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1    **Modulation of antigen-induced responses by serotonin and prostaglandin E<sub>2</sub> via EP<sub>1</sub>**  
2    **and EP<sub>4</sub> receptors in the peripheral rat lung**

3

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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<sub>1</sub> receptor antagonist ONO-8713 reduced the contractions, whereas the EP<sub>4</sub> receptor antagonist L-161,982 significantly increased the contractile response to ovalbumin. The 5-HT<sub>2A</sub> 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<sub>2</sub> is the main prostanoid involved in the early allergic airway response in the rat lung. PGE<sub>2</sub> appears to act both as a primary mediator of antigen-induced airway contraction via the EP<sub>4</sub> receptor and as a downstream modulator of serotonin-induced bronchoconstriction via the EP<sub>1</sub> 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<sub>2</sub> (TXA<sub>2</sub>), prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), 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<sub>2</sub> 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 et al., 2011). TXA<sub>2</sub> may also cause airway hyperresponsiveness (Held and Uhlig, 2000) and contributes to cytokine-induced bronchoconstriction (Martin et al., 2001). PGD<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> are diverse (Coleman et al., 1994). Recent findings indicate that PGE<sub>2</sub> has its bronchodilatory effect mainly via the EP<sub>4</sub> 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-HT<sub>2A</sub> 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 ( $220 \pm 20$  g) obtained from Charles River (Sulzfeld, Germany) and kept under controlled conditions ( $22^{\circ}\text{C}$ , 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  $250 \pm 20$   $\mu\text{m}$  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 ( $1.36 \pm 0.28$   $\text{mm}^2$ ) were selected, covered with 1 ml of medium and fixed with a nylon thread attached to a

platinum wire to avoid movements and allow relaxation of the slice (Schleputz et al., 2011). Images were recorded by an analogue (JAI 2040; JAI Pulnix, Alzenau, Germany) or a digital camera (IRB640; Visitron Systems, Munich, Germany) controlled by the software program Optimas 6.5 (Optimas, Bothell, WA). A control picture was taken before addition of any agonists or antagonists and frames were recorded every 30 sec. A time interval of 5 minutes for cumulatively given doses and 20 minutes for single doses were used. The images were analyzed by the image analysis program Optimas 6.5 (Optimas, Bothell, WA).

### *2.3. Sensitization for antigen studies*

For antigen studies with ovalbumin, the lung slices were incubated over night with cell culture medium containing 1% serum from actively sensitized rats, as previously done (Wohlsen et al., 2001). The medium was not changed until the following day. All other lung slices were maintained in standard cell culture medium. Control studies were performed to verify that 1% serum (of sensitized rats) did not interfere with responses induced by other agonists and that ovalbumin did not show any effect in non-sensitised slices.

### *2.4. Study design*

Airway contractions to ovalbumin, PGD<sub>2</sub>, PGE<sub>2</sub>, the thromboxane receptor analogue u46619, serotonin and methacholine were studied in rat PCLS. Effects of selective COX inhibitors and selective EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub>, EP<sub>4</sub> and TP receptor antagonists on airway tone were assessed. The release of serotonin and prostanoids after ovalbumin-stimulation were



139 analysed in the supernatant. A single concentration of ovalbumin (10 µg/ml) was used for  
140 antigen-induced contractions. This concentration was selected from a cumulative  
141 concentration-response curve (0.01-1000 µg/ml of ovalbumin) (Fig 1A). The single dose  
142 (10 µg/ml) of ovalbumin produced a strong, stable and reproducible bronchoconstriction  
143 with the same maximum airway contraction as generated by cumulative challenge of  
144 ovalbumin (Fig 1B).

#### 146 2.5. *Measurements of released mediators*

147 Supernatant (0.5 ml) of six incubated PCLS (weight 0.03 g/slice) was collected and  
148 immediately frozen at -80°C. The samples were taken at three different time points. First  
149 from unchallenged slices to obtain initial mediator release, thereafter, 20 minutes from  
150 pre-treatment with the different drugs and finally 20 minutes after the slices were  
151 challenged with 10 µg/ml ovalbumin. Enzyme immunoassays of TXA<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub> and  
152 serotonin were performed according to the manufacturer's instructions. TXA<sub>2</sub> was  
153 measured as the stable metabolite TXB<sub>2</sub> and PGD<sub>2</sub> as PGD<sub>2</sub>-mox. The assay detection  
154 limits for the different mediators were 7.8 pg/ml for TXB<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub> and 50 pg/ml for  
155 serotonin. The enzyme immunoassay specificity for the different mediators to interfere  
156 with each other was less than 0.01% for PGE<sub>2</sub>, PGD<sub>2</sub>-mox and serotonin, whereas the  
157 antibody tracer for TXB<sub>2</sub> cross reacted with PGD<sub>2</sub> (0.53%) and with PGE<sub>2</sub> (0.09%).

