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Modulation of antigen-induced responses by serotonin and prostaglandin E_2 via EP_1 and EP_4 receptors in the peripheral rat lung

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Abstract

The cyclooxygenase (COX) pathway and prostanoids may critically contribute to the early allergic airway response. In the rat lung, serotonin (5-HT) is a major mediator of antigen-induced contractions. The aim of this study was therefore to examine the relative role of the COX pathway and serotonin for antigen-induced contractions in the rat lung. Airway responses were studied in rat precision cut lung slices (PCLS). Lung slices were stimulated with ovalbumin or serotonin after pretreatment with COX inhibitors or specific TP or EP receptor antagonists. Changes in airway size (contractions/relaxations) were measured by a digital video camera. The supernatants were analysed for changes in prostaglandin and serotonin release. Airway contractions to ovalbumin were attenuated by the unselective COX inhibitor indomethacin, the selective COX-1 inhibitor FR-122047 and COX-2 inhibitor celecoxib. The EP_1 receptor antagonist ONO-8713 reduced the contractions, whereas the EP_4 receptor antagonist L-161,982 significantly increased the contractile response to ovalbumin. The 5-HT_{2A} receptor antagonist ketanserin completely inhibited the ovalbumin-induced contractions. The different COX inhibitors decreased the production of prostaglandins but did not affect the synthesis of serotonin. The serotonin-induced bronchoconstriction was attenuated by celecoxib and ONO-8713, but not by methacholine. Taken together, our data indicate that PGE_2 is the main prostanoid involved in the early allergic airway response in the rat lung. PGE_2 appears to act both as a primary mediator of antigen-induced airway contraction via the EP_4 receptor and as a downstream modulator of serotonin-induced bronchoconstriction via the EP_1 receptor. **Keywords:** Airway smooth muscle, contraction, ovalbumin, precision-cut lung slices, prostaglandins, serotonin

1. Introduction

Airway obstructions play an important role in the development of symptoms associated with remodelling processes and loss of lung function in asthma (Janson, 2010). Standard therapy with anti-inflammatory corticosteroids and bronchodilators does not fully prevent airway obstructions and bronchoconstriction in severe asthma (Holgate and Polosa, 2006), requiring new therapeutic approaches to treat this disease state. Prostanoids, such as thromboxane A_2 (TXA_2), prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2), are involved in various physiological and pathophysiological processes in the lung and play a critical role in asthma (Rolin et al., 2006). Prostanoids are generated from arachidonic acid and converted to PG via cyclooxygenase (COX) (Vane, 1971). The COX enzyme exists in two isoforms; COX-1 (Picot et al., 1994) is constitutively expressed and is involved in regulation of physiological responses and homeostasis, COX-2 (Kurumbail et al., 1996) is mostly inducible and related to inflammation (FitzGerald, 2003). The prostanoids contribute to the asthmatic airway responses in different ways. TXA_2 is involved in allergen-induced asthmatic responses by activation of TP receptors (Manning et al., 1991) and thereby induction of both airway and vascular smooth muscle constrictions (Larsson el al., 2011). TXA_2 may also cause airway hyperresponsiveness (Held and Uhlig, 2000) and contributes to cytokine-induced bronchoconstriction (Martin et al., 2001). PGD_2 is a pro-inflammatory mediator of allergic asthma (Matsuoka et al., 2000), a marker of mast cell activation (Dahlen and Kumlin, 2004) and induces airway and vascular smooth muscle contractions via the TP receptor (Armour et al., 1989; Beasley et al., 1989; Johnston et al., 1995; Larsson et al., 2011; McKenniff et al., 1991). PGE_2 is implicated to have a beneficial role in the lung (Pavord and Tattersfield, 1995;

Vancheri et al., 2004), since this prostanoid may maintain airway tone (Tilley et al., 2003) and attenuate allergic airway responses (Hartney et al., 2006; Martin et al., 2002). However, owing to the existence of various EP receptors the potential actions of PGE_2 are diverse (Coleman et al., 1994). Recent findings indicate that PGE_2 has its bronchodilatory effect mainly via the EP_4 receptor in man (Benyahia et al., 2012; Buckley et al., 2011). Prostanoids are implicated in the early allergic airway response in different species; rat (Dahlback et al., 1984), guinea pig (Larsson et al, 2005, 2011) and man (Benyahia et al., 2012; Buckley et al., 2011; Ressmeyer et al., 2006). However, in the rat lung, serotonin (5-hydroxytryptamine, 5-HT) is the major mediator released from mast cells granules during an allergen response. The release of serotonin induces potent contractions via the $5\text{-}HT_{2A}$ receptor (Dahlback et al., 1984; Wohlsen et al., 2001). Inhibition of the COX pathway enhanced the early allergic response in guinea pig lung (Larsson et al., 2005) and induced airway hyperresponsiveness in murine lung (Peebles et al., 2002). It is unknown whether this modulation is a general mechanism of the early allergic response. The purpose of this study was to examine the contribution of the COX pathway and prostanoids to the early allergic airway response in relation to the effects of serotonin in the rat lung.

2. Methods

2.1. Animals

Precision-cut lung slices (PCLS) were prepared from 8-week-old Wistar rats (220 ± 20 g) obtained from Charles River (Sulzfeld, Germany) and kept under controlled conditions (22°C, 55% humidity and 12-h day/night rhythm). Animal experiments were approved by the local ethics committee.

2.2. Precision-cut lung slices

Rat PCLS were prepared as previously described (Wohlsen et al., 2001). Rats were sacrificed by an overdose of pentobarbital i.p. (60 mg/kg). The trachea was prepared and fixed with a tracheal cannula. The lungs were filled with pre-warmed agarose solution (0.75%) via the trachea and subsequently chilled with ice. The heart-lung package was removed and put on ice to allow the agarose to further cool and solidify. The lung lobes were separated and cut into 5 to 10 mm thick tissue segments from which cores were made along the airways with a coring tool. The cores were cut into 250 ± 20 µm thick slices with a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL). Slices were incubated in minimal essential medium, which was changed every half hour for the first two hours and then every hour for the next two hours to remove the agarose and inflammatory mediators from the airways. For the experiments, slices with airways that had intact surrounding epithelium were moved to 24-well plates and covered with 1 ml of medium. The airways were imaged and digitized using a digital video camera. For measurements, slices with comparable airway size (1.36 ± 0.28 mm^2) were selected, covered with 1 ml of medium and fixed with a nylon thread attached to a

116 platinum wire to avoid movements and allow relaxation of the slice (Schleputz et al.,
117 2011). Images were recorded by an analogue (JAI 2040; JAI Pulnix, Alzenau, Germany)
118 or a digital camera (IRB640; Visitron Systems, Munich, Germany) controlled by the
119 software program Optimas 6.5 (Optimas, Bothell, WA). A control picture was taken
120 before addition of any agonists or antagonists and frames were recorded every 30 sec. A
121 time interval of 5 minutes for cumulatively given doses and 20 minutes for single doses
122 were used. The images were analyzed by the image analysis program Optimas 6.5
123 (Optimas, Bothell, WA).
124
125 *2.3. Sensitization for antigen studies*
126 For antigen studies with ovalbumin, the lung slices were incubated over night with cell
127 culture medium containing 1% serum from actively sensitized rats, as previously done
128 (Wohlsen et al., 2001). The medium was not changed until the following day. All other
129 lung slices were maintained in standard cell culture medium. Control studies were
130 performed to verify that 1% serum (of sensitized rats) did not interfere with responses
131 induced by other agonists and that ovalbumin did not show any effect in non-sensitised
132 slices.
133
134 *2.4. Study design*
135 Airway contractions to ovalbumin, PGD_2, PGE_2, the thromboxane receptor analogue
136 u46619, serotonin and methacholine were studied in rat PCLS. Effects of selective COX
137 inhibitors and selective EP_1, EP_2, EP_3, EP_4 and TP receptor antagonists on airway tone
138 were assessed. The release of serotonin and prostanoids after ovalbumin-stimulation were

analysed in the supernatant. A single concentration of ovalbumin (10 µg/ml) was used for antigen-induced contractions. This concentration was selected from a cumulative concentration-response curve (0.01-1000 µg/ml of ovalbumin) (Fig 1A). The single dose (10 µg/ml) of ovalbumin produced a strong, stable and reproducible bronchoconstriction with the same maximum airway contraction as generated by cumulative challenge of ovalbumin (Fig 1B).

