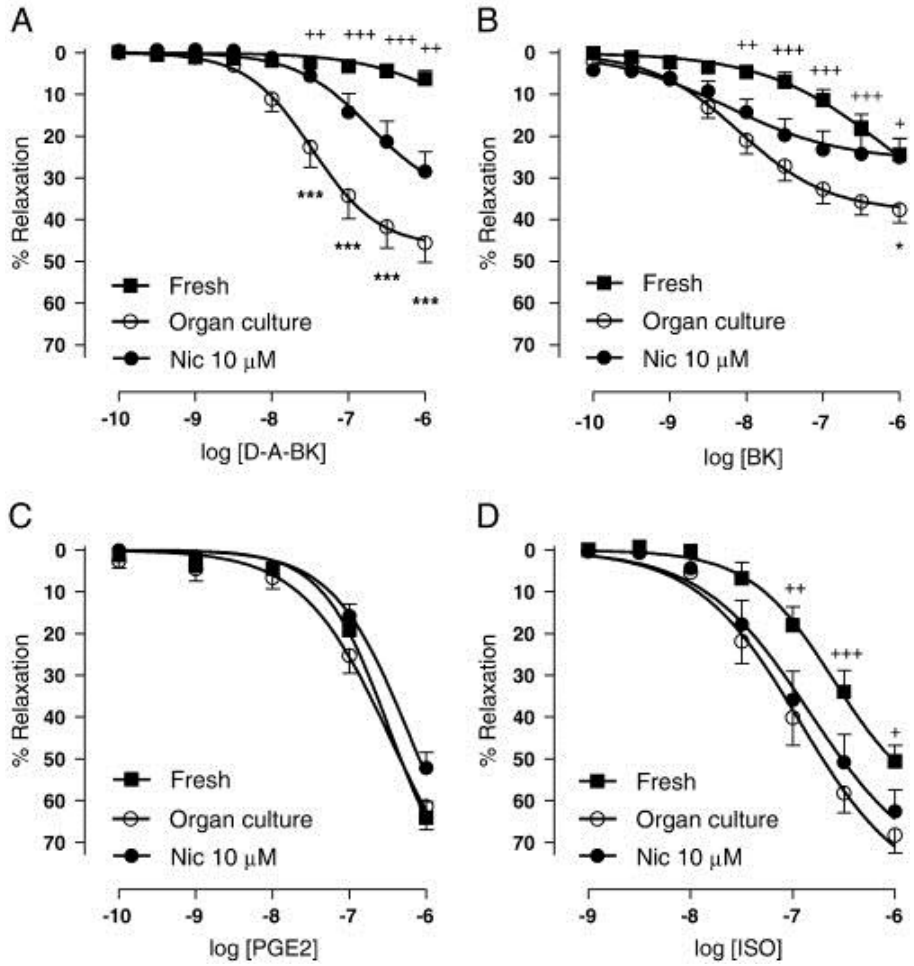

Figure 7. One-day nicotine exposure on airway relaxations. Cumulative dose–response relaxation curves to des-Arg^9-bradykinin (A), bradykinin (B), PGE_2 (C) and isoprenaline (D) were performed after pre-contraction with 1 μM carbachol. Each data point was derived from 7 to 18 segments. Statistical analysis was performed using two-way ANOVA with Bonferroni's *post-hoc* analysis. Culture vs Nic; *** $P < 0.001$. Culture vs Fresh. $^{+}$ $P < 0.05$; $^{++}$ $P < 0.01$; $^{+++}$ $P < 0.001$.

Fresh segments barely responded to des-Arg^9-bradykinin (6.3 ± 2.0% relaxation, at 10^{-6} M of agonist, Fig. 7A), exhibited limited relaxation to bradykinin (24.4 ± 3.9% relaxation, at 10^{-6} M of agonist, Fig. 7B), but relaxed markedly in response to PGE_2 (64.2 ± 3.9% relaxation, at 10^{-6} M of agonist, Fig. 7C) and isoprenaline (50.7 ± 3.8% relaxation, at 10^{-6} M of agonist, Fig. 7D). The organ culture procedure *per se* greatly increased the relaxation response to bradykinin and des-Arg^9-bradykinin (Fig. 7A-B), modestly affected isoprenaline relaxations (Fig. 7D) and did not alter relaxations induced by PGE_2 (Fig. 7C).

Nicotine suppressed the organ-culture-enhanced relaxations induced by des-Arg^9-bradykinin and bradykinin, leaving PGE_2- and isoprenaline-induced relaxations unaltered (Figs. 7A–D). Nicotine by itself produced nearly negligible relaxations regardless of group (Fresh: 9.3 ± 3.7% relaxation, n = 5; Organ culture: 6.4 ± 1.5% relaxation, n = 7; Nic: 7.3 ± 2.3% relaxation, n = 4, at 10^{-6} M of agonist).

4.2.2 Long-term nicotine exposure

Murine tracheal segments were cultured for 4 days in serum-free DMEM medium in the presence of nicotine (10 μM) or vehicle (0.1% DMSO). Their relaxation responses to bradykinin and des-Arg^9-bradykinin were characterized after pre-contraction with 1 μM carbachol in the absence of COX inhibitors. Neither B_1 nor B_2 receptor-mediated relaxations were affected by long-term nicotine exposure *in vitro* (Fig. 8).

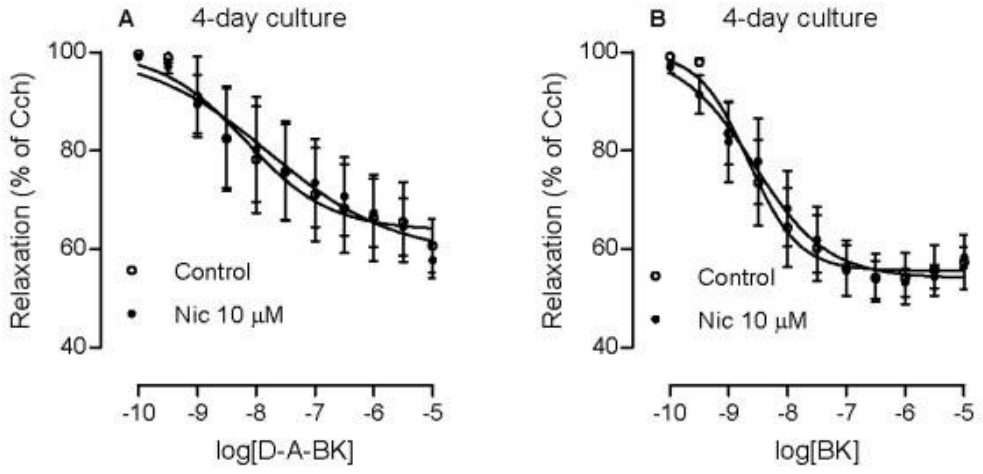

Figure 8. Four-day nicotine exposure on kinin receptor-mediated airway relaxations. Each data point is derived from 6-8 experiments and data is presented as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA with Bonferroni's *post hoc* analysis. Control vs Nic. No significant differences were found.

4.2.3 Role of epithelium

The epithelial layers of the tracheal segments were gently scraped away prior to organ culture with vehicle (0.1% DMSO) or nicotine (10 μM) for one day. This process nearly completely abolished des-Arg^9-bradykinin- (Fig. 9A) and bradykinin- (Fig. 9B) induced relaxations in both organ cultured and nicotine-treated segments which shows that kinin-receptor mediated relaxations are epithelium-dependent.

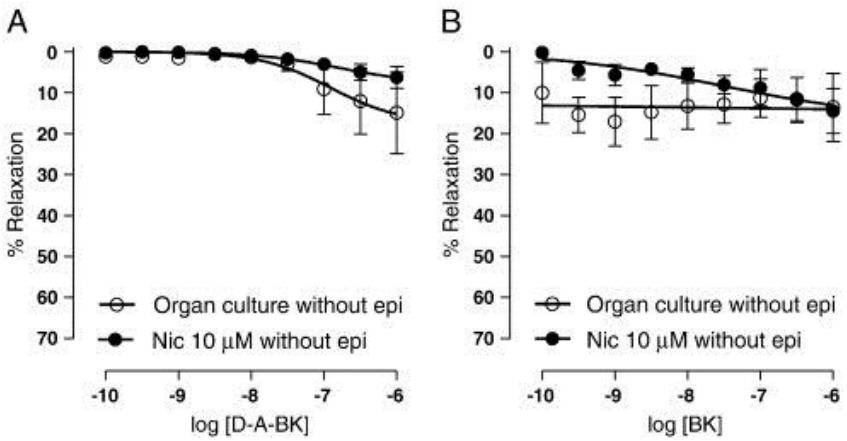

Figure 9. Effect of mechanical epithelial removal on kinin-receptor mediated airway relaxations. Each data point was derived from 3 to 5 segments. All data were presented as mean ± S.E.M.

4.2.4 Involvement of COX pathways

Kinin receptor agonist induced airway relaxations are dependent on the airway epithelium as well as on COX activity and EP receptors (Barnes, 1992). To further dissect the underlying mechanisms, segments were treated with indomethacin (2 μM), DuP-697 (selective COX-2 inhibitor, 5 nM), piroxicam (selective COX-1 inhibitor, 5 nM) or PF04418948 (selective EP_2 receptor inhibitor, 10 nM) for 30 min in the organ bath. Relaxations were then induced by des-Arg^9-bradykinin or bradykinin after pre-contraction with 1 μM carbachol (Fig. 10).

The levels of COX-1, COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) mRNA and protein expressions were examined after 1-day organ culture with/without nicotine exposure. COX-1, COX-2 and mPGES-1 were upregulated after 1-day of organ culture compared to fresh trachea (Fig. 11).

The increase in COX-2 mRNA expression following organ culture was the most prominent (Fig. 11, Fresh: 0.022 ± 0.004, n = 5; Organ culture: 2.31 ± 0.53, n = 5, relative to house-keeping gene Hprt1). Staining for COX-2 protein with immunohistochemistry revealed that

COX-2 protein was mostly amplified on the smooth muscle, but not on the epithelium (data not shown). Nicotine also showed a tendency to decrease organ-culture-induced COX-2, but without reaching statistical significance on mRNA (Fig. 11, Organ culture: 2.3 ± 0.5, n = 5; Nic: 1.5 ± 0.3, n = 5; P > 0.05, relative to house-keeping gene Hprt1) and protein levels (data not shown).