#### 159 2.6. *RT-PCR analysis*

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

162 Düren, Germany) automated on a QIAcube roboter (QIAGEN GmbH, Hilden, Germany).  
163 RNA was quantified in buffered 10 mM TRIS-HCl, pH 7.5, using a NanoDrop 1000  
164 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham (MA), USA). For reverse  
165 transcription 274 ng of total RNA was added to 1 µl of oligo(dT)<sub>15</sub> Primer (0.5 µg/µl)  
166 (Invitrogen, Karlsruhe, Germany) and water to a total volume of 12 µl. Samples were  
167 incubated for 10 minutes at 65°C to linearize the RNA. 4 µl buffer (5x), 2 µl dNTP (10  
168 mM), 1 µl Rnasin (40U/µl) and 1 µl M-MLV RT (H<sup>-</sup>) (200U/µl) (all substances from  
169 Promega GmbH, Mannheim, Germany) were added and RNA was reverse transcribed for  
170 90 minutes at 40°C. This was followed by a 2 minutes heat-inactivation step at 95°C. 20  
171 µl of water was added afterwards to a final volume of 40 µl per sample. All incubation  
172 steps were performed on a Biometra UNO II Thermocycler (Biometra GmbH, Göttingen,  
173 Germany). For real-time qPCR 1 µl of cDNA was incubated as template with 0.5 µl  
174 forward primer (6.25 µM) (Eurofins MWG GmbH, Ebersberg, Germany), 0.5 µl reverse  
175 primer (6.25 µM), 5 µL SYBR-Green I Mastermix (Roche-Diagnostics GmbH,  
176 Mannheim, Germany) and 3 µL water according to manufacturer's instructions in a  
177 LightCycler 480 (Roche-Diagnostics GmbH). Following primer pairs were used for 5-  
178 HT<sub>2A</sub>-receptor (gene symbol: Htr2a, NCBI Reference Sequence: NM\_017254.1): sense  
179 5'-CCA CCA ACT ATT TCC TGA TGT C-3' antisense 5'-GCA CAT CCA GGT AAA  
180 TCC AG-3' and for Beta-2-microglobulin (gene symbol: B2m, NCBI Reference  
181 Sequence: NM\_012512.2): sense 5'-CCG TGA TCT TTC TGG TGC TTG TCT-3'  
182 antisense 5'-ATC GGT CTC GGT GGG TGT GAA T-3'. Quantification after real-time  
183 qPCR was performed with Cp values, acquired via the Second Derivative Maximum  
184 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<sup>®</sup>) 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<sub>2</sub>, PGE<sub>2</sub>, 9,11-dideoxy-9 $\alpha$ ,11 $\alpha$ -methanoepoxy PGF<sub>2 $\alpha$</sub>  (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<sub>2</sub>, PGD<sub>2</sub>-mox and PGE<sub>2</sub> 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  $\pm$  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<sub>2</sub>, PGE<sub>2</sub> and TXA<sub>2</sub> analogue u46619 were tested on non-sensitized slices to evaluate the attenuated bronchoconstriction induced by the different COX-inhibitors. PGD<sub>2</sub> (10 µM;  $n=3$ ) and PGE<sub>2</sub> (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<sub>1</sub>, EP<sub>2</sub> and EP<sub>4</sub> (*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<sub>2</sub> may have diverse roles acting on both contractile EP receptors (mainly EP<sub>1</sub>, but also EP<sub>3</sub>) and relaxant EP receptors (EP<sub>2</sub> and EP<sub>4</sub>) (Buckley et al., 2011). EP<sub>1</sub> receptor antagonist ONO 8713 (Norel et al., 1999), EP<sub>2</sub> receptor antagonist AH6809, EP<sub>3</sub> receptor antagonist L-798,106 and EP<sub>4</sub> 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<sub>1</sub> receptor antagonist ONO-8713 (10 µM) significantly reduced the ovalbumin-induced contractions ( $P=0.004$ ; Fig 3A), whereas the EP<sub>4</sub> receptor

antagonist L-161,982 (0.5  $\mu$ M) significantly increased the ovalbumin-induced contractions ( $P=0.042$ ; Fig 3D) whereas pre-treatment with EP<sub>2</sub> receptor antagonist AH6809 (5  $\mu$ M) or EP<sub>3</sub> receptor antagonist L-798,106 (0.5  $\mu$ M) or TP receptor antagonist BAYu3405 (10  $\mu$ M), 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<sub>2A</sub> receptor antagonist ketanserin. Ketanserin (0.1  $\mu$ M;  $P=0.005$ ) 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<sub>2</sub>, TXB<sub>2</sub> and PGD<sub>2</sub> that were not changed after stimulation by ovalbumin. The COX inhibitors indomethacin (10  $\mu$ M) and celecoxib (10  $\mu$ M) significantly decreased the formation of PGE<sub>2</sub>, TXB<sub>2</sub> and PGD<sub>2</sub>, whereas the COX-1 inhibitor FR-122047 (5  $\mu$ M) significantly reduced the synthesis of TXB<sub>2</sub> (Fig 4C) and PGD<sub>2</sub> (Fig 4D), but not PGE<sub>2</sub> (Fig 4B). The formation of serotonin was significantly increased after addition of ovalbumin 10  $\mu$ g/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  $\mu$ M) significantly attenuated the airway contraction induced by serotonin (0.01-10  $\mu$ M;  $P=0.02$ ; Fig 5A) and shifted the concentration-response to the right ( $pEC_{50}$ :  $6.22 \pm 0.01$  vs control  $pEC_{50}$ :  $6.40 \pm 0.05$ ;  $P=0.008$ ). FR-122047 (5  $\mu$ M) had no effect on serotonin-induced bronchoconstriction (ns; Fig 5B), whereas celecoxib (10  $\mu$ M) decreased the response to serotonin ( $P=0.001$ ; Fig 5C) and shifted the concentration-response curve to the right ( $pEC_{50}$ :  $5.89 \pm 0.08$  vs control  $pEC_{50}$ :  $6.12 \pm 0.07$ ;  $P=0.03$ ). Pretreatment with the  $EP_1$  receptor antagonist ONO-8713 (10  $\mu$ M) attenuated the contractile response to cumulative doses of serotonin ( $P=0.01$ ; Fig 5D) and shifted the concentration-response to the right ( $pEC_{50}$ :  $5.66 \pm 0.07$  vs control  $pEC_{50}$ :  $6.27 \pm 0.11$ ;  $P=0.003$ ). The  $EP_2$  receptor antagonist AH6809 (5  $\mu$ M) 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  $\mu$ M) shifted the concentration-response curve to the right ( $pEC_{50}$ :  $5.89 \pm 0.07$  vs control  $pEC_{50}$ :  $6.15 \pm 0.06$ ;  $P=0.004$ ; Fig 5G). Neither the  $EP_3$  receptor antagonist L-798,106 (0.5  $\mu$ M; Fig 5F) nor the TP receptor antagonist BAYu3405 (10  $\mu$ M; Fig 5H) had any significant effects on the contractions induced by serotonin. Analysis of the 5-HT<sub>2A</sub> receptor expression indicated that COX-inhibition with indomethacin or celecoxib enhanced the expression of the 5-HT<sub>2A</sub> receptor after 4h (Fig 6).