2.5. Measurements of released mediators

Supernatant (0.5 ml) of six incubated PCLS (weight 0.03 g/slice) was collected and immediately frozen at -80°C. The samples were taken at three different time points. First from unchallenged slices to obtain initial mediator release, thereafter, 20 minutes from pre-treatment with the different drugs and finally 20 minutes after the slices were challenged with 10 µg/ml ovalbumin. Enzyme immunoassays of TXA_2, PGD_2, PGE_2 and serotonin were performed according to the manufacturer's instructions. TXA_2 was measured as the stable metabolite TXB_2 and PGD_2 as PGD_2-mox. The assay detection limits for the different mediators were 7.8 pg/ml for TXB_2, PGD_2, PGE_2 and 50 pg/ml for serotonin. The enzyme immunoassay specificity for the different mediators to interfere with each other was less than 0.01% for PGE_2, PGD_2-mox and serotonin, whereas the antibody tracer for TXB_2 cross reacted with PGD_2 (0.53%) and with PGE_2 (0.09%).

2.6. RT-PCR analysis

PCLS were snap-frozen and pounded in liquid nitrogen. Total RNA was isolated from 30 mg lung powder with a NucleoSpin RNA II Kit (Machery Nagel GmbH & Co. KG,

Düren, Germany) automated on a QIAcube roboter (QIAGEN GmbH, Hilden, Germany). RNA was quantified in buffered 10 mM TRIS-HCl, pH 7.5, using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham (MA), USA). For reverse transcription 274 ng of total RNA was added to 1 µl of oligo$(dT)_{15}$ Primer (0.5 µg/µl) (Invitrogen, Karlsruhe, Germany) and water to a total volume of 12 µl. Samples were incubated for 10 minutes at 65°C to linearize the RNA. 4 µl buffer (5x), 2 µl dNTP (10 mM), 1 µl Rnasin (40U/µl) and 1 µl M-MLV RT (H^-) (200U/µl) (all substances from Promega GmbH, Mannheim, Germany) were added and RNA was reverse transcribed for 90 minutes at 40°C. This was followed by a 2 minutes heat-inactivation step at 95°C. 20 µl of water was added afterwards to a final volume of 40 µl per sample. All incubation steps were performed on a Biometra UNO II Thermocycler (Biometra GmbH, Göttingen, Germany). For real-time qPCR 1 µl of cDNA was incubated as template with 0.5 µl forward primer (6.25 µM) (Eurofins MWG GmbH, Ebersberg, Germany), 0.5 µl reverse primer (6.25 µM), 5 µL SYBR-Green I Mastermix (Roche-Diagnostics GmbH, Mannheim, Germany) and 3 µL water according to manufacturer's instructions in a LightCycler 480 (Roche-Diagnostics GmbH). Following primer pairs were used for 5-HT_{2A}-receptor (gene symbol: Htr2a, NCBI Reference Sequence: NM_017254.1): sense 5'-CCA CCA ACT ATT TCC TGA TGT C-3' antisense 5'-GCA CAT CCA GGT AAA TCC AG-3' and for Beta-2-microglobulin (gene symbol: B2m, NCBI Reference Sequence: NM_012512.2): sense 5'-CCG TGA TCT TTC TGG TGC TTG TCT-3' antisense 5'-ATC GGT CTC GGT GGG TGT GAA T-3'. Quantification after real-time qPCR was performed with Cp values, acquired via the Second Derivative Maximum method. Advanced relative quantification was performed with the LightCycler 480

Software 1.5 SP3 (Roche-Diagnostics GmbH) and efficiency-corrected by in-run standard curves using the Roche Applied Science E-Method (Tellmann, 2006). Data were referenced first to the correspondent housekeeping gene B2m and normalized to the mean of the experimental control. Real-time qPCR quality control was performed by in-run negative controls, Melting Curve profiles using the LightCycler 480 Software and product separation in agarose gels.

2.7. Drugs

Indomethacin, ovalbumin (chicken egg albumin, grade V), serotonin, ketanserin and dimethylsulfoxid (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). 4-[5-(4-methylphenyl)-3-(trifluoromethyl) pyrazol-1-yl]benzenesulfonamide (Celecoxib; Celebrex®) was obtained from Pfizer (CA). 1-[4,5-bis(4-methoxyphenyl)-2-thiazoyl)carbonyl]-4-methylpiperazine hydrochloride (FR-122047), 3R-[[(4-fluorophenyl)sulfonyl]amino]-1,2,3,4-tetrahydro-9H-carbazole-9-propanoic acid (BAYu3405, Ramatroban) was purchased from Bayer AG (Wuppertal, Germany). PGD_2, PGE_2, 9,11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$ (U46619), 9-oxo-6-propan-2-yloxyxanthene-2-carboxylic acid (AH6809) and N-[[4'-[[3-butyl-1,5-dihydro-5-oxo-1-[2(trifluoromethyl)-phenyl]-4H-1,2,4-triazol-4-yl]methyl][1,1'-biphenyl]-2-yl]sulfonyl]-3-methyl-2-thiophenecarboxamide (L-161,982) were bought from Cayman Chemical (Ann Arbor, MI, USA). (E)-3-[4-[[2-(furan-2-ylsulfonyl-(2-methylpropyl)amino)-5-(trifluoromethyl)phenoxy]methyl]phenyl]prop-2-enoic acid (ONO-8713) (Norel et al., 2004) was a generous gift from ONO Pharmaceutical CO. LTD (Osaka, Japan). (E)-N-(5-bromo-2-methoxyphenyl)sulfonyl-3-[2-(naphthalen-2-ylmethyl)phenyl]prop-2-enamide

(L-798,106) were bought from Tocris Bioscience, Bristol, UK. The EIA kits for TXB_2, PGD_2-mox and PGE_2 were obtained from Cayman Chemicals (Ann Arbor, MI). ELISA kit for serotonin was purchased from IBL-Hamburg (Hamburg, Germany). FR-122047, ONO-8713, BAYu3405 and celecoxib were dissolved in DMSO. The final concentration of DMSO or ethanol never exceeded 0.3% (v/v) and did not influence the induced contractions. The other drugs were dissolved and diluted in distilled water. Dilutions of drugs were freshly made from the stocks for each experiment. The drugs were present in the medium fluid during the remaining experiment. Pretreatments were given 20 minutes before the addition of ovalbumin, serotonin or methacholine.

2.8. Calculations and Statistics

Airway area before addition of any drug was defined as 100%. Airway contractions were expressed as the percentage decrease in airway area compared to the initial airway area. All data are presented as mean ± standard error of the mean (S.E.M.). In all experiments the numbers of n represent animals and not the number of slices. Time courses were analyzed by the area under the curve (AUC). Enzyme immune assay measurements and allergen-induced bronchoconstriction curves were analyzed by Student t-test (two curves or bars) or by analyses of variances (ANOVA, more than two curves or bars) followed by Bonferroni's post-hoc test. Concentration-response curves to methacholine and serotonin were analyzed by comparison of sigmoid curves with Prism 5 (Graphpad, San Diego, USA). A p-value of less than 0.05 was considered significant. The Statistic program JMP 5.1 (Cary, NC, USA) was used to calculate the power of the experiments.

3. Results

3.1. Effect of the COX pathway on ovalbumin-induced contractions

Ovalbumin (10 µg/ml) generated a stable and reproducible airway bronchoconstriction (Fig 1A). The antigen-induced contractions to ovalbumin (10 µg/ml) were significantly attenuated by the unselective COX inhibitor indomethacin (10 µM; P=0.012; Fig 2A), the selective COX-1 inhibitor FR-122047 (5 µM; P=0.016; Fig 2B) and the selective COX-2 inhibitor celecoxib (10 µM; P=0.044; Fig 2C). PGD_2, PGE_2 and TXA_2 analogue u46619 were tested on non-sensitized slices to evaluate the attenuated bronchoconstriction induced by the different COX-inhibitors. PGD_2 (10 µM; n=3) and PGE_2 (10 µM; n=3) did not induce any bronchoconstriction or dilatory effects in the rat PCLS *(Table 1)*, nor did the specific EP receptor agonists for EP_1, EP_2 and EP_4 *(data not shown)*, whereas the TP receptor analogue u46619 (50 µM; n=3) induced airway contractions that were completely blocked by the TP receptor antagonist BAYu3405 (10 µM; n=3; $P<0.05$; *Table 1*). In line, only the TP receptor agonist u46619 induced contractions in the lung slices. However, both EP and TP receptor activation has been shown to be involved in allergen-mediated airway contractions. PGE_2 may have diverse roles acting on both contractile EP receptors (mainly EP_1, but also EP_3) and relaxant EP receptors (EP_2 and EP_4) (Buckley et al., 2011). EP_1 receptor antagonist ONO 8713 (Norel at al., 1999), EP_2 receptor antagonist AH6809, EP_3 receptor antagonist L-798,106 and EP_4 receptor antagonist L-161,982 and the TP receptor antagonist BAYu3405 were therefore tested on ovalbumin-induced contractions to further characterise the contractile response after COX inhibition. The EP_1 receptor antagonist ONO-8713 (10 µM) significantly reduced the ovalbumin-induced contractions (P=0.004; Fig 3A), whereas the EP_4 receptor

antagonist L-161,982 (0.5 µM) significantly increased the ovalbumin-induced contractions (P=0.042; Fig 3D) whereas pre-treatment with EP_2 receptor antagonist AH6809 (5 µM) or EP_3 receptor antagonist L-798,106 (0.5 µM) or TP receptor antagonist BAYu3405 (10 µM), had no significant effect (Fig 3B, C, E). Since serotonin is known as a major mediator of this particular ovalbumin model, the inhibitory effect of the COX inhibitors and the EP receptor antagonists was compared with the effect of the 5-HT_{2A} receptor antagonist ketanserin. Ketanserin (0.1 µM; P=0.005) completely inhibited the contractile response to ovalbumin (Fig 3F).