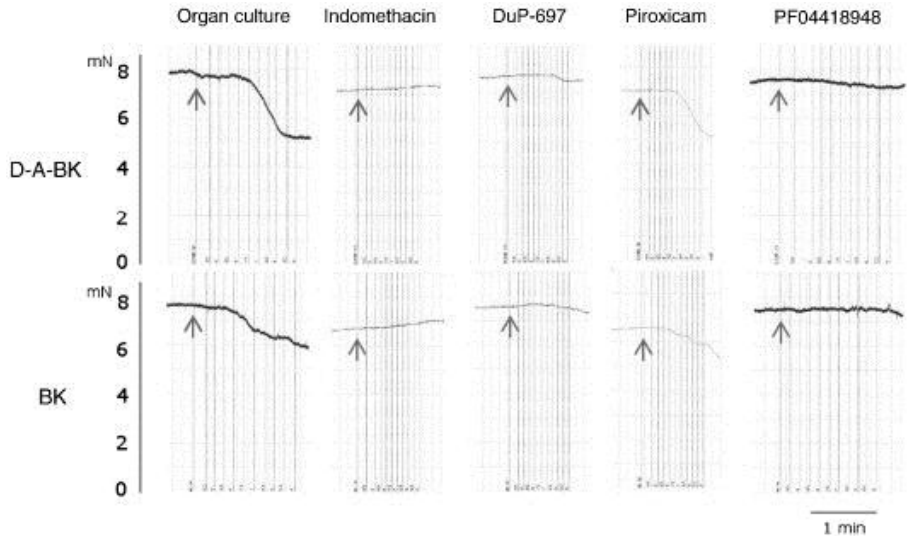

Figure 10. Effects of COX- and EP_2 receptor inhibition on airway relaxations. Typical relaxation trace after each inhibitor is presented. Arrows denote when the first dose of relaxation agent is added. Each experiment is carried out individually and repeated 3–6 times with similar results.

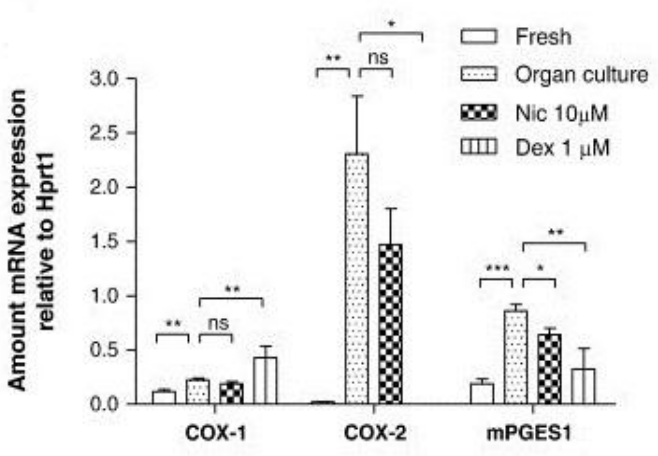

Figure 11. Effects of organ culture, nicotine (Nic) and dexamethasone (Dex) on mRNA expressions. Each data point is derived from 3 to 8 segments and data is presented as mean ± S.E.M. Statistical analysis was performed using unpaired Student's t-test. Fresh vs Organ culture, Organ culture vs Nic 10 μM, Organ culture vs Dex 1 μM; * P < 0.05; ** P < 0.01; *** P < 0.001.

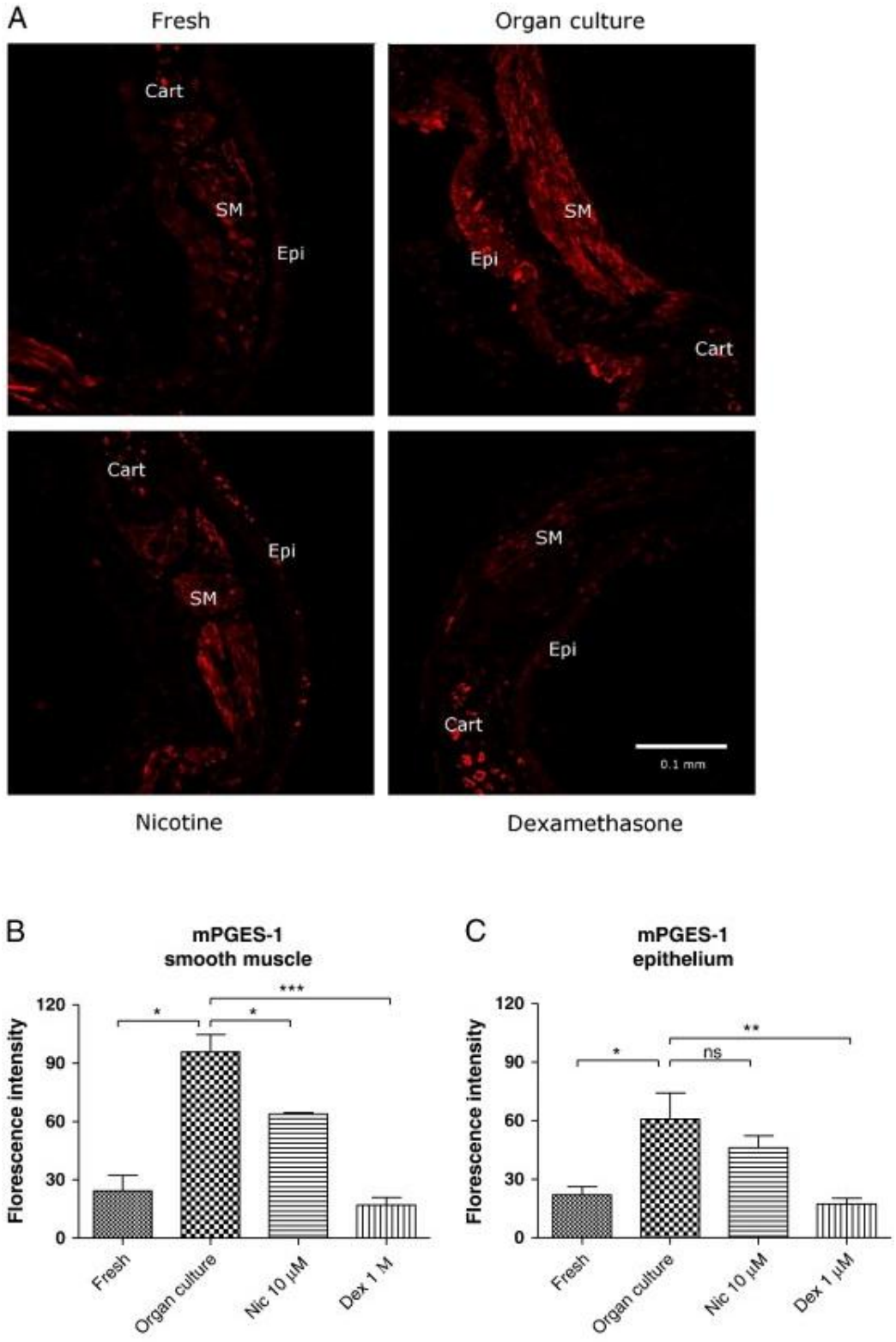

Figure 12. Effects of organ culture, nicotine (Nic) and dexamethasone on mPGES-1 protein expressions. Epi = epithelium; SM = smooth muscle; and Cart = cartilage. Each data point is derived from 3 to 4 samples. One-way ANOVA with Bonferroni's *post-hoc* analysis is performed. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, ns = no significance.

COX-1 mRNA expression increased 2 folds following organ culture (Fig. 11, Fresh: 0.12 ± 0.02, n = 7; Organ culture: 0.22 ± 0.02, n = 8, relative to house-keeping gene Hprt1). However, despite the changes in mRNA expressions (Fig. 11), COX-1 protein expressions were unaltered between groups (data not shown).

Fresh segments expressed relatively low levels of mPGES-1 protein (Fig. 12A) on the epithelium, and the smooth muscle. Organ culture markedly increased the mPGES-1 expression on both the epithelium (Fig. 12C) and the smooth muscle (Fig. 12B). Nicotine significantly decreased the mPGES-1 mRNA expression (Fig. 11, Organ culture: 0.86 ± 0.06, n = 6; Nic: 0.64 ± 0.06, n = 6; $P < 0.05$, relative to house-keeping gene Hprt1) as well as protein expression on the smooth muscle (Fig. 12B), and showed a tendency to decrease on the epithelium (Fig. 12C).

4.2.5 Comments

In contrast to long-term nicotine induced increase in ASM contractility, short-term nicotine induced decrease in epithelium-dependent airway relaxation was not mediated by changes in the kinin receptor expression, but rather on changes in the downstream mediators. The decreased kinin-receptor-mediated relaxation induced by nicotine was paralleled by decreased mPGES-1 mRNA and protein expressions, leaving kinin B_1, B_2 and COX-1 mRNA unaltered. A tendency towards a decrease in COX-2 mRNA and protein expressions were seen, but it did not reach statistical significance. Neither PGE_2- nor isoprenaline-induced relaxations were affected by nicotine exposure, suggesting that the downstream EP-receptor sensitivity and the relaxation-machinery of the tracheal rings were intact. It is therefore most likely that the decreased relaxation was caused by a decrease in PGE_2 production.

Bradykinin and des-Arg^9-bradykinin cause airway relaxations via stimulation of the kinin B_2 and B_1 receptors respectively, with subsequent activation of COX-pathways and production of airway relaxing prostaglandins (Barnes, 1992; Li *et al.*, 1998). In our study, selective inhibition of COX-2 with DuP-697 and non-selective inhibition of COX-1 and COX-2 with indomethacin completely abolished the relaxation induced by des-Arg^9-bradykinin and bradykinin, without affecting PGE_2 relaxations. COX-1 inhibition with the selective inhibitor piroxicam at previously shown effective concentrations (Bachar *et al.*, 2005b) did not affect bradykinin and des-Arg^9-bradykinin-induced relaxations. The central role of COX-2 in our model agrees well with reports indicating that in human (Daham *et al.*, 2011) and guinea pig (Safholm *et al.*, 2013) PGE_2 is mainly produced by COX-2 and not COX-1. However, increases in PGE_2 are not always accompanied by increased COX-2 (Kuroda *et al.*, 2003).

mPGES-1 is an inducible enzyme that is often upregulated, at both mRNA and protein levels, simultaneously with COX-2 to increase PGE_2 biosynthesis under inflammatory conditions, and this upregulation can be abolished by dexamethasone (Murakami *et al.*, 2000). PGE_2 stimulates EP1 receptors that mediate Ca^{2+} mobilization, EP3 receptors that inhibit adenylate

cyclase, and EP_2 and EP_4 receptors that activate adenylate cyclase (Sugimoto *et al.*, 2007). Previous studies show that the EP_2 subtype is mainly responsible for smooth muscle relaxation (Tilley *et al.*, 2003). However, though often upregulated simultaneously, the kinetics for the induction of COX-2 and mPGES-1 expressions have been shown to be distinct (Stichtenoth *et al.*, 2001), suggesting different transcriptional regulation mechanisms. This is in line with our observations that nicotine significantly inhibited mPGES-1 mRNA and protein expressions, but only showed a tendency to decrease COX-2.

