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Toll-like receptor 7 activation reduces the contractile response of airway smooth muscle

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ABSTRACT

Viral respiratory infections are a major cause of asthma exacerbations. The mechanisms by which such infections aggravate airway inflammation and hyperresponsiveness are complex and not fully understood. Toll-like receptor 7 is particularly relevant in the defence against common respiratory viruses, as it recognizes single-stranded viral RNA. The present study was designed to investigate the effect of Toll-like receptor 7 stimulation on airway smooth muscle reactivity. The presence of Toll-like receptor 7 within guinea pig airways was ensured with immunohistochemistry. The effects induced by 3 days of culture of tracheal segments with the Toll-like receptor 7 agonist R837 or the Toll-like receptor 7/8 agonist R848 were evaluated in a myograph organ bath system. The intracellular mechanisms involved were dissected using inhibitors of intracellular mitogen-activated protein kinase (MAPK) activity. Toll-like receptor 7 immunoreactivity was observed across the epithelial cell layer and in the airway smooth muscle cells. Treatment with R837 and R848 reduced the airway contractile responses to 5-hydroxytryptamine (5-HT). This effect was abolished upon treatment with inhibitors of the p38 MAPK pathway and nuclear factor (NF)-κB pathways. According to the present model, activation of Toll-like receptor 7 might prevent development of airway hyperresponsiveness by acting on the airway smooth muscle. The presented data support the idea that individuals with defect Toll-like receptor 7 function might be more prone to respond to virus infections with asthmatic exacerbations. Further, they suggest that inhaled Toll-like receptor 7 ligand might be an effective treatment alternative for asthma.

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1. Introduction

Asthma is a common disorder of the respiratory tract characterized by inflammation, hyperresponsiveness and remodelling (Ward et al., 2002). Airway hyperresponsiveness is defined as an exaggerated airway narrowing in response to bronchoconstrictor agents (Okazawa et al., 1996). It is believed to be connected to ongoing airway inflammation, and there is a strong link to viral infections (Shelhamer et al., 1995; Tauro et al., 2008). The mechanisms behind this relation remain unclear.

Toll-like receptors are a set of intracellular and extracellular receptors which recognize microbial patterns. So far, ten different Toll-like receptors, Toll-like receptors 1–10, have been identified in humans. These receptors are crucial in the defence against pathogens (Medzhitov, 2001). The Toll-like receptors that primarily recognize viral products are Toll-like receptors 3, -7, -8 and -9. Of these Toll-like receptor 7 is of special interest since synthetic Toll-like receptor 7/Toll-like receptor 8 ligands seem to be effective in preventing the development of the asthmatic phenotype in *in vivo* models of asthma (Quarcoo et al., 2004; Sel et al., 2007). These ligands have also been demonstrated to inhibit the inflammatory reaction in response to allergens when the asthmatic phenotype has been established (Moisan et al., 2006; Sel et al., 2007). Further, Toll-like receptor 7/8 ligands appear to inhibit the increase in airway smooth muscle mass and the development of goblet cell hyperplasia, which are known to be associated with chronic asthma (Camateros et al., 2007).

We have previously developed a murine model of isolated airways and used it to demonstrate that long-term exposure to inflammatory mediators may alter the contractile responses to 5-hydroxytryptamine (5-HT) and other contractile mediators (Adner et al., 2002; Bryborn et al., 2004; Zhang et al., 2004, 2007). In the airways, 5-HT is released by pulmonary neuroendocrine cells, platelets and, in some species, mast cells (Buckner et al., 1991; Cazzola and Matera, 2000). While the involvement of 5-HT in the development of airway hyperresponsiveness is debated, an anti-asthmatic effect of the 5-HT inhibitor ketanserin has been described in asthmatic patients (Cazzola et al., 1990) and increased levels of free 5-HT have been correlated to asthma severity (Lechin et al., 1996). Furthermore, the plasma concentration of free 5-HT increases during asthma exacerbations (Lechin et al., 1996), all justifying our use of 5-HT for evaluation of airway hyperreactivity. Increasing evidence suggests that deficiencies in the TLR system may be a cause of disease or aggravate an ongoing

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disease process (Abdollahi-Roodsaz et al., 2008; Zhang et al., 2006). If toll-like receptor 7 activation induces a protective effect against virus infection, a deficient Toll-like receptor 7 response or lack of proper activation in response to viruses could be an underlying cause of exacerbation of airway disease.