3.2. Synthesis of serotonin and prostanoids after challenge with ovalbumin

The supernatant was analysed to verify if there were any changes in generation of serotonin and prostaglandins after ovalbumin stimulation and COX inhibition. The medium contained relatively high levels of PGE_2, TXB_2 and PGD_2 that were not changed after stimulation by ovalbumin. The COX inhibitors indomethacin (10 µM) and celecoxib (10 µM) significantly decreased the formation of PGE_2, TXB_2 and PGD_2, whereas the COX-1 inhibitor FR-122047 (5 µM) significantly reduced the synthesis of TXB_2 (Fig 4C) and PGD_2 (Fig 4D), but not PGE_2 (Fig 4B). The formation of serotonin was significantly increased after addition of ovalbumin 10 µg/ml (P=0.032). Notably, its synthesis or release was not affected by the different COX inhibitors (Fig 4A). In addition, pre-incubation with COX inhibitors or EP receptor antagonists did not change the initial airway size in PCLS, indicating no direct effect on airway tone.

3.3. Effect of the COX pathway on serotonin-induced contractions

Since serotonin was the major contractile mediator of the antigen-induced response, the effect of COX and prostanoids on serotonin-induced contractions was also evaluated to investigate potential downstream modulations. Indomethacin (10 µM) significantly attenuated the airway contraction induced by serotonin (0.01-10 µM; P=0.02; Fig 5A) and shifted the concentration-response to the right (pEC_{50}: 6.22 ± 0.01 *vs* control pEC_{50}: 6.40 ± 0.05; P=0.008). FR-122047 (5 µM) had no effect on serotonin-induced bronchoconstriction (ns; Fig 5B), whereas celecoxib (10 µM) decreased the response to serotonin (P=0.001; Fig 5C) and shifted the concentration-response curve to the right (pEC_{50}: 5.89 ± 0.08 *vs* control pEC_{50}: 6.12 ± 0.07; P=0.03). Pretreatment with the EP_1 receptor antagonist ONO-8713 (10 µM) attenuated the contractile response to cumulative doses of serotonin (P=0.01; Fig 5D) and shifted the concentration-response to the right (pEC_{50}: 5.66 ± 0.07 *vs* control pEC_{50}: 6.27 ± 0.11; P=0.003). The EP_2 receptor antagonist AH6809 (5 µM) also attenuated the contractile response to cumulative doses of serotonin and shifted the concentration-response to the right, and the bottom of the concentration-response curves was unequal (P=0.02; Fig 5E), showing differences in potency and efficacy. Also the EP_4 receptor antagonist L-161982 (0.5 µM) shifted the concentration-response curve to the right (pEC_{50}: 5.89 ± 0.07 *vs* control pEC_{50}: 6.15 ± 0.06; P=0.004; Fig 5G). Neither the EP_3 receptor antagonist L-798,106 (0.5 µM; Fig 5F) nor the TP receptor antagonist BAYu3405 (10 µM; Fig 5H) had any significant effects on the contractions induced by serotonin. Analysis of the 5-HT_{2A} receptor expression indicated that COX-inhibition with indomethacin or celecoxib enhanced the expression of the 5-HT_{2A} receptor after 4h (Fig 6).

3.4. Effect of COX inhibition and EP_1 receptor antagonism on methacholine-induced contractions

To determine if the effect of COX inhibition and EP_1 receptor antagonism was specific to serotonin rather than a general property of rat airways, methacholine-induced bronchoconstriction was evaluated in the presence and absence of the COX inhibitor indomethacin (10 μM) or the EP_1 receptor antagonist ONO-8713 (10 μM). The contractions to methacholine were not altered by either indomethacin or ONO-8713 (Fig 7).

4. Discussion

In this study, we present evidence that in the early allergic airway response in rat; especially PGE_2 may act both as a primary mediator of antigen-induced airway contraction via COX and the EP_1 and EP_4 receptors and as a downstream modulator of serotonin-induced bronchoconstriction via COX-2 and the EP_1 receptor after antigen challenge. Previous studies have indicated that prostanoids and serotonin are involved in the early allergic airway response in the rat (Dahlback et al., 1984; Hele et al., 2001; Nagase et al., 1996; Wohlsen et al., 2001). The relative contributions of these mediators, however, remained uncertain. In the rat PCLS, the antigen-induced contractions to ovalbumin were significantly attenuated by selective COX-1 and COX-2 inhibitors, suggesting a role for both isoenzymes in the peripheral rat lung during the early allergic airway response. Both COX-1 and COX-2 have been shown to be constitutively expressed in the normal rat lung (Ermert et al., 1998b) with high enzyme activity (Baber et al., 2003; Ermert et al., 1998a), suggesting a crucial role for COX isoenzymes in the regulation of pulmonary responses. The beneficial effect of COX inhibition during the antigen response in the rat PCLS was somewhat surprising and opposite to other studies, since in other models COX inhibition resulted in airway hyperresponsiveness and increased contractility (Larsson et al., 2005; Peebles et al., 2002; Watts and Cohen, 1993). On the other hand, leukotrienes, the major mediators in COX-sensitive asthma, play only a minor role in rat PCLS (Wohlsen et al., 2001), which may explain the influence on the AHR and contractility. In addition, passively sensitized PCLS represents a mast-cell dependent model to study mainly early allergic airway responses (Ressmeyer et al., 2006; Wohlsen et al., 2001).

To understand the attenuated antigen-induced bronchoconstriction after COX-inhibition, the effect of the prostanoids PGD_2, PGE_2 and thromboxane was investigated on rat airway tone, where only the TP receptor agonist u46619 induced some contractions. Prostanoid receptors show considerable versatility and may mediate bronchoconstriction via both TP and EP_1 receptors (Lydford and McKechnie, 1994) and bronchodilatory effects through DP_1, EP_2 and EP_4 receptors (Hartney et al., 2006; Norel et al., 2004; Tilley et al., 2003). Therefore, to further investigate the reduced contractile response after COX-inhibition, we focused on the prostanoid receptors $EP_{1\text{-}4}$ and the TP receptor. Interestingly, the EP_1 receptor antagonist ONO-8713 attenuated the antigen-induced airway contraction, whereas the EP_4 receptor antagonist L-161,982 potently increased the contractions to ovalbumin. This data implicate that PGE_2 may modulate the early allergic airway response in rat lungs in two ways, mainly *via* activation of relaxant EP_4 receptors but also in part *via* activation of contractile EP_1 receptors. Recent findings indicate that PGE_2 has its bronchodilatory effect mainly via the EP_4 receptor in man (Benyahia et al., 2012; Buckley et al., 2011). Notably, the beneficial relaxant effect of PGE_2 via EP_4 receptor in this study correlated with the results obtained in human. Focusing on the different EP receptors on mast cells, there is little information about the distribution on mast cells. Feng and colleagues (Feng et al., 2006) have characterized the EP receptors on human mast cells. Interestingly they only found expression of $EP_{1,\,2,\,3}$ receptor mRNA. From their view of EP receptor activity, increasing cAMP via EP_2 and EP_4, seems to be important, whereas the role of EP_3, which acts via increase of calcium, is only minor. Also in our study the EP_3 receptor does not appear to have a direct or indirect effect on mast cell activation, which is again in line with the findings of Feng et

355 al, who found different EP_3-receptor subtypes, which can either be coupled to the G-
356 protein Gs or Gi (Feng et al., 2006).

