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Nicotine impairs cyclooxygenase-2-dependent kinin-receptor-mediated murine airway relaxations

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ABSTRACT

Introduction: Cigarette smoke induces local inflammation and airway hyperreactivity. In asthmatics, it worsens the symptoms and increases the risk for exacerbation. The present study investigates the effects of nicotine on airway relaxations in isolated murine tracheal segments.

Methods: Segments were cultured for 24 h in the presence of vehicle, nicotine (10 μM) and/or dexamethasone (1 μM). Airway relaxations were assessed in myographs after pre-contraction with carbachol (1 μM). Kinin receptors, cyclooxygenase (COX) and inflammatory mediator expressions were assessed by real-time PCR and confocal-microscopy-based immunohistochemistry.

Results: The organ culture procedure markedly increased bradykinin- (selective B_2 receptor agonist) and des-Arg^9-bradykinin- (selective B_1 receptor agonist) induced relaxations, and slightly increased relaxation induced by isoprenaline, but not that induced by PGE_2. The kinin receptor mediated relaxations were epithelium-, COX-2- and EP2-receptor-dependent and accompanied by drastically enhanced mRNA levels of kinin receptors, as well as inflammatory mediators MCP-1 and iNOS. Increase in COX-2 and mPGES-1 was verified both at mRNA and protein levels.

Nicotine selectively suppressed the organ-culture-enhanced relaxations induced by des-Arg^9-bradykinin and bradykinin, at the same time reducing mPGES-1 mRNA and protein expressions. α7-nicotinic acetylcholine receptor inhibitors α-bungarotoxin and MG624 both blocked the nicotine effects on kinin B_2 receptors, but not those on B_1. Dexamethasone completely abolished kinin-induced relaxations.

Conclusion: It is tempting to conclude that a local inflammatory process *per se* could have a bronchoprotective component by increasing COX-2 mediated airway relaxations and that nicotine could impede this safety mechanism. Dexamethasone further reduced airway inflammation together with relaxations. This might contribute to the steroid resistance seen in some patients with asthma.

Introduction

Active smokers, particularly females, are at greater risk of developing asthma than non-smokers and parental smoking strongly contributes to the development of asthma in children (Polosa and Thomson, 2013). It is also clear that smoke-addicted asthmatics exhibit a generally more severe disease and respond less well to local corticosteroid treatment than non-smokers (Comhair et al., 2011; Stapleton et al., 2011). In spite of this, smoking is still widespread among asthmatics (Vozoris and Stanbrook, 2011). One reason for this might be the highly addictive nature of nicotine. Besides cardiovascular and neural effects, nicotine is also known to affect the immune system and induce both pro-inflammatory (Vassallo et al., 2008; Wang et al., 2012) and anti-inflammatory effects (Wang et al., 2003). Acute nicotine exposure is believed to cause an initial mild airway relaxation (Streck et al., 2010), while long term exposure has been shown to increase airway hyperreactivity (AHR) in *in vivo* animal models (Sandberg et al., 2011; Sekhon et al., 2001). The effect of exposure to different types of cigarette smoke extract has been studied extensively *in vitro* (Andersson et al., 2004; Krimmer and Oliver, 2011; C.B. Xu et al., 2010), while studies on pure nicotine are less common. We have previously reported that long-term (4-day) exposure to nicotine *in vitro* contributes to the development of AHR by enhancing murine airway contractile responses to kinin receptor agonists *via* transcriptional upregulation of kinin B_1 and B_2 receptors. We have also found that dexamethasone suppresses transcription of kinin receptors and subsequently down-regulates kinin receptor-mediated airway contractions (Y. Xu et al., 2010).

Inhaled bradykinin causes a potent bronchoconstriction in asthmatic patients, but it has no effect even in high concentration in healthy individuals (Barnes, 1992). Thus, bradykinin has occasionally even been suggested as a marker, more sensitive than methacholine,

Abbreviations: AHR, airway hyperreactivity; COX, cyclooxygenase; mPGES-1, microsomal prostaglandin E synthase 1; iNOS, inducible NO synthase; MCP-1, monocyte chemotactic protein-1.

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for demonstrating AHR (Berman et al., 1995; Suguikawa et al., 2009). In guinea pig, chronic exposure to tobacco smoke increases the airway reactivity to bradykinin without altering the response to methacholine and histamine (Bergren, 2001), suggesting a special role for kinins in smoke-induced AHR. In murine models, the kinin receptors mediate both bronchoconstriction and epithelium-dependent airway relaxations, dependent on the localization of the receptor and the pre-contractile state. Relaxations have been shown to be caused *via* release of the bronchodilator prostaglandin (PG) E_2 (Li et al., 1998), a product of cyclooxygenase (COX).

We have previously, using an *in vitro* organ culture model of isolated murine tracheal segments, shown that nicotine enhances kinin receptor mediated contractions (Y. Xu et al., 2010), but the knowledge about the role of nicotine on kinin-receptor mediated COX-dependent airway relaxation is limited. Thus, the present study investigates the latter using a similar model.

Materials and methods

Tissue preparation and organ culture

10-week old male BALB/c mice were euthanized by cervical dislocation. Their trachea were harvested and dissected free of adhering tissue in Dulbecco's modified Eagle's medium (DMEM) with low glucose. Isolated trachea were cut into ring segments containing 2 cartilages for *in vitro* pharmacology or kept intact for real-time PCR studies. Organ culture was performed as previously described (Y. Xu et al., 2010) for 24 h in the presence of nicotine (10 μM), vehicle (0.1% DMSO) and/or dexamethasone (1 μM), with/without inhibitors. Segments from different mice were randomly assigned to the different groups to control for regional variation of the trachea. For the epithelium removal studies, the epithelia from the trachea segments were mechanically scraped away gently prior to organ culture. Selective α7 nicotinic receptor inhibitors MG624 (Gotti et al., 1998) and α-bungarotoxin (Zhang et al., 1994) were added in organ culture for evaluation of the role of the α7 subunit in mediating nicotine's effects. The experimental protocol was approved by the Stockholm north animals' ethics review board (Diary nr N153/11).