The intention of the present study was to use our *in vitro* model to investigate if Toll-like receptor 7 might have any direct effects on 5-HT-induced smooth muscle contractions.

2. Materials and methods

2.1. Materials

2-[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid (Indomethacin), Krebs–Henseleit buffer and 5-HT were purchased from Sigma (St. Louis, MO, USA). 1-(2-Methylpropyl)imidazo [4,5-c]quinolin-4-amine (R837/Imiquimod) and 1-[4-amino-2-(ethoxymethyl)imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol (R848/Resiquimod) were obtained from Invivogen (San Diego, CA, USA). 4-[4-(4-Fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4yl]pyridine (SB203580), 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) and Anthra(1,9-cd)pyrazol-6 (2H)-1 (SP600125) were purchased from Tocris Cookson (Avonmouth, UK). *N*-[(Phenylmethoxy)carbonyl]-L-leucyl-*N*-[(1*S*)-1-formyl-3-methylbutyl]-L-leucinamide (MG-132) was purchased from Alexis Biochemicals (Lausen, Switzerland). Dulbecco's modified eagle's medium (DMEM)/F12 and penicillin–streptomycin were obtained from Gibco (Carlsbad, CA, USA). The Toll-like receptor 7 antibody came from Acris (Herford, Germany). The study was approved by the local ethical committee responsible for Karolinska Institutet, and all animals were kept according to regulations.

2.2. Immunohistochemistry

For immunohistochemistry, freshly obtained guinea pig tracheas were fixed in 4% buffered formaldehyde, embedded in paraffin, and cut in sections. The sections were deparaffinized by immersion in xylene, and were rehydrated in graded ethanol and deionized water. The antigens were retrieved by heating the slides with target retrieval solution (Dako), and the cells in the tissue sections were subsequently permeabilized using Triton-X (1% in tris-buffered saline) for 20 min. Antibody staining was carried out in a moisture chamber using EnVision™ Doublestain system kit (Dako) for mouse or rabbit 3,3′-Diaminobenzidine (DAB)-staining. Briefly, the slides were incubated with a peroxidase blocker, followed by washing and the addition of primary antibody. The primary antibody used was rabbit polyclonal anti-Toll-like receptor 7. Antibody vehicle (Dako) was used to rule out background staining. The slides were exposed to the primary antibody for 2 h. Detection of binding was carried out through the addition of a marked polymer, followed by washing and the addition of substrate-chromogen. The slides were rehydrated in 95% ethanol, pure ethanol, and xylene. Lastly, the slides were mounted with Pertex (Histolab, Gothenburg, Sweden) and were allowed to dry over night before analysis.

2.3. Organ culture

Female Dunkin Hartley guinea pigs (200 g; Charles River, Germany) were euthanized through asphyxiation with CO_2. The tracheas were removed and immediately placed in ice-cold Krebs Henseleit buffer (143 mM Na^+, 5.9 mM K^+, 1.5 mM Ca^{2+}, 2.5 mM Mg^{2+}, 128 mM Cl^-, 1.2 mM $H_2PO_4^{2-}$, 1.2 mM SO_4^{2-}, 25 mM HCO_3^-, and 10 mM D-glucose). They were subsequently dissected free from adherent tissue, and were divided in segments 1–2 cartilage rings large, and rinsed. In a subset of experiments, the airway epithelium was removed from the trachea by perfusion with 0.1% Triton X-100, before dividing the trachea into segments (Zhang et al., 2007). The segments were then cultured with R837 (0.05 μg/ml, 0.5 μg/ml or 5 μg/ml), with R848 (0.05 μg/ml, 0.5 μg/ml or 5 μg/ml), or as untreated controls for 3 days in 1 ml DMEM/F-12 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin in 48-well plates. To ensure that 3 days was the most suitable length of culture, segments were also cultured with R837 (5 μg/ml) for 0, 1, and 2 days.