357 In addition, also the TP receptor antagonist partly affected the antigen-response,
358 indicating that TP receptors may contribute as well. Both TXA_2 and PGD_2 are known to
359 mediate airway contractions via the TP receptor (Larsson et al., 2011; McKenniff et al.,
360 1991), whereas PGD_2 may also cause bronchodilation via the DP_1 receptor (Larsson et
361 al., 2011; Norel et al., 1999). Despite the pharmacological evidence of prostanoid
362 involvement in the early allergic airway response, supernatant levels of TXB_2, PGD_2 or
363 PGE_2 were not increased after antigen challenge. COX inhibition significantly but not
364 completely reduced the generation of the prostanoids in the rat PCLS. The release of
365 serotonin was significantly increased after addition of ovalbumin and remained
366 unaffected by the different COX inhibitors. These data raised the hypothesis that
367 prostanoids may act as modulators of airway responsiveness. Since the 5-HT receptor
368 antagonist ketanserin completely blocked the antigen-induced contractions in the rat
369 PCLS, we hypothesised that serotonin is the main mediator of EAR and that the COX
370 metabolites may modulate the serotonin response. We observed that serotonin-induced
371 bronchoconstriction was attenuated by selective COX-2 inhibition and EP_1 antagonism,
372 whereas in contrast to the antigen-induced response, COX-1 inhibition had no effect. The
373 EP_2 and EP_4 receptor antagonist may also affect the serotonin-induced constriction. This
374 may result is probably depending on the fact that the EP_2 receptor antagonist has similar
375 affinity to the EP_1 receptor (Buckley et al., 2011). However, apart from the EP_3 receptor
376 antagonist, the other EP receptor antagonists shifted the concentration- response curve to
377 the right. This is in line with the dilatory response via EP_2 and EP_4 on smooth muscle

cells. For EP_1 antagonism, the strongest effect was found, when both EP_2 and EP_4 receptors were triggered by endogenous PGE_2 produced by the PCLS during challenge. In cases where either the EP_2 or EP_4 receptor was blocked this relaxation was reduced. Again the EP_3 receptor seems to play a minor role also in smooth muscle cells. Interestingly, the $EP_{2\text{-}4}$ receptors have been found on human smooth muscle cells (Mori et al., 2011). From our data, we would assume that the EP_1 receptor must have a role on smooth muscle cells or mast cells, maybe only in the rat species.

Our data support the notion that the generation of PGE_2 was due to COX-2, as the potent COX-1 inhibitor FR-122047 (Ochi and Goto, 2002) showed no effect on PGE_2 production in this study. In line with this, PGE_2 has been described to be generated in high amounts by the COX-2 pathway in alveolar epithelium cells and airway smooth muscle cells (Belvisi et al., 1997). The present findings suggest that during the EAR, serotonin-induced bronchoconstriction is enhanced by COX-2 derived PGE_2 acting on EP_1 receptors in the rat lung. As the methacholine-induced bronchoconstriction was not altered by either COX inhibition or EP_1 antagonism, the interaction between serotonin and PGE_2 appears to be specific for serotonin. Similar findings have been reported from other disease models (Sato et al., 2000; Xie et al., 2003), that also implicated that 5-HT responses may in part be mediated by the release of prostaglandins and associated with COX-2 expression. It is possible that PGE_2, formed either in response to allergen or 5-HT receptor activation, interacts at the cellular signalling level with $5\text{-}HT_{2A}$ receptor-induced responses (Berg et al., 1998; Selbie and Hill, 1998). This hypothesis is supported by the present finding that COX-inhibition enhanced $5\text{-}HT_{2A}$ receptor expression in the rat PCLS.

Thus, while there is some evidence that the COX-2-derived prostanoids might at least to some extent have been produced in epithelial and smooth muscle cells, it is tempting to speculate that the effect of the COX-1 inhibitor was occurring in mast cells that contain both COX-1 and COX-2 (Ermert et al., 1998b). This speculation is based on our observation that the COX-1 inhibitor had no effect on serotonin-induced bronchoconstriction or 5-HT2AR expression in the present study, and on the finding that COX-2 inhibitors had no effect on antigen-induced release of PGD_2 from rat mast cells (Lau and Stenton, 1998). Unfortunately, high basal levels of PGE_2, TXB_2 and PGD_2 in the supernatant of the lung slices, made it difficult to interpret the findings in Fig 4, although the reduced levels of TXB_2 and PGD_2 in FR122047-treated slices could be explained by the inhibition of COX-1 in mast cells. By note, high levels of PGE_2 are typical in asthmatic situation, where increased levels of PGE_2 have been measured in lung tissue and bronchoalveolar lavage fluid (Aggarwal et al., 2010; Krawiec et al., 2001), representing the pathophysiological situation of asthma.

5. Conclusions

The major aim of this study was to evaluate the role of COX isoenzymes and prostanoids in antigen-induced airway contractions of the peripheral rat lung. Since the preparation of PCLS is essentially the same in all species; this model also provides the opportunity to compare the early allergic airway response in different species. In guinea pig and human PCLS, both prostanoids and histamine contribute to the allergen-induced bronchoconstriction (Ressmeyer et al., 2006). In the rat PCLS, where the allergen-induced bronchoconstriction is almost exclusively mediated by serotonin, the antigen-

response appears to be modulated by locally formed prostanoids, in particular by PGE_2, derived from COX-2 and to some extent from COX-1. Apparently, the mechanisms by which prostanoids contribute to the early allergic airway response differ among species. In guinea pigs and humans, prostanoids are primary mediators of the antigen-induced bronchoconstriction (Larsson et al., 2005; Ressmeyer et al., 2006; Wohlsen et al., 2003), whereas in the rat lung prostanoids, and especially PGE_2, act both as primary mediators of the antigen-induced airway contraction and modulate the serotonin-induced bronchoconstriction. Interestingly, the EP_4 receptor has a bronchoprotective role during antigen exposure in this model which correlate with the bronchodilatory results obtained in man (Benyahia et al., 2012; Buckley et al., 2011), suggesting that the rat may be a promising test model for asthma therapy with EP_4 agonists.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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References

Aggarwal, S., Moodley, Y.P., Thompson, P.J., Misso, N.L., 2010. Prostaglandin E2 and cysteinyl leukotriene concentrations in sputum: association with asthma severity and eosinophilic inflammation. Clin Exp Allergy 40, 85-93.

Armour, C.L., Johnson, P.R., Alfredson, M.L., Black, J.L., 1989. Characterization of contractile prostanoid receptors on human airway smooth muscle. Eur J Pharmacol. 165, 215-222.

Baber, S.R., Champion, H.C., Bivalacqua, T.J., Hyman, A.L., Kadowitz, P.J., 2003. Role of cyclooxygenase-2 in the generation of vasoactive prostanoids in the rat pulmonary and systemic vascular beds. Circulation. 108, 896-901.

Beasley, R.C., Featherstone, R.L., Church, M.K., Rafferty, P., Varley, J.G., Harris, A., Robinson, C., Holgate, S.T., 1989. Effect of a thromboxane receptor antagonist on PGD2- and allergen-induced bronchoconstriction. J Appl Physiol. 66, 1685-1693.

Belvisi, M.G., Saunders, M.A., Haddad el, B., Hirst, S.J., Yacoub, M.H., Barnes, P.J., Mitchell, J.A., 1997. Induction of cyclo-oxygenase-2 by cytokines in human cultured airway smooth muscle cells: novel inflammatory role of this cell type. Br J Pharmacol. 120, 910-916.

Benyahia, C., Gomez, I., Kanyinda, L., Boukais, K., Danel, C., Leseche, G., Longrois, D., Norel, X., 2012. PGE(2) receptor (EP(4)) agonists: potent dilators of human bronchi and future asthma therapy? Pulm Pharmacol Ther 25, 115-118.

Berg, K.A., Maayani, S., Clarke, W.P., 1998. Interactions between effectors linked to serotonin receptors. Ann N Y Acad Sci. 861, 111-120.

Buckley, J., Birrell, M.A., Maher, S.A., Nials, A.T., Clarke, D.L., Belvisi, M.G., 2011. EP4 receptor as a new target for bronchodilator therapy. Thorax 66, 1029-1035.

Coleman, R.A., Smith, W.L., Narumiya, S., 1994. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 46, 205-229.

Dahlback, M., Bergstrand, H., Sorenby, L., 1984. Bronchial anaphylaxis in actively sensitized Sprague Dawley rats: studies on mediators involved. Acta Pharmacol Toxicol (Copenh). 55, 6-17.

Dahlen, S.E., Kumlin, M., 2004. Monitoring mast cell activation by prostaglandin D2 in vivo. Thorax. 59, 453-455.

Ermert, L., Ermert, M., Althoff, A., Merkle, M., Grimminger, F., Seeger, W., 1998a. Vasoregulatory prostanoid generation proceeds via cyclooxygenase-2 in noninflamed rat lungs. J Pharmacol Exp Ther. 286, 1309-1314.

Ermert, L., Ermert, M., Goppelt-Struebe, M., Walmrath, D., Grimminger, F., Steudel, W., Ghofrani, H.A., Homberger, C., Duncker, H., Seeger, W., 1998b. Cyclooxygenase isoenzyme localization and mRNA expression in rat lungs. Am J Respir Cell Mol Biol. 18, 479-488.

Feng, C., Beller, E.M., Bagga, S., Boyce, J.A., 2006. Human mast cells express multiple EP receptors for prostaglandin E2 that differentially modulate activation responses. Blood 107, 3243-3250.