It is worth noting that the effect of nicotine on murine airway relaxations is transient. After 4 days of organ culture, the difference between nicotine-treated and control segments disappears. At the same time, the morphology of the relaxation curve is drastically altered. While the relaxation curve after 1 day of organ culture has an inverse S-shape, the relaxation curve to bradykinin and des-Arg^9-bradykinin becomes more or less a straight line. Histological studies of cultured guinea pig bronchi showed that long-term organ culture (3 days or longer) caused submucosal swelling and decreased epithelial integrity, while the smooth muscle layer was unaffected. *In situ* detection of apoptosis by TUNEL assay and DAPI counterstaining revealed increases in apoptosis with time in the epithelial and submucosal layers, but not in the smooth muscle (Morin *et al.*, 2005). These findings suggest that normal epithelial, but not smooth muscle function is disrupted by long-term organ culture, making the long-term organ culture model unsuitable for studies of epithelial function.

4.3 NICOTINE ON NERVE-MEDIATED AIRWAY CONTRACTIONS (PAPER IV)

4.3.1 *In vitro* effects

Tracheal segments from mice were organ-cultured with nicotine for 1 or 4 days. Their contractile responses to increasing frequencies of EFS were recorded before and after 1 hr incubation with LPS. Nicotine *per se* did not have an effect on EFS-induced contractions neither after short- nor long-term exposure (Fig. 13).

LPS increased the contractile responses to EFS after 4 days regardless of nicotine exposure (Fig. 13B). The increases were most pronounced at the higher frequencies (~1.5-1.9 fold). However, nicotine-treated segments generally displayed lower contractile responses after LPS treatment than non-nicotine-treated ones (Fig. 13B). Responses to KCl and carbachol were similar in all groups (data not shown).

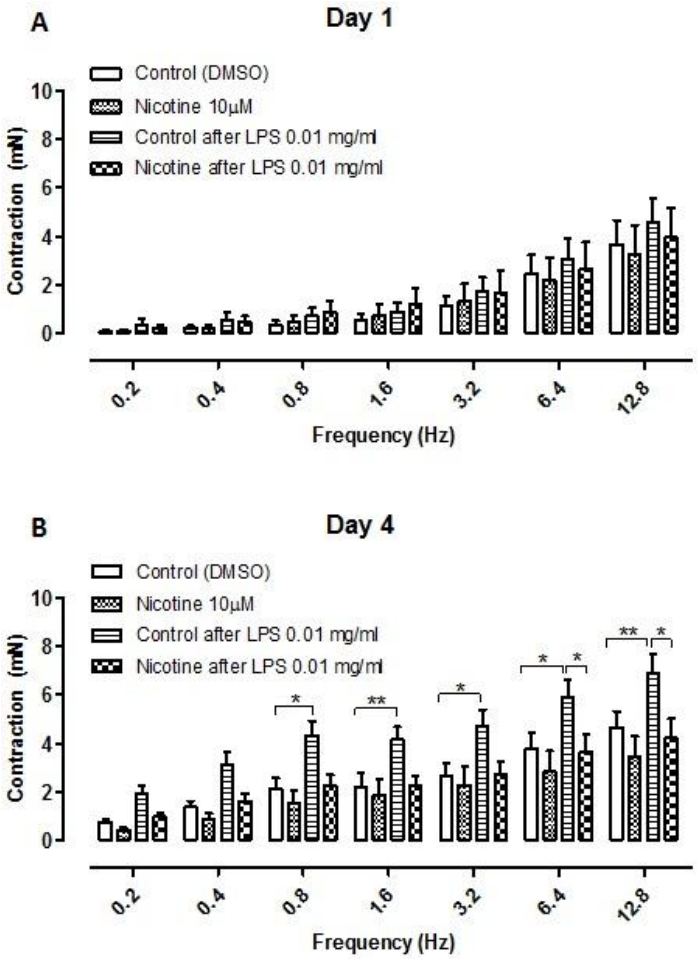

Figure 13. Effect of 1- and 4-days nicotine exposure *in vitro* on EFS-elicited airway contractions. Tracheal segments were cultured for 1 (A) or 4 (B) days with nicotine and pretreated with LPS for 1 hr prior to stimulation with EFS (0.2–12.8Hz) in the organ bath. Two-way ANOVA with Bonferroni *post hoc* analysis (unpaired) was used to compare Control vs Nicotine, and Control after LPS vs Nicotine after LPS. Two-way ANOVA with Bonferroni's *post hoc* analysis (paired) was used to compare Control vs Control after LPS and Nicotine vs Nicotine after LPS. * $p<0.05$, ** $p<0.01$. n=10–12 rings per group.

4.3.2 *In vivo* effects

Long-term (28 days) exposure to nicotine (24 mg/kg/day) delivered via a subcutaneous pump did not alter the airway mechanics *per se*, neither at baseline nor after methacholine (Mch) challenge. However, nicotine-treated mice that received intranasal LPS instillations displayed reduced responses to Mch in both central (Newtonian resistance, Rn, Fig. 14A) and peripheral (tissue damping, G, Fig. 14B) airways.

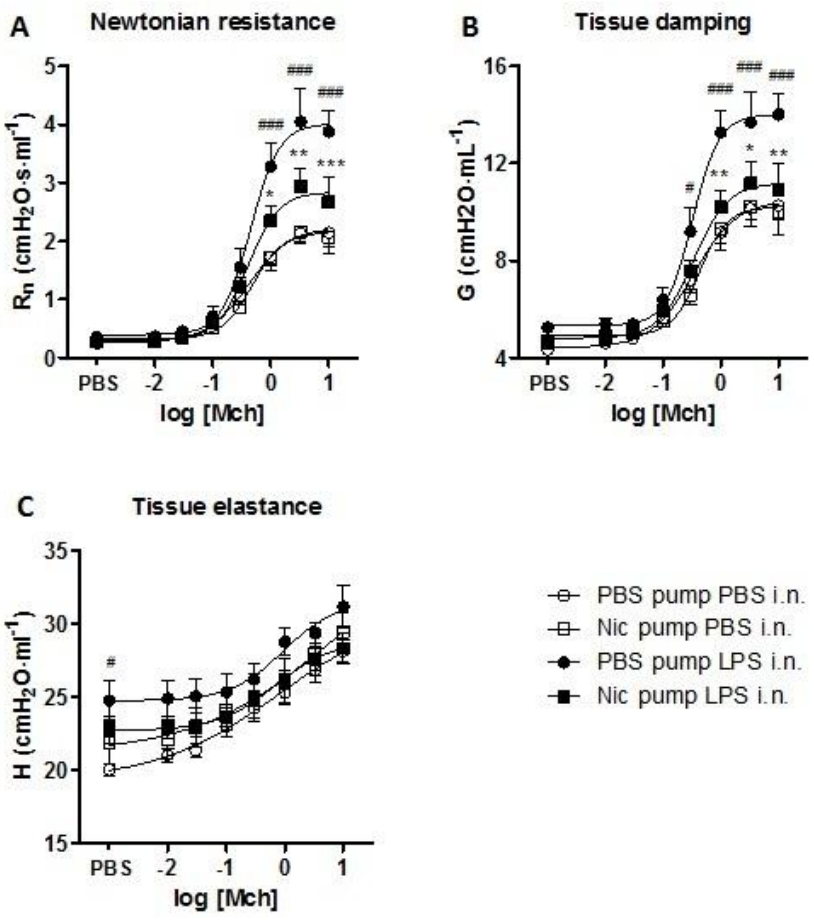

Figure 14. Airway mechanics after long-term *in vivo* nicotine exposure. One-way ANOVA with Bonferroni's *post hoc* analysis was performed for each individual methacholine dose. PBS pump LPS i.n. vs Nic pump LPS i.n, * $p<0.05$, ** $p<0.01$, *** $p<0.001$; PBS pump PBS i.n. vs PBS pump LPS i.n., $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$. n=8–12 animals per group.

Intranasal instillation of LPS (20 μl, 0.1 mg/ml) during three consecutive days enhanced the concentration-dependent increase in airway resistance caused by intravenous Mch in both central (Newtonian resistance, Rn, Fig 14A) and peripheral airways (tissue damping, G, Fig 14B). The LPS installations also raised the baseline tissue elastance (H, Fig 14C). The

increased AHR could partially be inhibited by intravenous pretreatment with the neurotoxin tetradotoxin (6 μg/kg) prior to Mch administration (data not shown).

4.3.3 Comments

The present study confirms that intranasal instillations of LPS enhance the concentration-dependent increase in airway resistance caused by intravenous Mch injections (Starkhammar *et al.*, 2012). We have demonstrated that long-term treatment with nicotine did not enhance the airway resistance *per se*, but markedly dampened the expected increase in airway resistance after acute LPS. These experiments were reproduced under *in vitro* conditions, using isolated murine tracheal segments. LPS caused a similar increase of the airway contractile response induced by EFS. Long-term, but not short-term culture of isolated tracheal segments with nicotine, dampened the LPS induced augmentation of the contractile EFS response, similar to what was seen for Mch *in vivo*.

The striking similarity between the *in vivo* effects of nicotine and LPS on the Mch-elicited AHR and the *in vitro* EFS-elicited AHR implicates the involvement of nerves in the former. EFS contractions in mice are known to be abolished by both the Na^+ channel blocker tetrodotoxin and the cholinergic antagonist atropine (Bachar *et al.*, 2005a) suggesting that this response is related to cholinergic nerve activity. The involvement of nerves in *in vivo* Mch-induced airway contractions is confirmed by a suppression of Newtonian resistance following acute treatment with tetrodotoxin. Other studies have suggested that activation of the transient receptor potential vanilloid type 1 (TRPV1) on sensory nerve contributes in this type of LPS-induced AHR in human bronchi (Calzetta *et al.*, 2015). Bilaterally vagotomized sheep do not exhibit any contractile response to Mch suggesting that neuronal mechanisms play a major role in this type of smooth muscle contraction *in vivo* (Wagner *et al.*, 1999).