300 3.4. *Effect of COX inhibition and EP<sub>1</sub> receptor antagonism on methacholine-induced*  
301 *contractions*

302 To determine if the effect of COX inhibition and EP<sub>1</sub> receptor antagonism was specific to  
303 serotonin rather than a general property of rat airways, methacholine-induced  
304 bronchoconstriction was evaluated in the presence and absence of the COX inhibitor  
305 indomethacin (10 µM) or the EP<sub>1</sub> receptor antagonist ONO-8713 (10 µM). The  
306 contractions to methacholine were not altered by either indomethacin or ONO-8713 (Fig  
307 7).



#### 4. Discussion

In this study, we present evidence that in the early allergic airway response in rat; especially PGE<sub>2</sub> may act both as a primary mediator of antigen-induced airway contraction via COX and the EP<sub>1</sub> and EP<sub>4</sub> receptors and as a downstream modulator of serotonin-induced bronchoconstriction via COX-2 and the EP<sub>1</sub> 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).

332 To understand the attenuated antigen-induced bronchoconstriction after COX-  
333 inhibition, the effect of the prostanoids PGD<sub>2</sub>, PGE<sub>2</sub> and thromboxane was investigated  
334 on rat airway tone, where only the TP receptor agonist u46619 induced some  
335 contractions. Prostanoid receptors show considerable versatility and may mediate  
336 bronchoconstriction via both TP and EP<sub>1</sub> receptors (Lydford and McKechnie, 1994) and  
337 bronchodilatory effects through DP<sub>1</sub>, EP<sub>2</sub> and EP<sub>4</sub> receptors (Hartney et al., 2006; Norel  
338 et al., 2004; Tilley et al., 2003). Therefore, to further investigate the reduced contractile  
339 response after COX-inhibition, we focused on the prostanoid receptors EP<sub>1-4</sub> and the TP  
340 receptor. Interestingly, the EP<sub>1</sub> receptor antagonist ONO-8713 attenuated the antigen-  
341 induced airway contraction, whereas the EP<sub>4</sub> receptor antagonist L-161,982 potently  
342 increased the contractions to ovalbumin. This data implicate that PGE<sub>2</sub> may modulate the  
343 early allergic airway response in rat lungs in two ways, mainly *via* activation of relaxant  
344 EP<sub>4</sub> receptors but also in part *via* activation of contractile EP<sub>1</sub> receptors. Recent findings  
345 indicate that PGE<sub>2</sub> has its bronchodilatory effect mainly via the EP<sub>4</sub> receptor in man  
346 (Benyahia et al., 2012; Buckley et al., 2011). Notably, the beneficial relaxant effect of  
347 PGE<sub>2</sub> via EP<sub>4</sub> receptor in this study correlated with the results obtained in human.  
348 Focusing on the different EP receptors on mast cells, there is little information about the  
349 distribution on mast cells. Feng and colleagues (Feng et al., 2006) have characterized the  
350 EP receptors on human mast cells. Interestingly they only found expression of EP<sub>1, 2, 3</sub>  
351 receptor mRNA. From their view of EP receptor activity, increasing cAMP via EP<sub>2</sub> and  
352 EP<sub>4</sub>, seems to be important, whereas the role of EP<sub>3</sub>, which acts via increase of calcium,  
353 is only minor. Also in our study the EP<sub>3</sub> receptor does not appear to have a direct or  
354 indirect effect on mast cell activation, which is again in line with the findings of Feng et

al, who found different EP<sub>3</sub>-receptor subtypes, which can either be coupled to the G-protein G<sub>s</sub> or G<sub>i</sub> (Feng et al., 2006).