FitzGerald, G.A., 2003. COX-2 and beyond: Approaches to prostaglandin inhibition in human disease. Nat Rev Drug Discov. 2, 879-890.

Hartney, J.M., Coggins, K.G., Tilley, S.L., Jania, L.A., Lovgren, A.K., Audoly, L.P., Koller, B.H., 2006. Prostaglandin E2 protects lower airways against bronchoconstriction. Am J Physiol Lung Cell Mol Physiol. 290, L105-113.

Held, H.D., Uhlig, S., 2000. Mechanisms of endotoxin-induced airway and pulmonary vascular hyperreactivity in mice. Am J Respir Crit Care Med. 162, 1547-1552.

Hele, D.J., Birrell, M.A., Webber, S.E., Foster, M.L., Belvisi, M.G., 2001. Mediator involvement in antigen-induced bronchospasm and microvascular leakage in the airways of ovalbumin sensitized Brown Norway rats. Br J Pharmacol. 132, 481-488.

Holgate, S.T., Polosa, R., 2006. The mechanisms, diagnosis, and management of severe asthma in adults. Lancet 368, 780-793.

Janson, C., 2010. The importance of airway remodelling in the natural course of asthma. Clin Respir J 4 Suppl 1, 28-34.

Johnston, S.L., Freezer, N.J., Ritter, W., O'Toole, S., Howarth, P.H., 1995. Prostaglandin D2-induced bronchoconstriction is mediated only in part by the thromboxane prostanoid receptor. Eur Respir J. 8, 411-415.

Krawiec, M.E., Westcott, J.Y., Chu, H.W., Balzar, S., Trudeau, J.B., Schwartz, L.B., Wenzel, S.E., 2001. Persistent wheezing in very young children is associated with lower respiratory inflammation. Am J Respir Crit Care Med 163, 1338-1343.

Kurumbail, R.G., Stevens, A.M., Gierse, J.K., McDonald, J.J., Stegeman, R.A., Pak, J.Y., Gildehaus, D., Miyashiro, J.M., Penning, T.D., Seibert, K., Isakson, P.C., Stallings, W.C., 1996. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. Nature. 384, 644-648.

514 Larsson, A.K., Back, M., Hjoberg, J., Dahlen, S.E., 2005. Inhibition of nitric-oxide
515 synthase enhances antigen-induced contractions and increases release of
516 cysteinyl-leukotrienes in guinea pig lung parenchyma: nitric oxide as a protective
517 factor. J Pharmacol Exp Ther. 315, 458-465.
518 Larsson, A.K., Hagfjard, A., Dahlen, S.E., Adner, M., 2011. Prostaglandin D induces
519 contractions through activation of TP receptors in peripheral lung tissue from the
520 guinea pig. Eur J Pharmacol 669, 136-142.
521 Lau, H.Y., Stenton, G.R., 1998. Effects of non-steroidal anti-inflammatory drugs and
522 cyclooxygenase-2 specific inhibitors on mediator release from rat peritoneal mast
523 cells. Inflamm Res. 47 Suppl 1, S22-23.
524 Lydford, S.J., McKechnie, K., 1994. Characterization of the prostaglandin E2 sensitive
525 (EP)-receptor in the rat isolated trachea. Br J Pharmacol 112, 133-136.
526 Manning, P.J., Stevens, W.H., Cockcroft, D.W., O'Byrne, P.M., 1991. The role of
527 thromboxane in allergen-induced asthmatic responses. Eur Respir J. 4, 667-672.
528 Martin, C., Uhlig, S., Ullrich, V., 2001. Cytokine-induced bronchoconstriction in
529 precision-cut lung slices is dependent upon cyclooxygenase-2 and thromboxane
530 receptor activation. Am J Respir Cell Mol Biol. 24, 139-145.
531 Martin, J.G., Suzuki, M., Maghni, K., Pantano, R., Ramos-Barbon, D., Ihaku, D., Nantel,
532 F., Denis, D., Hamid, Q., Powell, W.S., 2002. The immunomodulatory actions of
533 prostaglandin E2 on allergic airway responses in the rat. J Immunol. 169, 3963-
534 3969.
535 Matsuoka, T., Hirata, M., Tanaka, H., Takahashi, Y., Murata, T., Kabashima, K.,
536 Sugimoto, Y., Kobayashi, T., Ushikubi, F., Aze, Y., Eguchi, N., Urade, Y.,

537 Yoshida, N., Kimura, K., Mizoguchi, A., Honda, Y., Nagai, H., Narumiya, S., 2000.
538 Prostaglandin D2 as a mediator of allergic asthma. Science. 287, 2013-2017.
539 McKenniff, M.G., Norman, P., Cuthbert, N.J., Gardiner, P.J., 1991. BAY u3405, a potent
540 and selective thromboxane A2 receptor antagonist on airway smooth muscle in
541 vitro. Br J Pharmacol. 104, 585-590.
542 Mori, A., Ito, S., Morioka, M., Aso, H., Kondo, M., Sokabe, M., Hasegawa, Y., 2011.
543 Effects of specific prostanoid EP receptor agonists on cell proliferation and
544 intracellular Ca(2+) concentrations in human airway smooth muscle cells. Eur J
545 Pharmacol. 659, 72-78.
546 Nagase, T., Dallaire, M.J., Ludwig, M.S., 1996. Airway and tissue behavior during early
547 response in sensitized rats: role of 5-HT and LTD4. J Appl Physiol. 80, 583-590.
548 Norel, X., de Montpreville, V., Brink, C., 2004. Vasoconstriction induced by activation
549 of EP1 and EP3 receptors in human lung: effects of ONO-AE-248, ONO-DI-004,
550 ONO-8711 or ONO-8713. Prostaglandins Other Lipid Mediat. 74, 101-112.
551 Norel, X., Walch, L., Labat, C., Gascard, J.P., Dulmet, E., Brink, C., 1999. Prostanoid
552 receptors involved in the relaxation of human bronchial preparations. Br J
553 Pharmacol 126, 867-872.
554 Ochi, T., Goto, T., 2002. Differential effect of FR122047, a selective cyclo-oxygenase-1
555 inhibitor, in rat chronic models of arthritis. Br J Pharmacol. 135, 782-788.
556 Pavord, I.D., Tattersfield, A.E., 1995. Bronchoprotective role for endogenous
557 prostaglandin E2. Lancet. 345, 436-438.
558 Peebles, R.S., Jr., Hashimoto, K., Morrow, J.D., Dworski, R., Collins, R.D., Hashimoto,
559 Y., Christman, J.W., Kang, K.H., Jarzecka, K., Furlong, J., Mitchell, D.B., Talati,

M., Graham, B.S., Sheller, J.R., 2002. Selective cyclooxygenase-1 and -2 inhibitors each increase allergic inflammation and airway hyperresponsiveness in mice. Am J Respir Crit Care Med. 165, 1154-1160.

Picot, D., Loll, P.J., Garavito, R.M., 1994. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. Nature. 367, 243-249.

Ressmeyer, A.R., Larsson, A.K., Vollmer, E., Dahlen, S.E., Uhlig, S., Martin, C., 2006. Characterisation of guinea pig precision-cut lung slices: comparison with human tissues. Eur Respir J. 28, 603-611.

Rolin, S., Masereel, B., Dogne, J.M., 2006. Prostanoids as pharmacological targets in COPD and asthma. Eur J Pharmacol. 533, 89-100.

Sato, K., Li, J., Metais, C., Bianchi, C., Sellke, F., 2000. Increased pulmonary vascular contraction to serotonin after cardiopulmonary bypass: role of cyclooxygenase. J Surg Res 90, 138-143.

Schleputz, M., Uhlig, S., Martin, C., 2011. Electric field stimulation of precision-cut lung slices. J Appl Physiol 110, 545-554.

Selbie, L.A., Hill, S.J., 1998. G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. Trends Pharmacol Sci. 19, 87-93.

Tellmann, G., 2006. The E-Method: a highly accurate technique for gene-expression analysis. Nature Methods 3, i - ii.

Tilley, S.L., Hartney, J.M., Erikson, C.J., Jania, C., Nguyen, M., Stock, J., McNeisch, J., Valancius, C., Panettieri, R.A., Jr., Penn, R.B., Koller, B.H., 2003. Receptors and pathways mediating the effects of prostaglandin E2 on airway tone. Am J Physiol Lung Cell Mol Physiol. 284, L599-606.

Vancheri, C., Mastruzzo, C., Sortino, M.A., Crimi, N., 2004. The lung as a privileged site for the beneficial actions of PGE2. Trends Immunol. 25, 40-46.

Vane, J.R., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol. 231, 232-235.

Watts, S.W., Cohen, M.L., 1993. Time-dependent improvement in guinea pig tracheal contractility: modification by cyclooxygenase inhibitors. J Pharmacol Exp Ther 266, 950-957.