4.4 COMBINED NICOTINE AND LPS ON AIRWAY CONTRACTIONS (PAPER III)

4.4.1 Effects of LPS

Trachea rings were cultured for 4 days in the presence of LPS at concentrations of 10^{-5}, 10^{-4}, and 10^{-3} mg/ml. LPS induced dose-dependent AHR to des-Arg^9-bradykinin (Fig. 15A) and bradykinin (Fig. 15B). LPS at 10^{-5} mg/ml had almost no effect, while 10^{-4} mg/ml and 10^{-3} mg/ml produced significant effects. The highest effect was reached with 10^{-3} mg/ml LPS for both bradykinin and des-Arg^9-bradykinin.

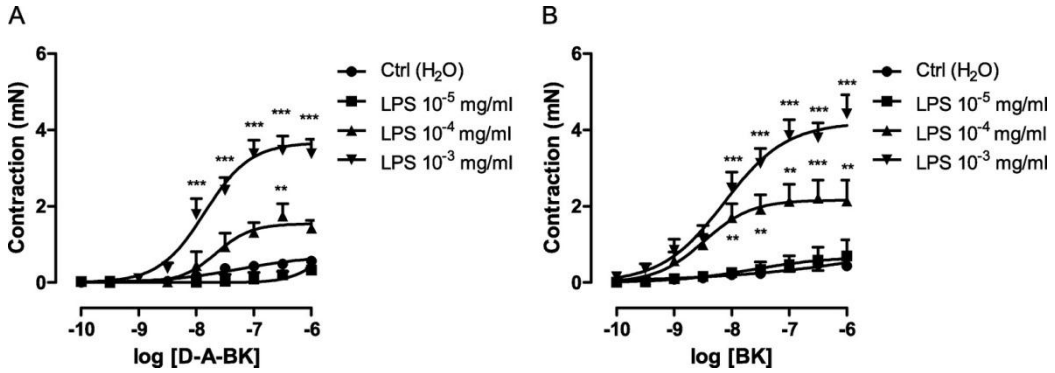

Figure 15. Concentration-dependent effects of LPS on kinin receptor agonist mediated airway contractions. D-A-BK: des-Arg^9-bradykinin. BK: bradykinin. Each data point was derived from 6 to 11 segments. All data were presented as mean ± SEM, LPS-treated versus control (Ctrl). P values were calculated using two-way ANOVA with Bonferroni's *post hoc* analysis. **P < 0.01; ***P < 0.001.

4.4.2 Effects of nicotine and LPS combined

Trachea rings were cultured for 4 days with LPS 10^{-5} mg/ml (Fig. 16A-B) or 10^{-4} mg/ml (Fig. 16C-D) with 10 μM nicotine or vehicle (DMSO). LPS at 10^{-5} mg/ml alone produced nearly no effect on bradykinin- and des-Arg^9-bradykinin–induced airway contractions, whereas the addition of nicotine increased the contractile responses to bradykinin (Fig. 16B) and des-Arg^9-bradykinin (Fig. 16A) but not carbachol and KCl (data not shown). Nicotine at 10^{-5} M *per se* also could increase the contractile response, but the magnitude was smaller than when nicotine and a low-concentration LPS were combined (Fig. 16).

A similar synergistic effect between nicotine and LPS was observed at an LPS concentration of 10^{-4} mg/ml. Although nicotine alone increased contractions produced by 10^{-6} M des-Arg^9-bradykinin by 74% (compared with control contractions) and LPS (10^{-4} mg/ml) increased it

by 80%, the combination of nicotine and LPS increased the contraction induced by 10^{-6} M des-Arg^{9}-bradykinin by 235% (Fig. 16C).

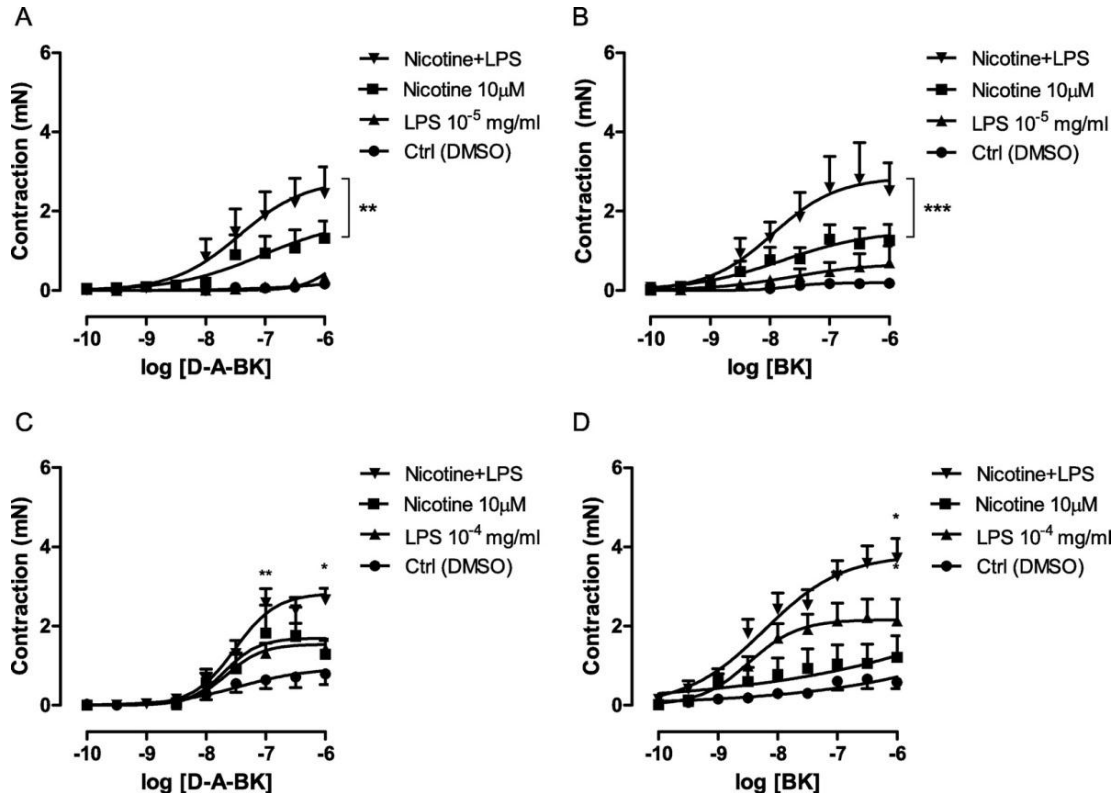

Figure 16. Effects of combined nicotine and LPS exposure on kinin receptor agonist mediated airway contractions. Each data point was derived from 5 to 7 segments. All data were presented as mean ± SEM, Nicotine+LPS versus Nicotine. P values were calculated using two-way ANOVA with Bonferroni's *post hoc* analysis. *P < 0.05; **P < 0.01; ***P < 0.001.

Nicotine alone increased the contraction produced by 10^{-6} M bradykinin by 109%, whereas LPS (10^{-4} mg/ml) alone increased the contraction by 268%. The combination of nicotine and LPS increased the contraction by 541%, thereby demonstrating a synergistic effect (Fig. 16D). However, when LPS concentration was as high as 10^{-3} mg/ml, the addition of nicotine failed to produce a further increase in contraction (data not shown).

4.4.3 Comments

In the present study, nicotine was used to mimic the effects of cigarette smoke exposure, and the presence of LPS mimicked bacterial infection. LPS was found to concentration-

dependently increase the contractile responses to the selective kinin B_2 and B_1 receptor agonists bradykinin and des-Arg^9-bradykinin without affecting carbachol- or KCl-induced contractions. Nicotine exaggerated the AHR to LPS, causing stronger contractions at lower concentrations. No such effects were seen when nicotine and LPS were given separately. This finding is in agreement with the clinical observation that smokers, regardless of whether they have COPD, are more sensitive to bacterial infections than non-smokers (Nikota *et al.*, 2012).

The current study confirms this idea by demonstrating that nicotine, in combination with low concentrations of LPS, induces a more potent response to kinin receptor agonists than the sum of the effects of the two substances given separately. In humans, inhalation of 5 μg of LPS is sufficient to induce a small but significant change in FEV1 in "healthy" smokers (Aul *et al.*, 2012), while healthy non-smokers only display a small but not significant decrease in FEV1 after 50 μg LPS (Michel *et al.*, 1997). In small animal *in vivo* experiments, LPS administered locally at 10^{-1} mg/ml elicit pulmonary inflammation and AHR (Starkhammar *et al.*, 2012). It is therefore important to note that nicotine enhances the response to LPS at concentrations more than 1,000-fold lower than that used *in vivo*. Hence, it is natural to assume that the high LPS concentrations produce contractions that are so strong (i.e., close to the segment's contraction maximal limit) that the effect induced by nicotine becomes hard to visualize.

4.5 NICOTINE ON TLR EXPRESSIONS (PAPERS III AND IV)

4.5.1 mRNA

The effects of nicotine on mRNA expressions of cell surface TLR in mice tracheal segments were examined under *in vitro* conditions. Four days of nicotine exposure (10 μM) increased the mRNA expression of TLR2 (1.5-fold; $P < 0.05$), TLR4 (1.8-fold; $P < 0.01$), and TLR6 (2.0-fold; $P < 0.01$) in the airways. TLR1 and -11 remained unchanged, whereas the mRNA expression for TLR5 decreased (−2.4-fold; $P < 0.01$) (Fig. 17A).

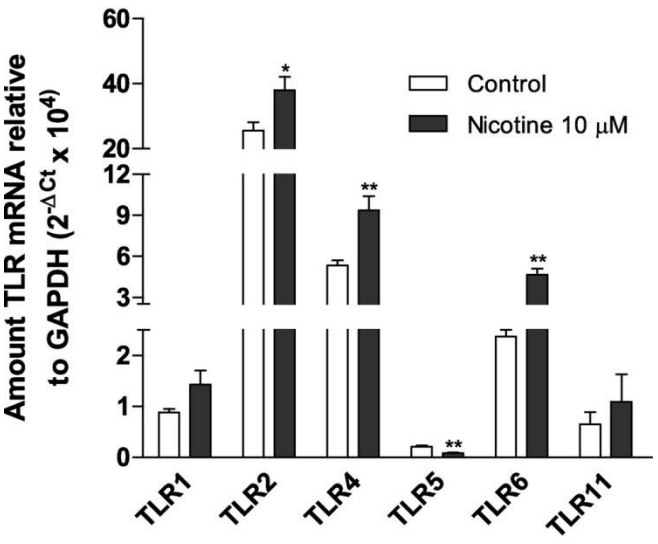

Figure 17. Effects of nicotine on cell surface TLR mRNA expressions. Tracheae were incubated in the presence of vehicle (DMSO, control) or nicotine (10 μM) for 4 days. Each data point is derived from three or four animals. P values were calculated using two-tailed unpaired Student's *t* test. *$P < 0.05$; **$P < 0.01$.