In addition, also the TP receptor antagonist partly affected the antigen-response, indicating that TP receptors may contribute as well. Both TXA<sub>2</sub> and PGD<sub>2</sub> are known to mediate airway contractions via the TP receptor (Larsson et al., 2011; McKenniff et al., 1991), whereas PGD<sub>2</sub> may also cause bronchodilation via the DP<sub>1</sub> receptor (Larsson et al., 2011; Norel et al., 1999). Despite the pharmacological evidence of prostanoid involvement in the early allergic airway response, supernatant levels of TXB<sub>2</sub>, PGD<sub>2</sub> or PGE<sub>2</sub> were not increased after antigen challenge. COX inhibition significantly but not completely reduced the generation of the prostanoids in the rat PCLS. The release of serotonin was significantly increased after addition of ovalbumin and remained unaffected by the different COX inhibitors. These data raised the hypothesis that prostanoids may act as modulators of airway responsiveness. Since the 5-HT receptor antagonist ketanserin completely blocked the antigen-induced contractions in the rat PCLS, we hypothesised that serotonin is the main mediator of EAR and that the COX metabolites may modulate the serotonin response. We observed that serotonin-induced bronchoconstriction was attenuated by selective COX-2 inhibition and EP<sub>1</sub> antagonism, whereas in contrast to the antigen-induced response, COX-1 inhibition had no effect. The EP<sub>2</sub> and EP<sub>4</sub> receptor antagonist may also affect the serotonin-induced constriction. This may result is probably depending on the fact that the EP<sub>2</sub> receptor antagonist has similar affinity to the EP<sub>1</sub> receptor (Buckley et al., 2011). However, apart from the EP<sub>3</sub> receptor antagonist, the other EP receptor antagonists shifted the concentration- response curve to the right. This is in line with the dilatory response via EP<sub>2</sub> and EP<sub>4</sub> on smooth muscle

cells. For EP<sub>1</sub> antagonism, the strongest effect was found, when both EP<sub>2</sub> and EP<sub>4</sub> receptors were triggered by endogenous PGE<sub>2</sub> produced by the PCLS during challenge. In cases where either the EP<sub>2</sub> or EP<sub>4</sub> receptor was blocked this relaxation was reduced. Again the EP<sub>3</sub> receptor seems to play a minor role also in smooth muscle cells. Interestingly, the EP<sub>2-4</sub> receptors have been found on human smooth muscle cells (Mori et al., 2011). From our data, we would assume that the EP<sub>1</sub> 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<sub>2</sub> was due to COX-2, as the potent COX-1 inhibitor FR-122047 (Ochi and Goto, 2002) showed no effect on PGE<sub>2</sub> production in this study. In line with this, PGE<sub>2</sub> 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<sub>2</sub> acting on EP<sub>1</sub> receptors in the rat lung. As the methacholine-induced bronchoconstriction was not altered by either COX inhibition or EP<sub>1</sub> antagonism, the interaction between serotonin and PGE<sub>2</sub> 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<sub>2</sub>, formed either in response to allergen or 5-HT receptor activation, interacts at the cellular signalling level with 5-HT<sub>2A</sub> receptor-induced responses (Berg et al., 1998; Selbie and Hill, 1998). This hypothesis is supported by the present finding that COX-inhibition enhanced 5-HT<sub>2A</sub> 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-HT<sub>2</sub>AR expression in the present study, and on the finding that COX-2 inhibitors had no effect on antigen-induced release of PGD<sub>2</sub> from rat mast cells (Lau and Stenton, 1998). Unfortunately, high basal levels of PGE<sub>2</sub>, TXB<sub>2</sub> and PGD<sub>2</sub> in the supernatant of the lung slices, made it difficult to interpret the findings in Fig 4, although the reduced levels of TXB<sub>2</sub> and PGD<sub>2</sub> in FR122047-treated slices could be explained by the inhibition of COX-1 in mast cells. By note, high levels of PGE<sub>2</sub> are typical in asthmatic situation, where increased levels of PGE<sub>2</sub> 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<sub>2</sub>, 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<sub>2</sub>, act both as primary mediators of the antigen-induced airway contraction and modulate the serotonin-induced bronchoconstriction. Interestingly, the EP<sub>4</sub> 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<sub>4</sub> agonists.

#### **Competing interests**

The authors declare that they have no competing interests.

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## Figure legends

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

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

**Fig 3.** Effect of EP, TP and 5-HT<sub>2A</sub> receptor antagonists on contractions to ovalbumin (10 µg/ml). Before airway contractions were induced by ovalbumin (10 µg/ml), the lung slices were pretreated with A) the EP<sub>1</sub> receptor antagonist ONO-8713 (10 µM, n=6; control n=6), B) the EP<sub>2</sub> receptor antagonist A6809 (5 µM, n=6; control n=6) C) the EP<sub>3</sub> receptor antagonist L-798,106 (0.5 µM, n=6; control n=6) D) the EP<sub>4</sub> receptor antagonist L-161,982 (0.5 µM, n=6; control n=6), E) the TP receptor antagonist BAYu3405 (10 µM, n=5; control n=5) or F) the 5-HT<sub>2A</sub> receptor antagonist ketanserin (0.1 µM, n=5; control n=5). Control slices are shown in black, experiments with the inhibitors in gray.

Bronchoconstriction is expressed as the decrease of airway area (%) compared to initial airway area. Data are presented as mean  $\pm$  S.E.M. \*\*,  $P < 0.01$ .