The tracheas were kept at 37 °C in a humidified 5% CO_2 air atmosphere, and were daily transferred to new wells with fresh medium and ligands. In a subset of experiments, segments were cultured in the presence of signal pathway inhibitors of p38 mitogen-activated protein kinase (MAPK) (SB203580; 10 μM), extracellular signal-regulated kinase (ERK)1/2 (PD98059; 10 μM), c-Jun N-terminal kinase (JNK) (SP600125; 10 μM), with proteasome inhibitor MG-132 (10 μM) which subsequently inhibits nuclear factor (NF)-κB, or with vehicle DMSO, along with R837 (5 μg/ml).

2.4. In vitro pharmacology

Tracheal segments were mounted on L-shaped prongs in a temperature-controlled (37 °C) myograph system (myograph 700MO; J.P. Trading, Aarhus, Denmark). One prong was connected to a force-displacement transducer for continuous recording of isometric tension by the Chart software (AD Instruments Ltd., Hasting, UK). The other prong was connected to a displacement device, allowing for adjustment of the distance between the two parallel prongs. During the course of one hour, the resting tension was gradually increased to 1.5 mN. The segments were continuously kept in Krebs–Henseleit buffer equilibrated with 5% CO_2 and 95% O_2. Indomethacin (3 μM) was present in the wells during the course of the experiment. Each segment was tested for viability with 60 mM KCl. Concentration–response curves were obtained for 5-HT. The method of studying airway contraction with a myograph system has been previously described and evaluated by Adner et al. (2002).

2.5. Data analysis

Data were compiled with the Chart software, and regression analysis was performed for concentration effect curves on each segment (GraphPad, San Diego, CA, USA). The values of maximal contraction (E_{max}) and pEC_{50} (negative logarithm of the concentration that induces half of the maximal effect) were compared using ANOVA with Dunnett's post-test or Student's t-test. A P-value less than 0.05 was considered significant. All data are presented as mean ± S.E.M., and n equals the number of segments in each data set.

3. Results

The presence of Toll-like receptor 7 in the guinea pig trachea was assessed using immunohistochemistry. Toll-like receptor 7 staining was observed across the epithelial cell layer and in the airway smooth muscle cells (Fig. 1). No immunoreactivity was observed when primary antibody was omitted.

For functional studies of Toll-like receptor 7, tracheal segments were treated for 3 days with R837 or R848 in various concentrations. All segments displayed a marked response to 60 mM K^+. Treatment with R837 did not affect the response to K^+ (60 mM), regardless of ligand concentration (R837 0.05 μg/ml: 4.5 ± 0.7 mN; R837 0.5 μg/ml: 4.5 ± 1.1 mN; 5 μg/ml: 5.2 ± 0.9 mN; untreated: 5.0 ± 0.5 mN; n = 8; Fig. 2A). Nor did R848 affect the contraction to K^+ (R848 0.05 μg/ml: 4.9 ± 1.0 mN; R848 0.5 μg/ml: 4.6 ± 0.8 mN; R848: 5 μg/ml: 4.6 ± 0.9 mN; untreated: 4.8 ± 0.8 mN; n = 8–9; Fig. 2B).

During normal conditions, 5-HT is a potent bronchoconstrictor in several species (Chung and Barnes, 1992; Watts et al., 1994). In the present system, both fresh and cultured guinea pig tracheal segments contracted strongly when 5-HT was added. Treatment for 3 days with

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