4.5.2 Proteins

After 4 days of organ culture with nicotine *in vitro*, immunohistochemistry revealed a dominating TLR4 staining of the smooth muscle cells during control conditions, with only a weak expression in the epithelium. Nicotine exposure increased the TLR4 expression at muscular and epithelial levels, with a more prominent increase in the latter (Fig. 18A-C). TLR6 staining was evenly divided between the smooth muscle and the epithelium at fresh state and increased in both the epithelium and smooth muscle after incubation with nicotine (Fig. 18D-F).

After 28 days of nicotine exposure via a subcutaneous nicotine pump *in vivo*, the fluorescence intensity, which reflects the amount of TLR4 on bronchial epithelial cells, was markedly increased compared to mice receiving PBS in their pumps (2.9 fold, $p<0.01$, Fig. 19A,B,D).

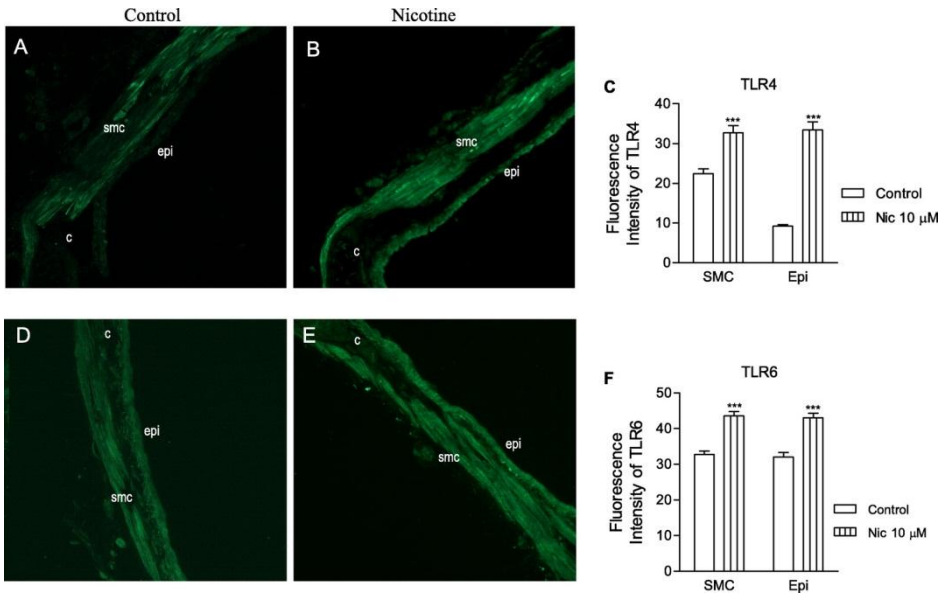

Figure 18. Effects of nicotine on TLR4 (A–C) and TLR6 (D–F) receptor protein expressions in isolated mouse airway. C, cartilage; Epi, epithelium; SMC, smooth muscle cells. Each data point is derived from 6 experiments. P values were calculated using two-tailed unpaired Student's *t* test, Control versus Nic. *** $P < 0.001$.

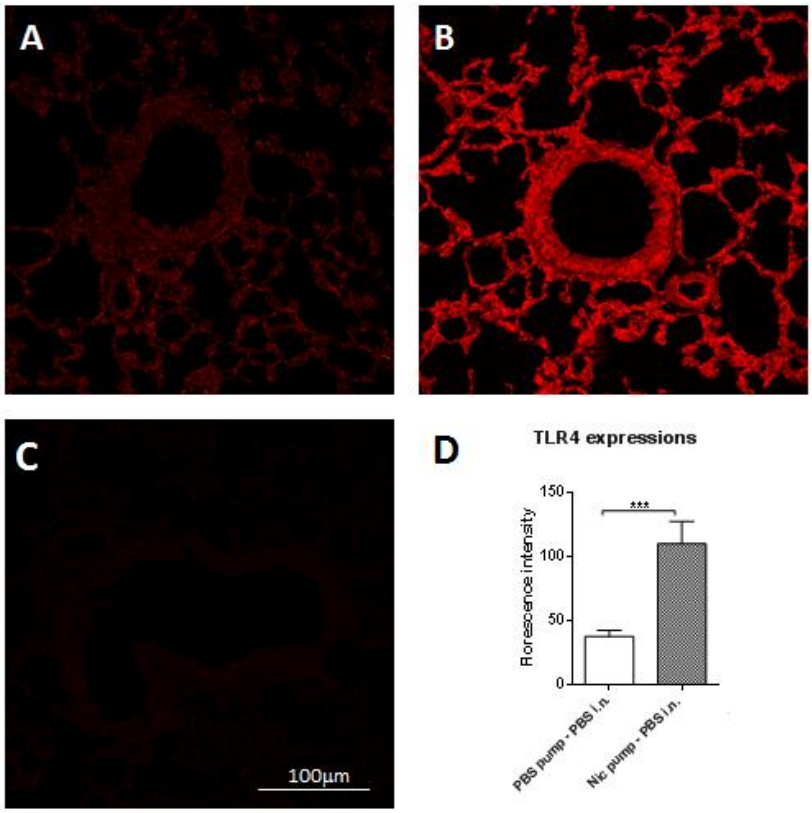

Figure 19. TLR4 expressions after long-term *in vivo* nicotine exposure. A: PBS pump PBS i.n.; B: Nic pump; C: Isotype control; D: TLR4 expression semi-quantified by florescence intensity. One-way ANOVA with Bonferroni *post hoc* analysis. PBS pump PBS i.n. vs Nic pump PBS i.n., *** $p<0.001$. n=5-6 animals.

4.5.3 Involvement of the JNK pathway

Specific pharmacological inhibitors for JNK (SP600125, 10 μM), ERK1/2 (U0126, 10 μM), and p38 (SB203589, 10 μM) pathways were used to explore the role of the MAPK pathways in nicotine-induced alteration of TLR expression. SP600125 abrogated the nicotine-induced alterations in the mRNA expression of TLR2, -4, -5, and -6, without affecting the TLR1 and -11 expressions (Fig. 20).

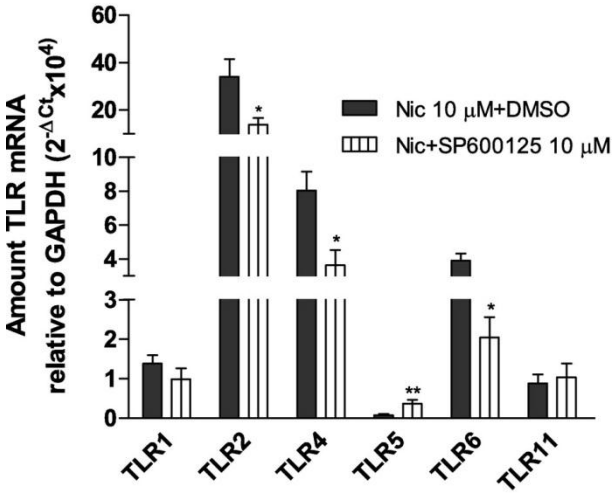

Figure 20. Effects of specific JNK inhibitor SP600125 on TLR mRNA expression. Each data point is derived from 4 to 7 animals. P values were calculated using two-tailed unpaired Student's *t* test. *P < 0.05, **P < 0.01.

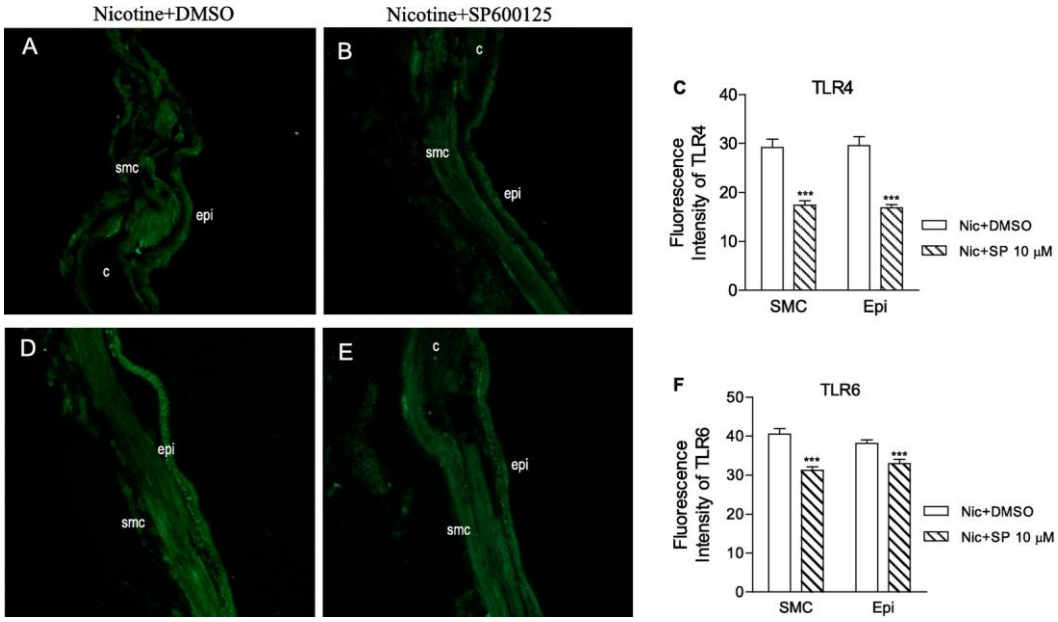

Figure 21. Effects of the specific JNK inhibitor SP600125 on TLR4 (A–C) and TLR6 (D–F) protein expressions. C, cartilage; Epi, epithelium; SMC, smooth muscle cells. Each data point is derived from six experiments. P values were calculated using two-tailed unpaired Student's *t* test, Nic+DMSO vs Nic+SP600125. ***P < 0.001.

U0126 and SB203580 had no significant effect on the TLR expressions (data not shown). Analysis of TLR4 protein expressions by immunohistochemistry verified that SP600125 depressed TLR4 (Fig. 21A-C) and TLR6 (Fig. 21D-F) protein expressions in both smooth muscle and epithelium.