**Fig 4.** The synthesis of A) serotonin, B) PGE<sub>2</sub>, C) TXB<sub>2</sub>, and D) PGD<sub>2</sub> was measured and compared with initial release in the medium supernatant after pretreatment with selective and unselective COX inhibitors and exposure to 10  $\mu$ g/ml ovalbumin. Control: Medium; Control: Ovalbumin 10  $\mu$ g/ml; Indomethacin: Indomethacin 10  $\mu$ M + ovalbumin 10  $\mu$ g/ml; FR122047: FR-122047 5  $\mu$ M + ovalbumin 10  $\mu$ g/ml; Celecoxib: Celecoxib 10  $\mu$ M + ovalbumin 10  $\mu$ g/ml. Data are expressed as the mean  $\pm$  S.E.M of 5 independent experiments \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

**Fig 5.** Effect of COX inhibition, EP and TP antagonists on contractions induced by cumulative doses of serotonin (0.01-10  $\mu$ M). Before airway contractions were induced by serotonin, the lung slices were pretreated with A) the COX inhibitor indomethacin (10  $\mu$ M, n=9; control n=9), B) the COX-1 inhibitor FR-122047 (5  $\mu$ M, n=5; control n=5), C) the COX-2 inhibitor celecoxib (10  $\mu$ M, n=5; control n=5), D) the EP<sub>1</sub> antagonist ONO-8713 (10  $\mu$ M, n=5; control n=5), E) the EP<sub>2</sub> receptor antagonist A6809 (5  $\mu$ M, n=5; control n=6) F) the EP<sub>3</sub> receptor antagonist L-798,106 (0.5  $\mu$ M, n=6; control n=6), G) the EP<sub>4</sub> receptor antagonist L-161,982 (0.5  $\mu$ M, n=6; control n=6) or H) the TP antagonist BAYu3405 (10  $\mu$ M, n=6; control n=6). Control slices are shown in black, experiments with the inhibitors in gray. Bronchoconstriction is expressed as the decrease of airway area (%) compared to initial airway area. Data are presented as mean  $\pm$  S.E.M. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

652

653 **Fig 6.** Receptor expression of 5-HT<sub>2A</sub>R. An incubation of PCLS with the EP<sub>1</sub> antagonist  
654 ONO-8713 (10  $\mu$ M), the COX-1 inhibitor FR-122047 (5  $\mu$ M), the unselective COX-  
655 inhibitor indomethacin (10  $\mu$ M) and the COX-2-inhibitor celecoxib (10  $\mu$ M) for 4h  
656 resulted in the change of the 5-HT<sub>2A</sub> receptor. Data were referenced to the housekeeping  
657 gene B2m and normalized to the mean of the experimental control. Data (n=5) are  
658 presented as mean  $\pm$  S.E.M. \*, P<0.05.

659

660 **Fig 7.** Effect of COX inhibition and EP<sub>1</sub> receptor antagonist on airway contractions to  
661 cumulative doses of methacholine (0.01-10  $\mu$ M). Before airway contractions were  
662 induced by methacholine, the lung slices were pretreated with indomethacin (10  $\mu$ M,  
663 n=4, gray) or ONO-8713 (10  $\mu$ M, n=4, gray dashes) compared to control (n=6, black).  
664 Bronchoconstriction is expressed as the decrease of airway area (%) compared to initial  
665 airway area. Data are presented as mean  $\pm$  S.E.M.



1    **Table 1.** Contractile responses to PGD<sub>2</sub>, PGE<sub>2</sub> and U46619 in ratPCLS

Agonists	Contractions (%)	SEM
PGD <sub>2</sub> (10 µM)	0	0.8
PGE <sub>2</sub> (10 µM)	0	1.1
U46619 (50 µM)	16	2.1
U46619 (50 µM) + BAYu3405 (10 µM) <sup>a</sup>	0	0.1

2

3    Airway contractions to PGD<sub>2</sub> (10 µM, n=3), PGE<sub>2</sub> (10 µM, n=3), TP receptor agonist

4    U46619 (50 µM, n=3) and U46619 (50 µM) in combination with the TP receptor

5    antagonist BAYu3405 (10 µM, n=3, <sup>a</sup>P<0.05) in rat PCLS. Contractions are expressed as

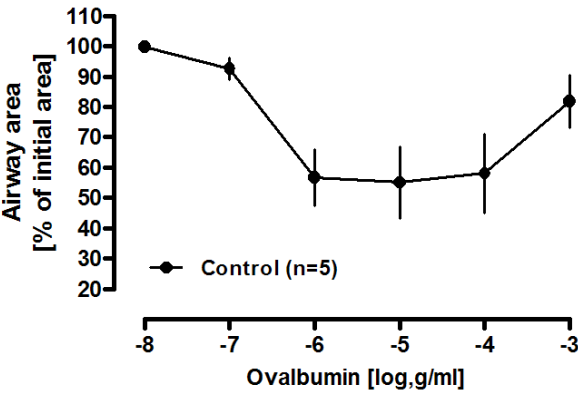
6    the decrease of airway area (%) compared to the initial airway area. Data are presented as

7    mean ± S.E.M.