In-vitro pharmacology

Isometric tension of the isolated trachea rings was measured and recorded as previously described (Bachar et al., 2005). In short, rings were mounted on two L-shaped prongs and immersed in 5 ml Krebs–Henseleit buffer solution. A basal tension of 0.8 mN, which was determined by previous experiments to be the optimal tension load (Adner et al., 2002), was gradually reached over the course of at least 90 min. Rings were pre-constricted with 1 μM carbachol (submaximal concentration producing a contraction corresponding to 70–80% of maximal carbachol contraction) until a stable plateau was reached. Concentration-effect curves of agonists were performed by cumulative administration.

For the COX-inhibition experiments, the selective COX-1 inhibitor piroxicam (5 nM) (Pairet et al., 1998), the selective COX-2 inhibitor DuP-697 (5 nM) (Seibert et al., 1996) or the non-selective COX-1 and COX-2 inhibitor indomethacin (2 μM) was added to the organ bath 30 min prior to pre-contraction. For EP2 receptor inhibition, a novel selective EP2 inhibitor PF04418948 (10 nM) (af Forselles et al., 2011) was added 30 min prior to pre-contraction.

Real-time quantitative PCR

Total RNA extraction, cDNA reverse transcription and real-time PCR thermal cycles were performed according to standard protocols (Y. Xu et al., 2010).

Specific primers for murine kinin B_1 and B_2 receptors, TNF-α, COX-1, iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Prime Express 2.0 software (Applied Biosystem, Forster City, CA, USA) and synthesized by DNA Technology A/S (Aarhus, Denmark). Their sequences are shown in Table 1. Primers for β-actin, Hprt1, MCP-1, mPGES-1 and COX-2 were purchased from SABiosciences.

Three different house-keeping genes, GAPDH, β-actin and Hprt1, were simultaneously evaluated. The relative amount of mRNA was expressed as the C_T values of target gene mRNA in relation to the C_T values for the most stable house-keeping gene, in the current case Hprt1, in the same sample.

Immunohistochemistry

After organ culture, the tracheal segments were immersed in a fixative solution consisting of phosphate-buffered formaldehyde for 3 h at 4 °C. The tissues then undergo dehydration in series of ethanol from 50% to 99.5%, followed by UltraClear and embedded in paraffin blocks. Sections are cut into 4 μm-thick slices using a microtome and mounted on SuperFrost Plus slides (Menzel GMBH & COKG, Braunschweig, Germany).

Immunohistochemistry was carried out using standard protocols after antigen retrieval in citrate buffer, protein block with 10% donkey serum and 1% bovine serum albumin (BSA) and membrane permeabilization with 1% Triton-X in phosphate-buffered saline (PBS). The sections were then incubated with the primary antibody overnight at 4 °C and the secondary antibody for 1 h at room temperature in darkness. Primary and secondary antibodies as well as the dilutions used were as following: mPGES-1 (1:60, rabbit, Abcam, Cambridge, United Kingdom), COX-1 (1:200, mouse, Abcam) and COX-2 (1:300, rabbit, Abcam). The appropriate secondary antibodies, donkey anti-rabbit IgG H&L conjugated to AlexaFluor® 647 (1:500, Abcam) or donkey anti-mouse IgG H&L conjugated to Alexa Fluor® 488 (1:500, Abcam) were used respectively. In the control experiments, either the primary antibody or the secondary antibody was omitted. The stained specimens were examined under a confocal microscope (Leica TCS SP2). The fluorescence intensity was measured and analyzed by ImageJ software http://rsb.info.nih.gov/ij.

To avoid systemic errors, specimen was cultured, fixated, stained, examined and scanned at the same time as the same batch, and the setting of the confocal microscope is kept unchanged throughout. This ensures comparability between the groups. The measurements are repeated for each specimen at 2 sections and the florescence density was measured at 5 randomly selected areas for both smooth muscle and epithelium, and the mean values were obtained.

Table 1
Sequences of specific PCR primers.

Primer		Sequences
TNF-α	Fwd	5′-GAC TCA AAT GGG CTT TCC GA-3′
[NM_013693]	Rev	5′-TCC AGC CTC ATT CTG AGA CAG AG-3′
COX-1	Fwd	5′-CAC TGG TGG ATG CCT TCT CT-3′
[NM_008969]	Rev	5′-TCT CGG GAC TCC TTG ATG AC-3′
iNOS	Fwd	5′-GGA ATG GAG ACT GTC CCA GCA-3′
[NM_010927]	Rev	5′-GTC ATG AGC AAA GGC GCA GA-3′
Kinin B_1 receptor	Fwd	5′-CCA TAG CAG AAA TCT ACC TGG CTA AC-3′
[NM_007539]	Rev	5′-GCC AGT TGA AAC GGT TCC-3′
Kinin B_2 receptor	Fwd	5′-ATG TTC AAC GTC ACC ACA CAA GTC-3′
[NM_009747]	Rev	5′-TGG ATG GCA TTG AGC CAA C-3′
GAPDH	Fwd	5′-CAT GGC CTT CCG TGT TCC TA-3′
[NM_008084]	Rev	5′-TGC TTC ACC ACC TTC TTG ATG-3′

Note: β-actin, Hprt1, COX-2, mPGES-1 and MCP-1 primers were purchased from SABioscience and their sequences are therefore not available in this table.