4.5.4 Comments

TLR activation is crucial for mounting a forceful immune response against invading pathogens. However, excessive activation of TLRs can also cause an overproduction of inflammatory cytokines and chemokines as well as an increase in AHR (Starkhammar *et al.*, 2012). Patients with fatal asthma have been shown to have a more pronounced airway expression of TLR3 and TLR4 than healthy control subjects (Ferreira *et al.*, 2012). On the other hand, a down-regulation of the TLRs might compromise the host's immune defense. The differential effects of nicotine on the expression of various TLRs might be a reflection of the complex nature of smoke-associated airway disease. TLRs are known to be able to form heterodimers with each other. For example, upregulated TLR4 and -6 can form heterodimers together with the co-receptor CD36. Similar formations have been shown to be of importance in atherosclerosis, a disease where cigarette smoking is known to be detrimental (Stewart *et al.*, 2010).

Nicotine *per se* increased the mRNA expressions of TLR2, -4, and -6, with TLR4 and -6 showing the largest fold increase. Further analysis of TLR4 and -6 revealed receptor protein increases in the epithelium and in the smooth muscle layer. An augmentation of the receptor density might explain the amplified LPS sensitivity. The present data are well in line with *in vivo* studies which showed that exposure to cigarette smoke increases the pulmonary expression of TLR2 and -4 in mice (Maes *et al.*, 2006).

The effect of nicotine in cell surface TLR mRNA expressions and TLR4 and 6 protein expressions were completely reversed by the inhibition of the MAPK-JNK pathway. The role of JNK-related mechanisms in many of the pathological processes in respiratory diseases has also been discussed in the recent decade (Bennett, 2006). However, clinical development of kinase inhibitors has primarily focused on p38 inhibitors (Adcock *et al.*, 2004). In animal studies, it has been found that the JNK inhibitor SP600125 can reduce allergic cellular inflammation and ASM proliferation (Eynott *et al.*, 2003) as well as allergen-induced AHR (Nath *et al.*, 2005). Our results suggest that JNK may play a special role in cigarette-smoke-induced pulmonary diseases.

4.6 CIGARETTE SMOKE EXPOSURE ON ALK EXPRESSIONS (PAPER V)

4.6.1 Cotinine assay

Twenty seven pairs of tonsils were collected from patients between 15 and 40 years old undergoing bilateral tonsillectomy for chronic tonsillitis at the Ear- nose- and throat clinic at Malmö University Hospital, Sweden.

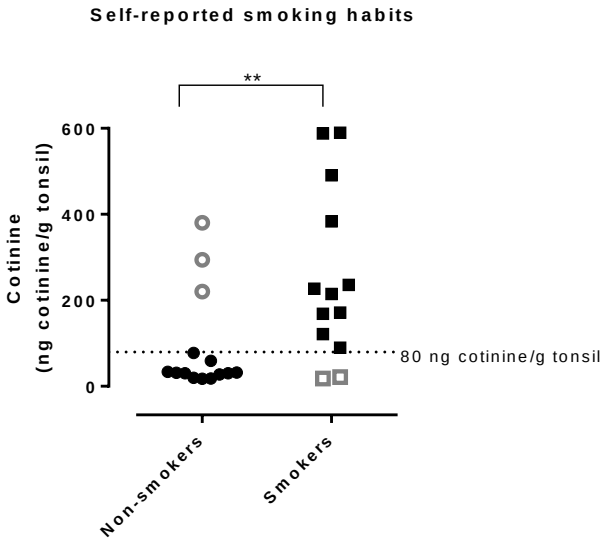

Figure 22. Local cotinine concentrations in tonsil tissue. Unpaired Student's *t* test was used to compare self-reported non-smokers with self-reported smokers. ** p<0.01.

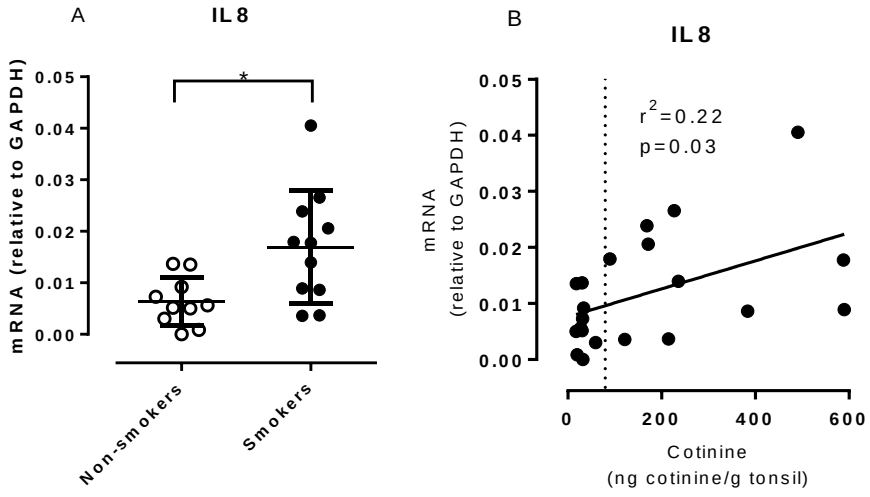

Figure 23. IL-8 mRNA expressions in cotinine-verified smokers and non-smokers. Unpaired Student's *t* test was used to compare IL-8 mRNA levels in non-smokers vs smokers (A). * p<0.05. Linear regression was performed relating IL-8 mRNA to local cotinine concentration (B).

The tonsils were homogenized, centrifuged and the supernatants were analyzed with cotinine ELISA. A cut-off level of 80 ng cotinine/g tonsil was used to distinguish smokers from non-smokers (Fig. 22). This level was chosen to minimize the number of excluded samples.

The mRNA of IL-8, a cytokine known to be upregulated by cigarette smoke (Agusti *et al.*, 2012; Lau *et al.*, 2012; Starrett *et al.*, 2011) was significantly higher in the cotinine-verified smoker group compared to the cotinine-verified non-smoker group (2.7 folds, $p<0.05$, Fig. 23A). A linear positive correlation was found between tonsillar cotinine levels and IL-8 mRNA levels ($r^2=0.22$, $p<0.05$, Fig. 23B).

4.6.2 ALK expressions

The mRNA expressions of ALK1 and 5 were relatively high in the tonsillar tissue, ALK 2, 3, and 4 were lower, while ALK7 was near to undetectable, ALK6 was not detectable at all. Significant increases in ALK1 (1.5 fold, $p<0.05$) and 2 (1.4 fold, $p<0.05$) mRNA levels were found in the cotinine-verified smoker group compared to cotinine-verified non-smoker group (Fig. 24A). Both ALK1 and 2 levels displayed linear positive correlations with tonsillar cotinine levels (ALK1: $r^2=0.23$, $p<0.05$, Fig. 24B; ALK2: $r^2=0.51$, $p<0.001$, Fig. 24C).

4.6.3 Comments

Despite the importance of smoking in a wide range of diseases, patient-reported smoking history is notoriously unreliable (Caraballo *et al.*, 2001; Florescu *et al.*, 2009; Hobbs *et al.*, 2005; Stelmach *et al.*, 2015). This is also reflected in the present study. There can be many reasons behind this discrepancy, including memory failure, recall bias, lack of knowledge, but also intentional false reporting especially when the social acceptance for smoking in the society is decreasing (Caraballo *et al.*, 2001). A valid estimation of the risks of smoke exposure, both active and passive is crucial for the assessment of risks associated with smoking. The present study suggests that cotinine measurements in homogenates of tonsils can be used to demonstrate local smoke exposure in the tissue. The obtained cotinine levels were positively correlated to tonsillar IL-8 mRNA levels. A clear correlation between smoke exposure and ALK1 and ALK2 mRNA levels was also seen.

ALKs are receptors for the transforming growth factor-beta (TGF-β) superfamily. They are involved in a range of cancer-related cellular processes including proliferation, differentiation, adhesion, migration and apoptosis (Graham *et al.*, 2006). Among the seven different ALKs found in humans, ALK1 and 5 are the most studied in the context of cancer. ALK1 and ALK5 (also termed TGF-βI receptor) exhibit antagonizing effects in endothelial cells. While ALK1 promotes growth and proliferation and thereby carcinogenesis, ALK5 inhibits the same processes (Jonker, 2014). ALK5 knockout mice develop spontaneous

squamous cell carcinoma (Honjo *et al.*, 2007). ALK5 expression is also known to be decreased in human head and neck squamous cell carcinoma tumor cells (Eisma *et al.*, 1996). At the same time, high levels of ALK1 in tumor tissue from patients with head and neck squamous cell carcinoma appear to be positively correlated to more advanced TNM stages as well as a poor prognosis (Chien *et al.*, 2013). Inhibitors of ALK1 are currently under clinical trials for the treatment of solid tumors (Cunha *et al.*, 2011; Jonker, 2014). Blocking ALK1 signaling using an ALK ligand trap ALK1-Fc in combination with cisplatin was recently found to inhibit tumor growth in murine head and neck cancer models more efficiently than chemotherapy alone (Hawinkels *et al.*, 2015). The present data supports the role for ALK in the poor prognoses that characterize tonsillar cancer among smokers.