8

Figure 1

Fig 1A



1B

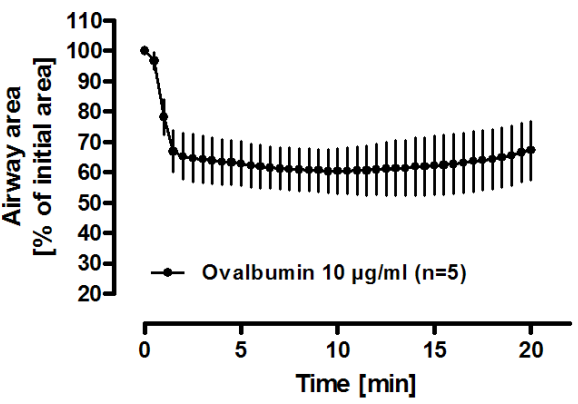
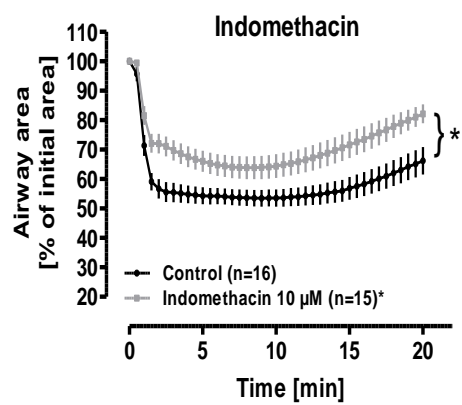
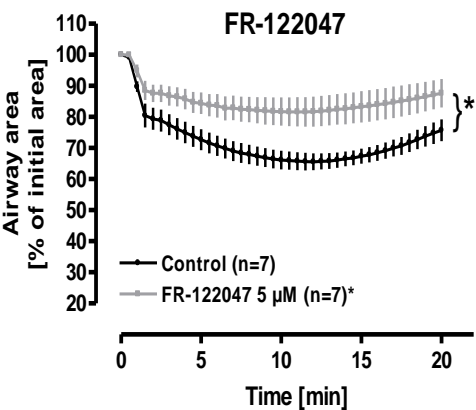