Wohlsen, A., Martin, C., Vollmer, E., Branscheid, D., Magnussen, H., Becker, W.M., Lepp, U., Uhlig, S., 2003. The early allergic response in small airways of human precision-cut lung slices. Eur Respir J. 21, 1024-1032.

Wohlsen, A., Uhlig, S., Martin, C., 2001. Immediate allergic response in small airways. Am J Respir Crit Care Med. 163, 1462-1469.

Xie, G., Wang, Y., Sharma, M., Gabriel, A., Mitchell, J., Xing, Y., Meuser, T., Palmer, P.P., 2003. 5-Hydroxytryptamine-induced plasma extravasation in the rat knee joint is mediated by multiple prostaglandins. Inflamm Res 52, 32-38.

606 **Figure legends**

607 **Fig 1**. Airway contractions to ovalbumin in rat PCLS. A) Contractions to cumulative
608 concentrations of ovalbumin (0.01-1000 µg/ml, n=5). B) Contractions to a single dose of
609 ovalbumin (10 µg/ml, n=5). Contractions are expressed as the decrease of airway area
610 (%) compared to the initial airway area. Data are presented as mean ± S.E.M.

611

612 **Fig 2.** Effect of COX inhibition on ovalbumin-induced bronchoconstriction. Before
613 airway contractions were induced by ovalbumin (10 µg/ml), the lung slices were
614 pretreated with A) the unselective COX inhibitor indomethacin (10 µM, n=15; control
615 n=16), B) the selective COX-1 inhibitor FR-122047 (5 µM, n=7; control n=7), C) the
616 selective COX-2 inhibitor celecoxib (10 µM, n=8; control n=8). Control slices are shown
617 in black, experiments with the inhibitors in gray. Bronchoconstriction is expressed as the
618 decrease of airway area (%) compared to the initial airway area. Data are presented as
619 mean ± S.E.M. *, $P<0.05$.

620

621 **Fig 3.** Effect of EP, TP and 5-HT_{2A} receptor antagonists on contractions to ovalbumin (10
622 µg/ml). Before airway contractions were induced by ovalbumin (10 µg/ml), the lung
623 slices were pretreated with A) the EP_1 receptor antagonist ONO-8713 (10 µM, n=6;
624 control n=6), B) the EP_2 receptor antagonist A6809 (5 µM, n=6; control n=6) C) the EP_3
625 receptor antagonist L-798,106 (0.5 µM, n=6; control n=6) D) the EP_4 receptor antagonist
626 L-161,982 (0.5 µM, n=6; control n=6), E) the TP receptor antagonist BAYu3405 (10 µM,
627 n=5; control n=5) or F) the 5-HT_{2A} receptor antagonist ketanserin (0.1 µM, n=5; control
628 n=5). Control slices are shown in black, experiments with the inhibitors in gray.

629 Bronchoconstriction is expressed as the decrease of airway area (%) compared to initial
630 airway area. Data are presented as mean ± S.E.M. **, P<0.01.
631
632 **Fig 4.** The synthesis of A) serotonin, B) PGE_2, C) TXB_2, and D) PGD_2 was measured and
633 compared with initial release in the medium supernatant after pretreatment with selective
634 and unselective COX inhibitors and exposure to 10 µg/ml ovalbumin. Control: Medium;
635 Control: Ovalbumin 10 µg/ml; Indomethacin: Indomethacin 10 µM + ovalbumin 10
636 µg/ml; FR122047: FR-122047 5 µM + ovalbumin 10 µg/ml; Celecoxib: Celecoxib 10
637 µM + ovalbumin 10 µg/ml. Data are expressed as the mean ± S.E.M of 5 independent
638 experiments *, P<0.05; **, P<0.01; ***, P<0.001.
639
640 **Fig 5.** Effect of COX inhibition, EP and TP antagonists on contractions induced by
641 cumulative doses of serotonin (0.01-10 µM). Before airway contractions were induced by
642 serotonin, the lung slices were pretreated with A) the COX inhibitor indomethacin (10
643 µM, n=9; control n=9), B) the COX-1 inhibitor FR-122047 (5 µM, n=5; control n=5), C)
644 the COX-2 inhibitor celecoxib (10 µM, n=5; control n=5), D) the EP_1 antagonist ONO-
645 8713 (10 µM, n=5; control n=5), E) the EP_2 receptor antagonist A6809 (5 µM, n=5;
646 control n=6) F) the EP_3 receptor antagonist L-798,106 (0.5 µM, n=6; control n=6), G) the
647 EP_4 receptor antagonist L-161,982 (0.5 µM, n=6; control n=6) or H) the TP antagonist
648 BAYu3405 (10 µM, n=6; control n=6). Control slices are shown in black, experiments
649 with the inhibitors in gray. Bronchoconstriction is expressed as the decrease of airway
650 area (%) compared to initial airway area. Data are presented as mean ± S.E.M. *, P<0.05;
651 **, P<0.01; ***, P<0.001.

Fig 6. Receptor expression of 5-HT2AR. An incubation of PCLS with the EP_1 antagonist ONO-8713 (10 µM), the COX-1 inhibitor FR-122047 (5 µM), the unselective COX-inhibitor indomethacin (10 µM) and the COX-2-inhibitor celecoxib (10 µM) for 4h resulted in the change of the 5-HT2A receptor. Data were referenced to the housekeeping gene B2m and normalized to the mean of the experimental control. Data (n=5) are presented as mean ± S.E.M. *, $P<0.05$.

Fig 7. Effect of COX inhibition and EP_1 receptor antagonist on airway contractions to cumulative doses of methacholine (0.01-10 µM). Before airway contractions were induced by methacholine, the lung slices were pretreated with indomethacin (10 µM, n=4, gray) or ONO-8713 (10 µM, n=4, gray dashes) compared to control (n=6, black). Bronchoconstriction is expressed as the decrease of airway area (%) compared to initial airway area. Data are presented as mean ± S.E.M.

Table 1. Contractile responses to PGD_2, PGE_2 and U46619 in ratPCLS

Agonists	Contractions (%)	SEM
PGD_2 (10 µM)	0	0.8
PGE_2 (10 µM)	0	1.1
U46619 (50 µM)	16	2.1
U46619 (50 µM) + BAYu3405 (10 µM)[a]	0	0.1

Airway contractions to PGD_2 (10 µM, n=3), PGE_2 (10 µM, n=3), TP receptor agonist U46619 (50 µM, n=3) and U46619 (50 µM) in combination with the TP receptor antagonist BAYu3405 (10 µM, n=3, [a]$P<0.05$) in rat PCLS. Contractions are expressed as the decrease of airway area (%) compared to the initial airway area. Data are presented as mean ± S.E.M.

Figure 1

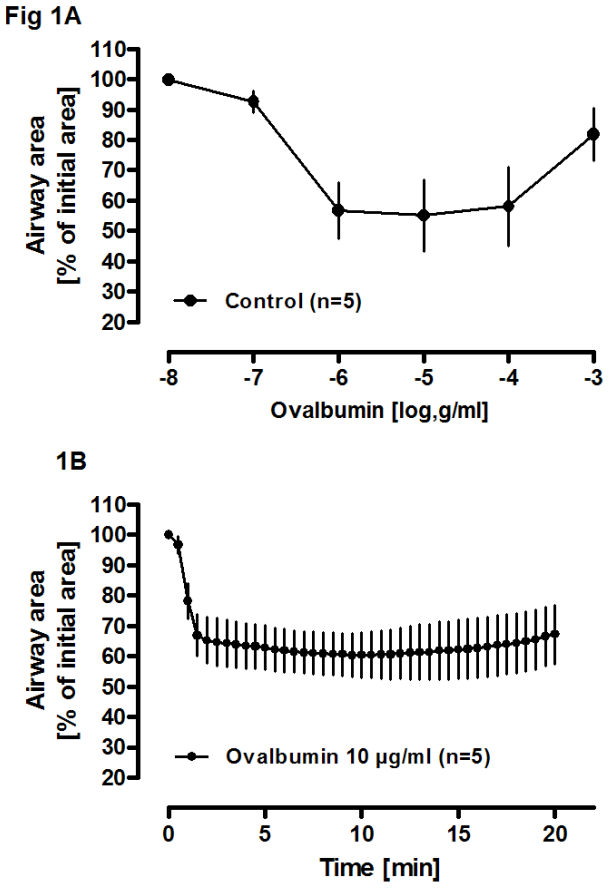
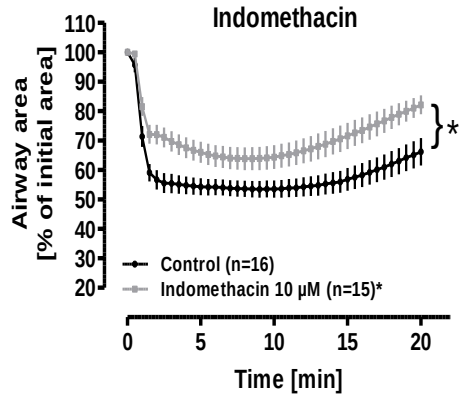