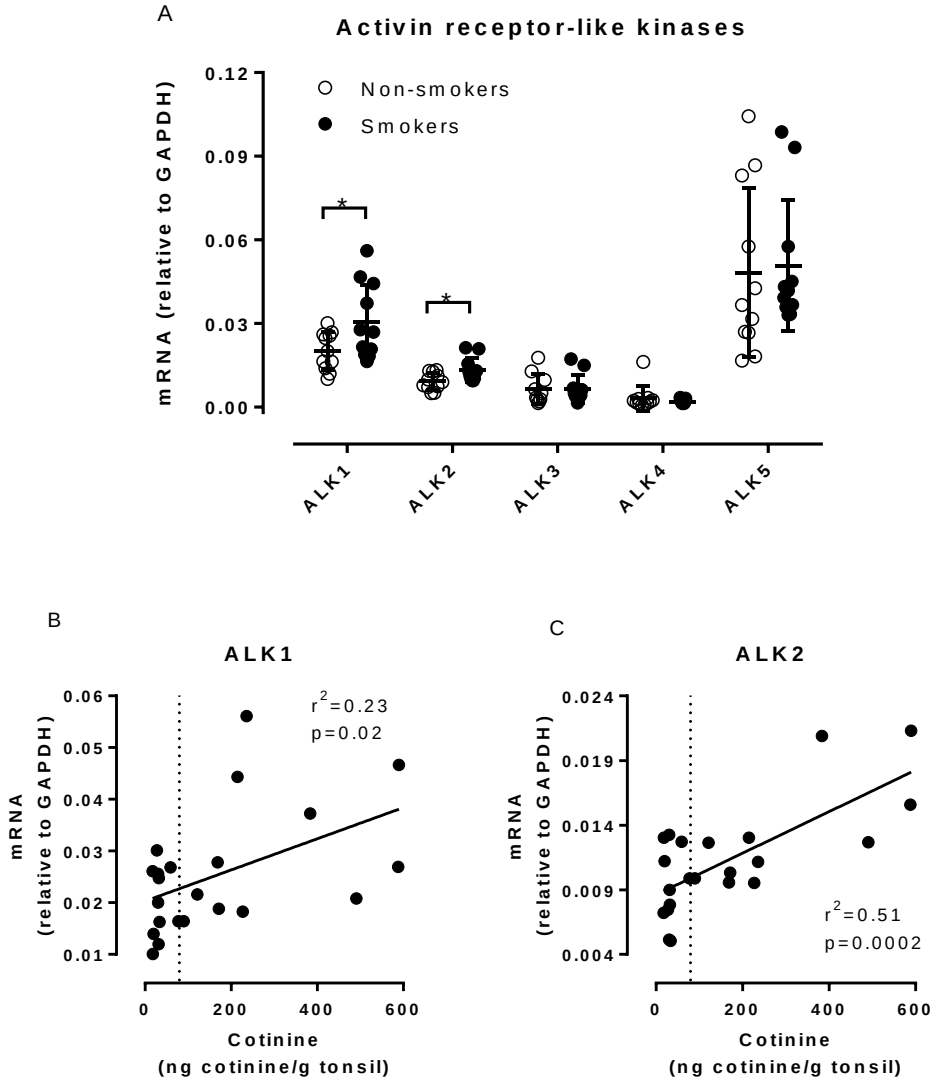

Figure 24. ALK mRNA levels in tonsils of cotinine-verified smokers and non-smokers. Unpaired Student's *t* test was used to compare individual ALK mRNA levels in non-smokers vs smokers (A). * $p<0.05$. Linear regression was performed relating ALK1 (B) and 2 (C) mRNA levels to tonsillar cotinine concentrations.

5 CONCLUSIONS

The present thesis has investigated the effects of nicotine and LPS, alone or in combination, on AHR and inflammation.

The main conclusions are the following:

- Long-term nicotine exposure *in vitro* enhances bradykinin- and des-Arg^9-bradykinin-induced airway contractions in murine tracheal segments. The nicotine effect is mediated by activation of neuronal nicotinic receptors which lead to transcriptional upregulation of kinin B_1 and B_2 receptors. This process depends on the activation of JNK- and PDE4-related intracellular signalling pathways.

- Short-term nicotine exposure *in vitro* impaired epithelium-dependent kinin-receptor mediated airway relaxations in murine tracheal segments. In addition, nicotine suppressed the mRNA and protein expression of mPGES-1 and showed a tendency to suppress COX-2. Addition of dexamethasone to the system did not reverse the effects of nicotine.

- Long-term nicotine exposure *in vitro* induced inflammation and upregulated the expression of TLR4 and 6 via the MAPK-JNK pathway. In addition, nicotine exaggerated LPS-induced AHR. This might explain the increased AHR often seen among cigarette smokers when confronted with bacterial infections.

- LPS increased AHR to EFS *in vitro* and to methacholine *in vivo*. Long-term nicotine exposure markedly dampened this LPS-induced AHR both *in vitro* and *in vivo*. The effect of nicotine was mimicked by tetrodotoxin, suggesting an important role for neuronal mechanisms in cigarette smoke-induced AHR *in vivo*.

- Measurement of cotinine in tonsil homogenate appears to be a useful and reliable method to objectify smoke exposure levels in tonsils. A direct correlation between smoke exposure and increased tonsillar expressions of IL-8, ALK1 and 2 were demonstrated, suggesting a role for ALK1 in the poor prognosis that characterize tonsillar cancer among smokers.

6 GENERAL DISCUSSION

6.1 WHY STUDY NICOTINE?

One might wonder why it is necessary to study the effects of nicotine, since everybody already knows that smoking is detrimental to health. After the Surgeon General report made headlines in the United States and the rest of the world in 1964, it has been known that cigarette smoking causes lung cancer, laryngeal cancer, chronic bronchitis and cardiovascular disease. Now, more than 50 years after the publication of this report, the knowledge about the spectrum of organ systems negatively affected by smoking has expanded, accompanied by drastic changes in attitudes towards smoking (Alberg *et al.*, 2014).

Indeed, the smoking prevalence is decreasing, especially in Western countries. In Sweden, the smoking prevalence has decreased from 27.7% to 12.7% among middle-aged men and 32.3% to 14.3% among women from the period 1990-1995 to the period 2002-2007. However, the use of the Swedish oral moist snuff, has steadily increased from 27.5% to 33.7% among middle-aged men and rapidly increased from 3.2% to 13.9% among middle-aged women (Norberg *et al.*, 2011). Also, it should not be forgotten that in non-Western countries such as China, the overall smoking prevalence (including both regular smokers and former smokers) is still over 70% among men (Paskett *et al.*, 2015).

Despite falling smoking prevalence, the availability of nicotine in the society is greater now than ever. Nicotine replacement products, such as nicotine gum, patch and spray, deliver pure nicotine through the oral or nasal mucosa or the skin and reduce the severity of withdrawal symptoms and craving of tobacco products. They are generally recommended as first-line treatment against tobacco dependence. Their side effects are small and they are often sold over-the-counter in most countries (Little *et al.*, 2015). Moreover, e-cigarettes were introduced to the European and American markets in 2006, and their sales increased dramatically around 2010, quickly surpassing nicotine replacement products and are predicted to surpass traditional cigarettes within 10 years. The regulatory status of e-cigarettes is still unclear in most countries and is a subject of debate (Fagerstrom *et al.*, 2015).

Given the high prevalence of nicotine exposure in the society today, it is therefore of interest to study the long-term physiological and pathophysiological effects of nicotine.

6.2 EXOGENOUS VS ENDOGENOUS NICOTINE RECEPTOR ACTIVATION

In the present thesis, nicotine receptor activation caused by exogenous nicotine that may derive from sources such as cigarettes, moist snuff or nicotine replacement products is discussed. The concentration of nicotine used in both the *in vitro* and *in vivo* studies corresponds to that seen among heavy smokers and moist snuff users. For the *in vitro* studies,

nicotine at a concentration of 10 μM was used. This level can roughly be compared to the "local exposure" level. In the saliva of smokers during smoking days, the level of nicotine measures up to 8 μM (Lindell *et al.*, 1993) and 34 μM is found in the induced sputum of smokers 5 min after smoking one cigarette (Clunes *et al.*, 2008).

For the *in vivo* studies, a level equivalent to "systemic exposure" was used. Subcutaneous osmotic pumps containing nicotine tartate salt produces a stable plasma nicotine concentration and have long been utilized for the study of chronic nicotine effects. Nicotine at 24 mg/kg/day supplied via a subcutaneous osmotic pump produces plasma cotinine concentration equivalent to that of a human smoker (Dickson et al., 2014). This seemingly high nicotine dosage is necessary due to the relative high nicotine metabolism in mice compared to other species and is commonly utilized in *in vivo* nicotine research (Matta et al., 2007).

It should be noted that the endogenous neurotransmitter acetylcholine can also bind to and activate nicotinic acetylcholine receptors. However, the mode of action is very different between normal physiological neurotransmission where the receptors are exposed to a sudden high concentration of an agonist and a chronic exposure with a sustained low agonist level. The former causes the receptors to rapidly change from resting to activated and then desensitization, while the latter can stabilize the receptor in an active or inactive state depending on the concentration of agonist (Fenster *et al.*, 1997; Jones *et al.*, 2012). The time and concentration of exposure is therefore of crucial importance for the effect of nicotine. This is also reflected by results from Paper I and II in the present thesis.

6.3 *IN VITRO* AND *IN VIVO* DIFFERENCES?

In Paper I, it was seen that long-term nicotine exposure in mice *in vitro* caused increased AHR measured by the myographs. This was not confirmed by the *in vivo* study in Paper IV where long-term exposure to nicotine alone did not alter AHR measured with flexiVent™.

At a first glance, the most obvious reason for this inconsistency is probably the difference between *in vitro* and *in vivo* environments. It is not inconceivable that a piece of tracheal tissue free from supporting tissue, neural connections and circulating inflammatory cells should react differently to that of a living mouse that has access to liver, kidney, brain and blood. However, there are several other differences between the two models that also need to be considered.

The differences between large and small airway in terms of function and physiology have been brought to attention during the recent decades (van der Wiel et al., 2013). On the other hand, the "united airway hypothesis" suggesting that the respiratory tract, all the way from the nose down to the alveoli, should be treated as one single entity, has also gained momentum (Grossman, 1997; Passalacqua *et al.*, 2001). In the present thesis, in the *in vitro*

model, the large extra pulmonary airway, trachea is used, while in the *in vivo* model, the contraction of the whole lung is tested, including both large and small intrapulmonary airways. However, by applying different oscillation frequencies and fitting into the constant phase model, the flexiVent^{TM} system computes three separate variables for airway resistance: Newtonian resistance (Rn), tissue damping (G) and tissue elastance (H), where Rn reflects AHR in the central airways and G reflects AHR in peripheral airways (Bates *et al.*, 2011). In the present study, the effects of nicotine and LPS on Rn and G nearly always follow the same pattern and no difference was seen between the central and peripheral airways.

It is worth noting that in the *in vitro* model, AHR to kinin receptor agonists was increased, but contractile responses to the muscarinic receptor agonist, carbachol remained unaltered. In the *in vivo* model, the muscarinic agonist methacholine was used to elicit airway contractile responses as methacholine is the golden standard used to demonstrate AHR in *in vivo* animal models and humans. If the responses to the muscarinic agonists carbachol and methacholine are compared *in vitro* and *in vivo*, the results are consistent, none of the responses were altered by nicotine.

The role of kinin receptor agonists in AHR is a subject of debate. Early studies have shown an extensive role for bradykinin in asthma. Increased bradykinin concentration was found in BAL of asthma patients. Bradykinin also specifically acted as a potent bronchoconstrictor in asthmatic patients, while it had no effect in normal individuals. (Barnes, 1992). Allergen-induced AHR to bradykinin was found to be more pronounced than that to methacholine in human subjects (Berman *et al.*, 1995). However, the failure of the inhaled selective kinin B_2 receptor inhibitor HOE140 to achieve clinically relevant symptom score improvements in a phase II trial for moderate to severe asthma (Akbary *et al.*, 1996) has dampened the interest for kinin receptors as a target for the treatment of asthma and/or COPD. In rats, HOE140 inhibits AHR to bradykinin, but not to acetylcholine (Huang *et al.*, 1999). HOE140 was later found to be efficient in the treatment of hereditary angioedema attacks (Cicardi *et al.*, 2010).