Fig 2A



2B



2C

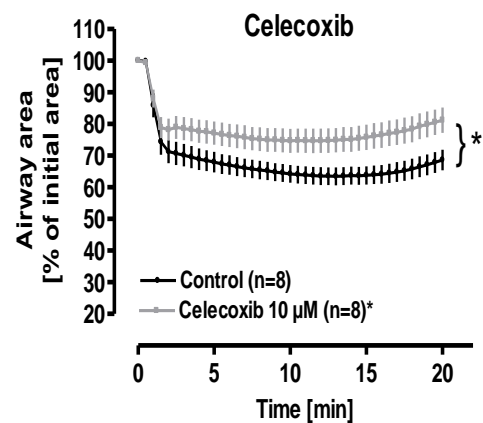


Figure 3

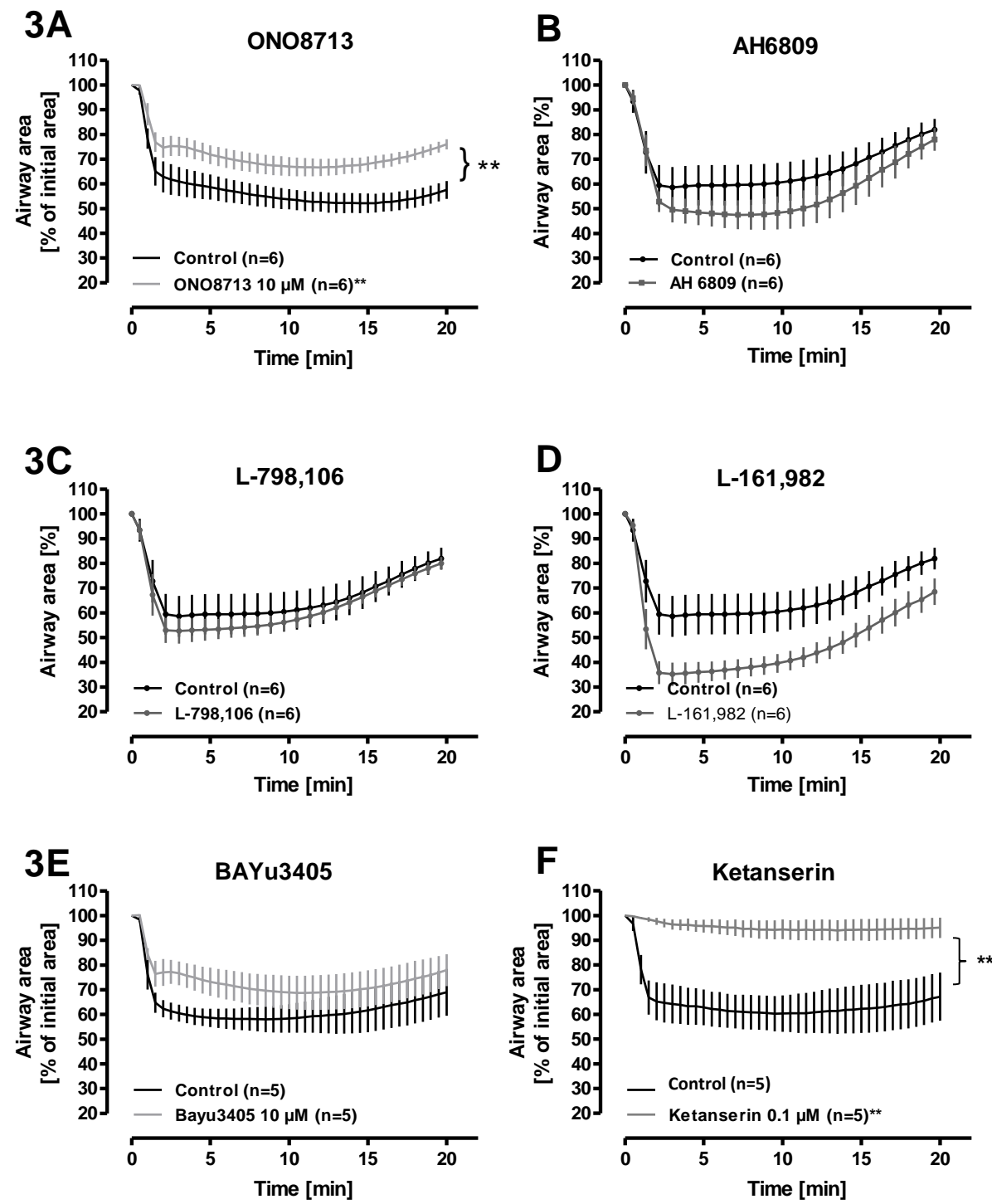


Figure 4

Fig 4