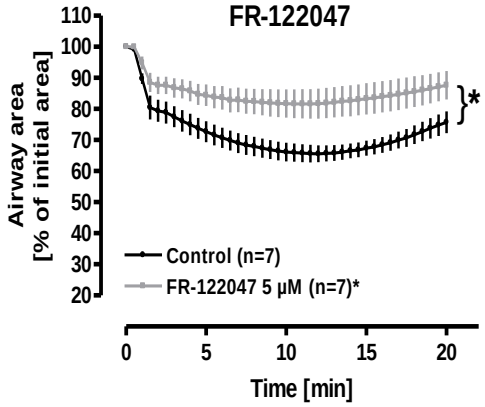
Fig 1A
Airway area
[% of initial area]
110
100
90
80
70
60
50
40
30
20
Control (n=5)
-8
-7
-6
-5
-4
-3
Ovalbumin [log,g/ml]
1B
Airway area
[% of initial area]
110
100
90
80
70
60
50
40
30
20
Ovalbumin 10 µg/ml (n=5)
0
5
10
15
20
Time [min]

Figure 2

Fig 2A



2B



2C

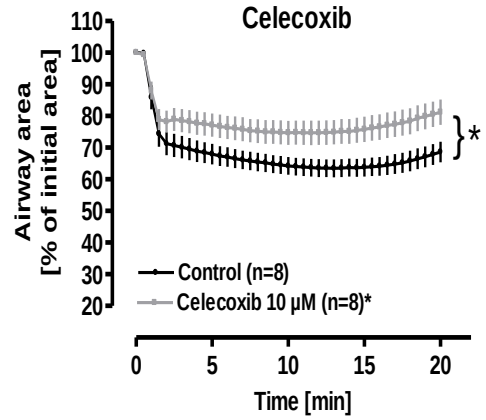


Figure 3

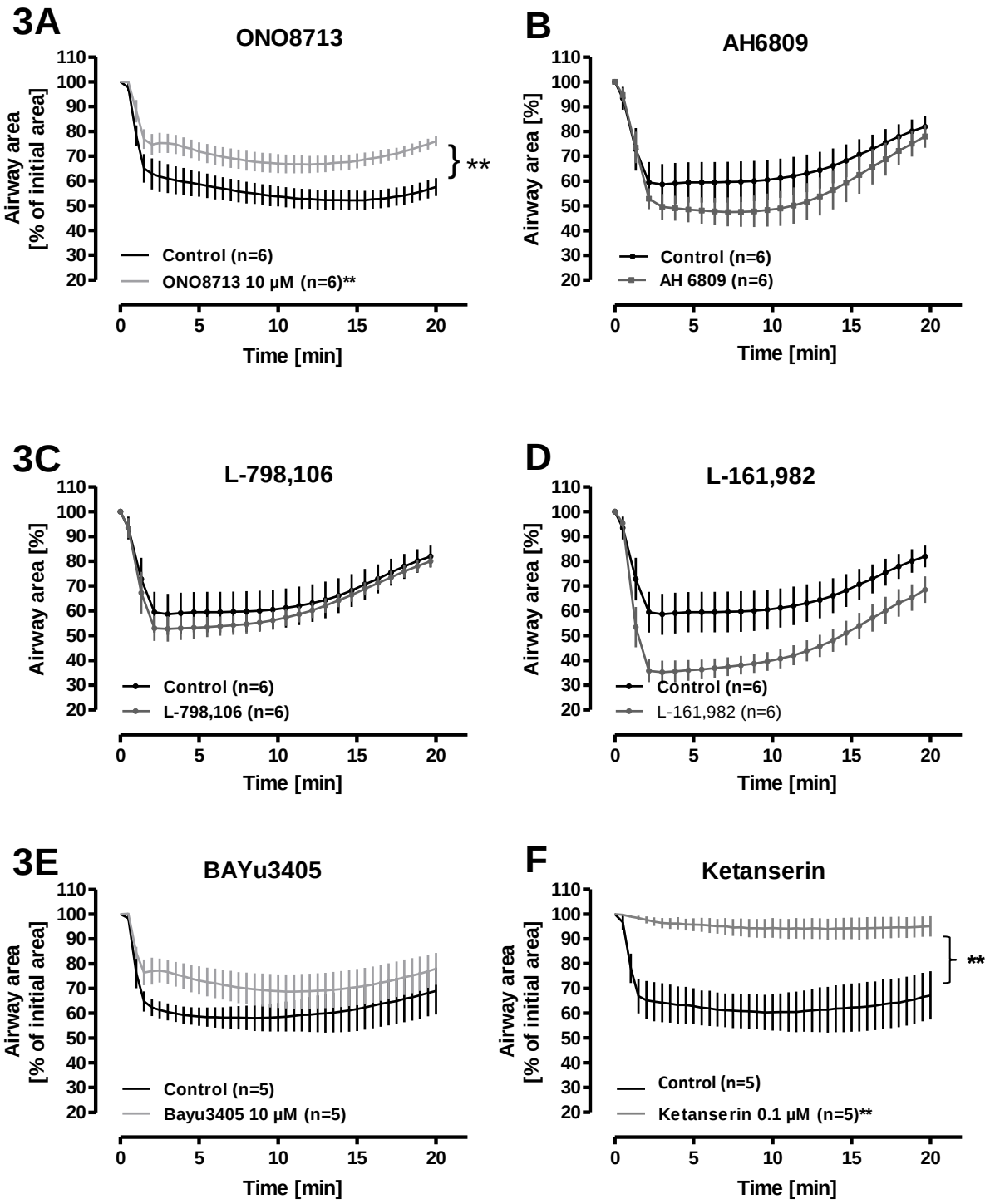

3A
ONO8713
Airway area [% of initial area]
Control (n=6)
ONO8713 10 µM (n=6)**
**
Time [min]
B
AH6809
Airway area [%]
Control (n=6)
AH 6809 (n=6)
Time [min]
3C
L-798,106
Airway area [%]
Control (n=6)
L-798,106 (n=6)
Time [min]
D
L-161,982
Airway area [%]
Control (n=6)
L-161,982 (n=6)
Time [min]
3E
BAYu3405
Airway area [% of initial area]
Control (n=5)
Bayu3405 10 µM (n=5)
Time [min]
F
Ketanserin
Airway area [% of initial area]
Control (n=5)
Ketanserin 0.1 µM (n=5)**
**
Time [min]