Stimulation of kinin receptors produces different effects dependent on the receptor localization. On the ASM, kinin receptors directly activates the inositol 1,4,5-trisphosphate (IP3) pathway increasing intracellular Ca^{2+} levels which subsequently activates the cellular contractile machinery (Billington *et al.*, 2003). On airway sensory nerves, kinin receptors activate the vagal ganglion and thereby initiate the cough reflex (Fox *et al.*, 1996; Grace *et al.*, 2012). On the airway epithelium, kinin receptors activate COX and stimulate the release of PGE_2 from airway epithelial cells which induces airway relaxation through EP receptor activation (Barnes, 1992). In *in vitro* studies, the production of PGE_2 was blocked with the non-selective COX inhibitor indomethacin, while this is not the case in *in vivo* studies or humans. The complex and sometimes contradictory nature of bradykinin's effects might be the reason behind the failure of the kinin receptor antagonist in clinical trials as an asthma drug.

6.4 TLR4 EXPRESSION AND RESPONSE TO LPS

TLRs are central players in the innate immune response. They are PRRs and recognize a variety of molecular structure of pathogens such as bacteria, virus and fungi.

In Paper III, both TLR4 mRNA and protein was found to be increased by long-term nicotine treatment *in vitro*. TLR4 protein was predominantly expressed on ASM in fresh segments, but after nicotine exposure for 4 days, its expression increased significantly both on ASM and epithelium, with a greater percentage increase on the epithelium. This nicotine-induced upregulation of TLR4 receptor was believed to be responsible for the synergistic effect observed between nicotine and LPS on AHR, making the tracheal segments more sensitive to low concentrations of LPS. However, in the *in vivo* model in Paper IV, despite an increase in the protein density of TLR4 on bronchial epithelium following long-term nicotine exposure, the AHR to LPS decreased in the nicotine-treated group.

The clear increase in TLR4 expression but decrease in LPS response seen *in vivo* is somewhat puzzling. One reason could be the fact that only epithelial TLR4 protein expression is semi-quantified with immunohistochemistry, while the expression on other structures such as ASM and nerves cannot be seen in the immunohistochemistry sections used. The ability of tetrodotoxin to inhibit the LPS-induced AHR *in vivo*, as well as the striking similarity between the effect of nicotine and LPS on EFS-responses *in vitro,* supports the role for nerves in the observed decreased LPS responses. Therefore, the TLR4 expression in bronchial epithelium might not be important for the development of LPS-induced AHR. This is supported by the recent finding that stimulation of TLR4 on different cellular compartments contributes to different inflammatory responses. While TLR4 on hematopoietic cells is responsible for the neutrophilic inflammation following LPS- and house dust mite lysates, TLR4 on airway epithelial cells is mainly responsible for the eosinophilic airway inflammation following allergen sensitization and challenge (McAlees *et al.*, 2015).

Another possible explanation for the difference in LPS responses might be due to the difference in LPS concentrations. In the *in vitro* system in paper III, a synergistic effect between nicotine and LPS was seen at LPS concentrations of 10^{-5} mg/ml and 10^{-4} mg/ml, but not 10^{-3} mg/ml. This was possibly due to the ability of high LPS concentrations to saturate the TLR4 receptors and thereby mask the differences in response caused by different TLR4 receptor density. A much higher LPS concentration (10^{-1} mg/ml) was used in the *in vivo* system in paper IV, compared to that used *in vitro*.

6.5 FUTURE ASPECTS

In the present thesis, the inhibition of MAPK JNK with SP600125 attenuated nicotine-induced AHR as well as the transcriptional upregulation of TLR *in vitro.* It might therefore be a promising target in the treatment of cigarette-smoke associated airway diseases.

It is interesting to note that despite the current interest in p38 inhibitors un the treatment of COPD (Norman, 2015), no changes in p38 phosphorylation were seen in the *in vitro* nicotine-exposure model and the upregulated TLR expression was selectively inhibited by the JNK inhibitor SP600125, but not by the p38 inhibitor SB203589. Our group has previously shown that cytokine-induced increase in AHR to bradykinin *in vitro* is also mediated via activation of JNK and the downstream transcription factor NF-κB which leads to increased expression of kinin receptors (Zhang *et al.*, 2005; Zhang *et al.*, 2004).

The effect of the specific JNK inhibitor SP600125 has been studied in animal models with somewhat different results. It was found to reduce allergic cellular inflammation and ASM proliferation, but not AHR in a rat model of chronic allergen exposure (Eynott *et al.*, 2003). While in another mice model, SP600125 was found to decrease allergen-induced AHR (Nath *et al.*, 2005).

Over 100 mediators have now been implicated in asthma and COPD inflammation. Blocking a single mediator is therefore unlikely to be very effective in this complex disease. So far, mediator antagonists have not proved to be very effective compared to drugs that have a broad spectrum of anti-inflammatory effects, such as glucocorticoids. MAPKs are therefore desirable targets as they are involved in a large number of intracellular activities including gene expression, apoptosis, differentiation, proliferation and oncogenesis. However, at the same time, with such a broad activity profile, it is not strange that inhibition of MAPK comes along with many side effects, a problem that troubles the clinical development of p38 inhibitors (Adcock *et al.*, 2004).

One possibility to reduce the side effects could be the use of a combination of drugs in lower doses. Paper I revealed cross-talk between the JNK and PDE4 pathways. The PDE4 inhibitor Roflumilast is already approved for the treatment of COPD in the clinic. A combination of a PDE4 inhibitor with a JNK inhibitor can theoretically lower the concentration of JNK inhibitor required and thereby possibly also lower the risk of side effects.

Paper IV highlighted the involvement of neuronal mechanisms in LPS-induced AHR and its modulation by long-term nicotine treatment. While one might not want to return to the 1950s and perform surgical denervation and vagotomy on asthma (Levine et al., 1950) and COPD (Abbott et al., 1953) patients, recent research find transient receptor potential channels to be promising targets. Transient receptor potential channels are mainly found on sensory nerve endings. Studies have shown a fivefold increase in the number of nerve profiles that express TRPV1 channels in airway biopsies from subjects with chronic cough compared with normal controls (Groneberg *et al.*, 2004). Recent study using optogenetics found that activation of

TRPV1 positive nerves in the vagal ganglion dramatically exacerbated AHR of inflamed airways, which shows that the AHR phenotype can be physiologically dissociated from the immune component. The TRPV1 blocker JNJ17203212 (Bhattacharya et al., 2007) and the transient receptor potential ankyrin 1 blocker GRC 17536 (Mukhopadhyay *et al.*, 2014; Ryan *et al.*, 2014) are currently under development as anti-tussives.

Paper V found ALK1 to be specifically upregulated in the tonsils of smokers. The effects of ALK1 inhibitors for the treatment of solid tumor cancer are currently under intensive study (Cunha *et al.*, 2011). Our results suggest that ALK1 inhibitors could be effective for the treatment of smokers with tonsillar cancer, a group of patients characterized by poor disease prognosis and low response to current therapy.

In conclusion, the results in the present thesis may contribute to the development of specific and personalized treatment strategies for patients exposed to cigarette smoke or nicotine.

7 POPULAR SCIENCE SUMMARY IN SWEDISH

Det är välkänt att tobaksrök kan orsaka och förvärra sjukdomar i luftvägar. Tobaksrök ger upphov till ökad inflammation och ökad retbarhet i luftrören, så kallad luftvägshyper-reaktivitet. Rökare svarar sämre på dagens behandlingar mot luftvägssjukdomar och cancer och därför är behoven av effektivare och individanpassade behandlingsstrategier stora.

Tobakrökens negativa inverkan på luftvägarna har inte alltid varit självklart. Astmacigaretter innehållande en blandning av olika örter och i många fall också tobak har används under minst hälften av 1900-talet för att förebygga och behandla astmaattacker. Detta vittnar om den komplexa syn som funnits på tobaksrökens effekter på luftvägarna. De exakta mekanismerna bakom tobaksrökens verkan på luftvägshyperreaktivitet och inflammation är fortfarande ofullständigt kartlagda.

Tobaksrök innehåller tusentals olika kemiska ämnen. Avhandlingen har valt att fokusera på två av de viktigaste komponenterna, nikotin och endotoxin. Den senare finns också i cellväggar hos bakterier och aktiverar det medfödda immunförsvaret. Effekter på lung-funktionen och på isolerade luftrör av dessa två ämnen enskilt eller tillsammans har studerats på möss. Effekterna av tobaksrök studerades också i tonsiller från rökare och matchade icke-rökare. Målet har varit att identifiera nyckelmolekyler i de signalvägar som är involverade i luftvägssjukdomar associerade med tobaksrök. Genom att selektivt hämma dessa signal-molekyler hoppas vi att nya effektivare behandlingsalternativ skall kunna utvecklas.

Delarbete 1-IV visar att korttidsexponering för nikotin minskar luftvägarnas förmåga att relaxera medan långtidsexponering ökar de glatta muskelcellernas sammandragningsförmåga. Långtidsexponering ökar också uttrycket av toll-lika receptorer på luftvägarna. Dessa receptorer är en del av vårt medfödda immunförsvar. Ökat toll-lika receptoruttryck ökar den glatta muskulaturens känslighet för långtidsendotoxinstimulering men minskar känsligheten för korttidsendotoxinstimulering på nerver. Möss som har exponerats för nikotin under 28 dagar minskar den endotoxin-inducerade luftvägshyperreaktiviteten. Detta antyder att nerverna kan spela en viktig roll i uppkomsten av luftvägshyperreaktivitet.

Delarbete V visar att tonsiller från rökare uttrycker höga nivå av interleukin 8 samt activin receptor-like kinase (ALK) 1 och 2. Nivåerna korrelerar med mängden lokal rökexponering. Interleukin 8 och ALK1 är involverade i såväl uppkomst som spridning av cancer och kan bidra till den negativa prognos som rökare med tonsillcancer har.

Sammanfattningsvis, bidrar både nikotin och endotoxin till utvecklingen av luftvägs-hyperreaktivitet. Nikotin modifierar dessutom det medfödda immunsystemet och påverkar på så sätt känsligheten för endotoxin. Tobaksrök förändrar ALK uttryck i tonsiller och tycks påverka cancerprognosen. Dessa resultat kan bidra till utvecklingen av individanpassade strategier för behandling av patienter som exponerats för tobaksrök eller ren nikotin, såväl aktivt som passivt.

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