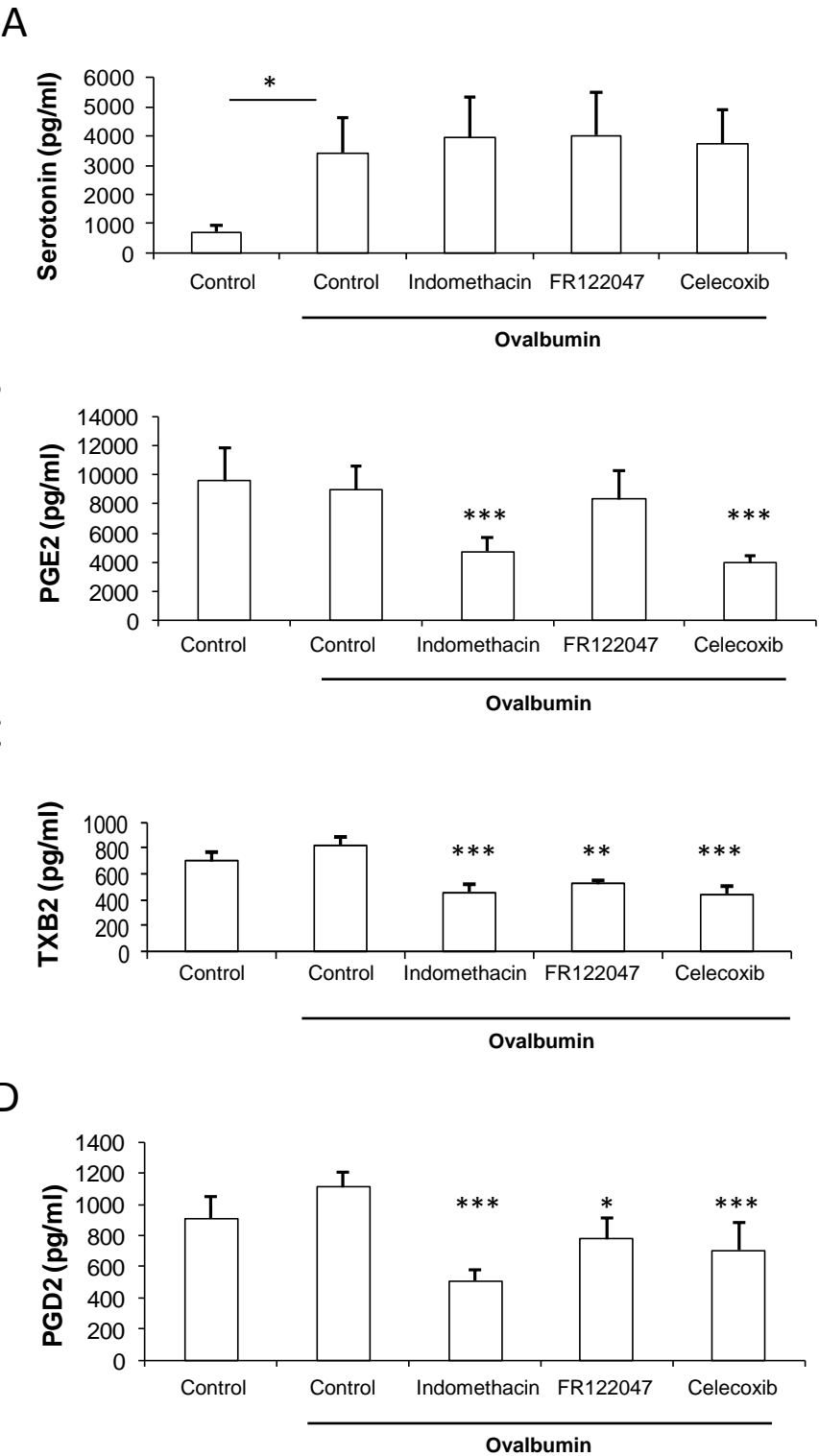


Figure 5

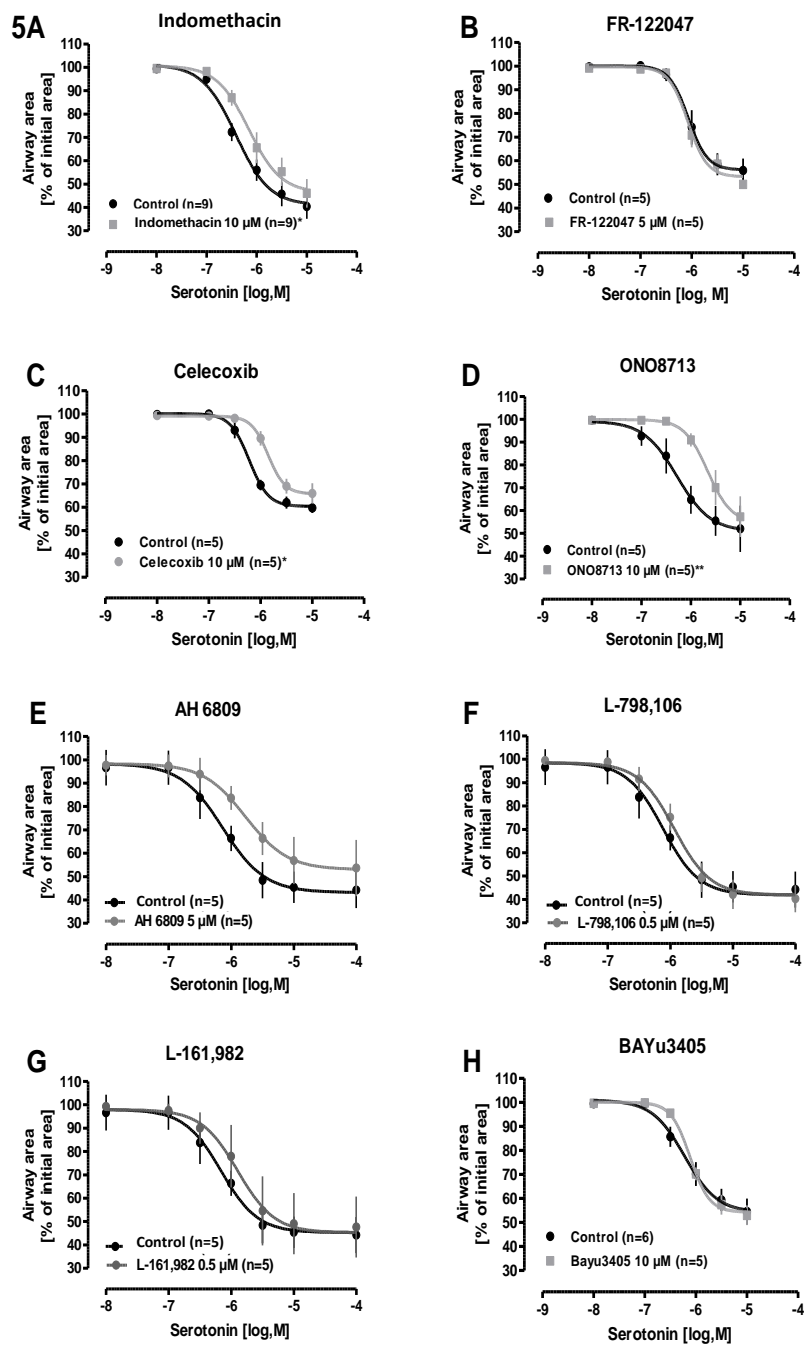


Fig 6

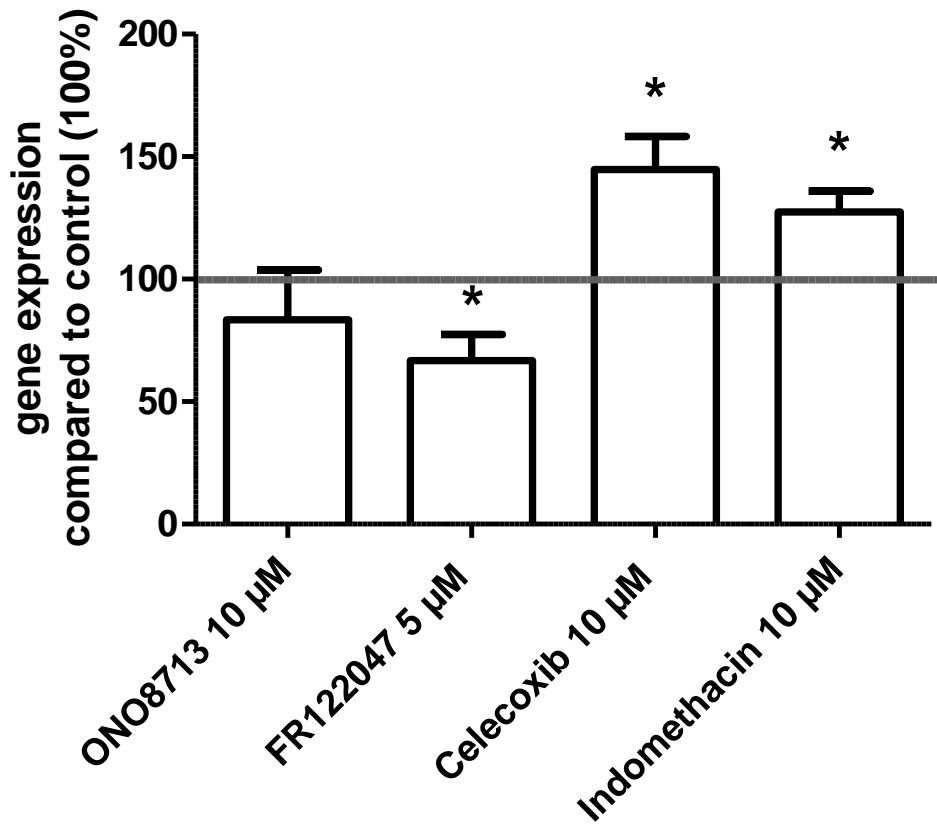


Figure 7

Fig 7