Figure 4

Fig 4

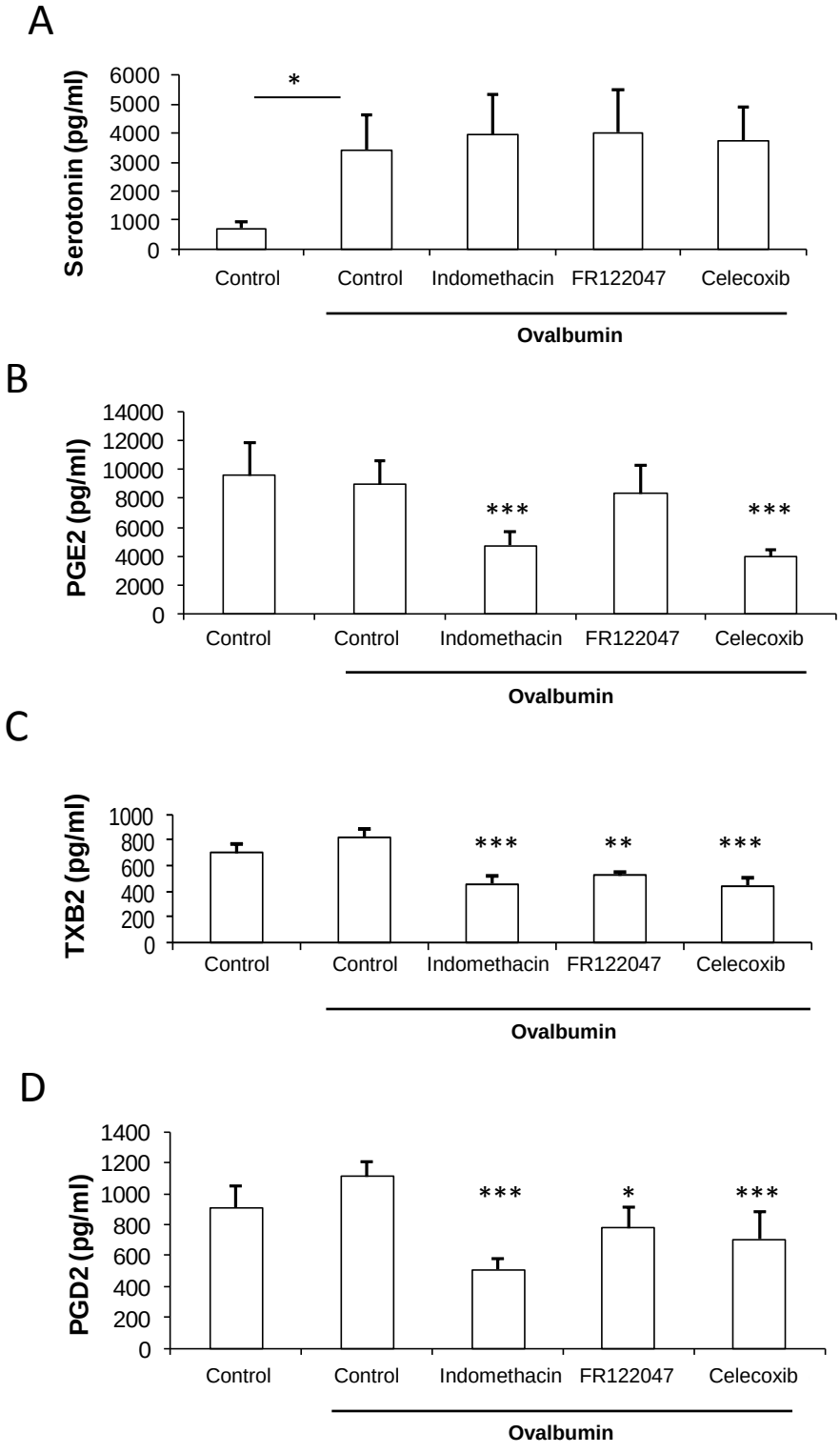


Figure 5

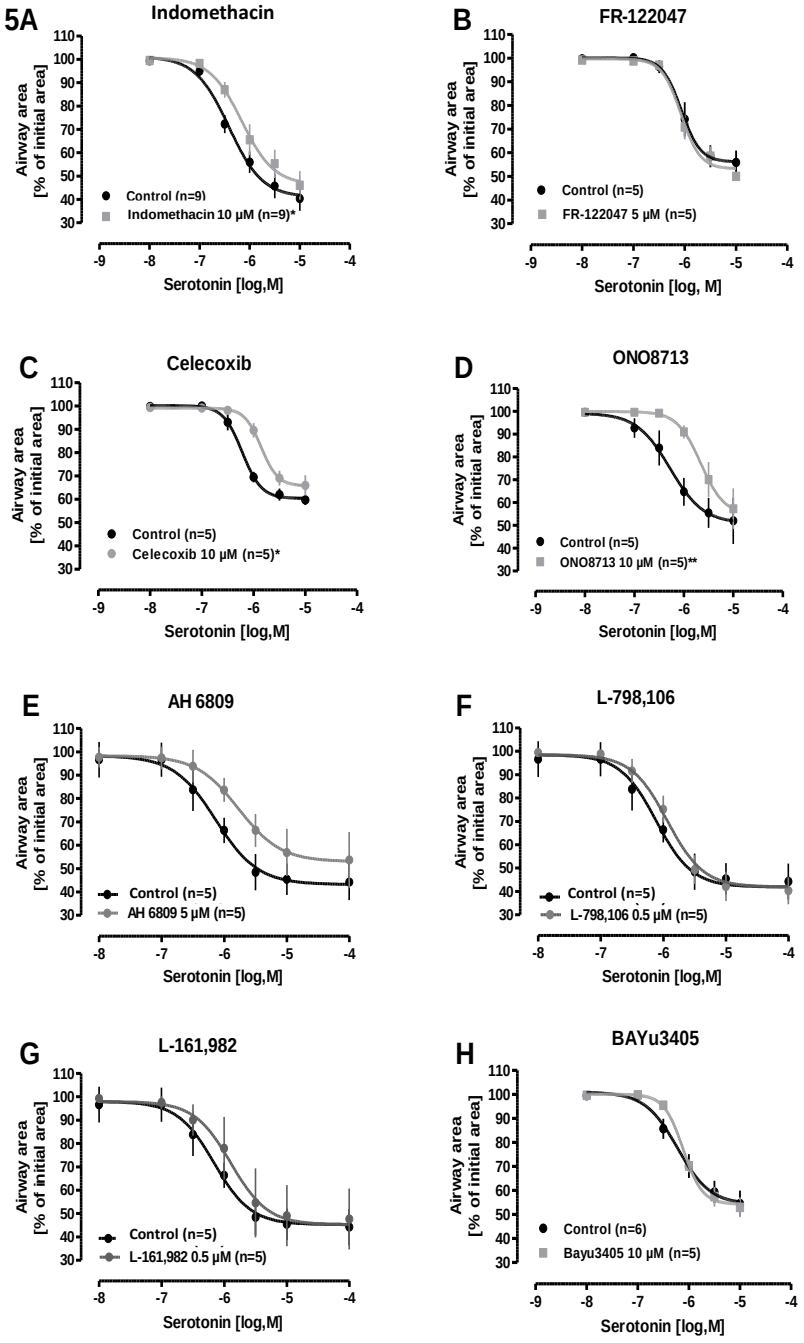

5A
Indomethacin
Airway area [% of initial area]
Control (n=9)
Indomethacin 10 µM (n=9)*
Serotonin [log,M]
B
FR-122047
Control (n=5)
FR-122047 5 µM (n=5)
Serotonin [log, M]
C
Celecoxib
Control (n=5)
Celecoxib 10 µM (n=5)*
Serotonin [log,M]
D
ONO8713
Control (n=5)
ONO8713 10 µM (n=5)**
Serotonin [log,M]
E
AH 6809
Control (n=5)
AH 6809 5 µM (n=5)
Serotonin [log,M]
F
L-798,106
Control (n=5)
L-798,106 0.5 µM (n=5)
Serotonin [log,M]
G
L-161,982
Control (n=5)
L-161,982 0.5 µM (n=5)
Serotonin [log,M]
H
BAYu3405
Control (n=6)
Bayu3405 10 µM (n=5)
Serotonin [log,M]

Figure 6

Fig 6

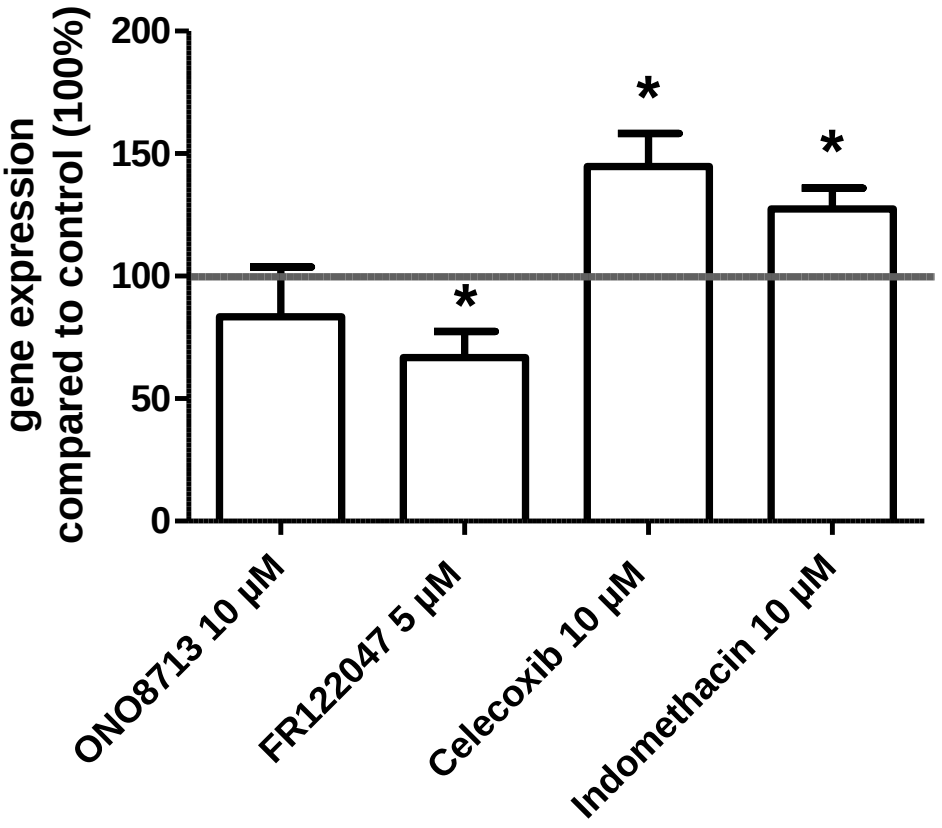


Figure 7

Fig 7