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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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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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24 **Abstract**

25 The cyclooxygenase (COX) pathway and prostanoids may critically contribute to the  
26 early allergic airway response. In the rat lung, serotonin (5-HT) is a major mediator of  
27 antigen-induced contractions. The aim of this study was therefore to examine the relative  
28 role of the COX pathway and serotonin for antigen-induced contractions in the rat lung.  
29 Airway responses were studied in rat precision cut lung slices (PCLS). Lung slices were  
30 stimulated with ovalbumin or serotonin after pretreatment with COX inhibitors or  
31 specific TP or EP receptor antagonists. Changes in airway size (contractions/relaxations)  
32 were measured by a digital video camera. The supernatants were analysed for changes in  
33 prostaglandin and serotonin release. Airway contractions to ovalbumin were attenuated  
34 by the unselective COX inhibitor indomethacin, the selective COX-1 inhibitor FR-  
35 122047 and COX-2 inhibitor celecoxib. The EP<sub>1</sub> receptor antagonist ONO-8713 reduced  
36 the contractions, whereas the EP<sub>4</sub> receptor antagonist L-161,982 significantly increased  
37 the contractile response to ovalbumin. The 5-HT<sub>2A</sub> receptor antagonist ketanserin  
38 completely inhibited the ovalbumin-induced contractions. The different COX inhibitors  
39 decreased the production of prostaglandins but did not affect the synthesis of serotonin.  
40 The serotonin-induced bronchoconstriction was attenuated by celecoxib and ONO-8713,  
41 but not by methacholine. Taken together, our data indicate that PGE<sub>2</sub> is the main  
42 prostanoid involved in the early allergic airway response in the rat lung. PGE<sub>2</sub> appears to  
43 act both as a primary mediator of antigen-induced airway contraction via the EP<sub>4</sub> receptor  
44 and as a downstream modulator of serotonin-induced bronchoconstriction via the EP<sub>1</sub>  
45 receptor.

45 **Keywords:** Airway smooth muscle,  
46 contraction, ovalbumin, precision-cut lung slices, prostaglandins, serotonin

47 **1. Introduction**

48 Airway obstructions play an important role in the development of symptoms associated  
49 with remodelling processes and loss of lung function in asthma (Janson, 2010). Standard  
50 therapy with anti-inflammatory corticosteroids and bronchodilators does not fully prevent  
51 airway obstructions and bronchoconstriction in severe asthma (Holgate and Polosa,  
52 2006), requiring new therapeutic approaches to treat this disease state. Prostanoids, such  
53 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  
54 involved in various physiological and pathophysiological processes in the lung and play a  
55 critical role in asthma (Rolin et al., 2006). Prostanoids are generated from arachidonic  
56 acid and converted to PG via cyclooxygenase (COX) (Vane, 1971). The COX enzyme  
57 exists in two isoforms; COX-1 (Picot et al., 1994) is constitutively expressed and is  
58 involved in regulation of physiological responses and homeostasis, COX-2 (Kurumbail et  
59 al., 1996) is mostly inducible and related to inflammation (FitzGerald, 2003). The  
60 prostanoids contribute to the asthmatic airway responses in different ways. TXA<sub>2</sub> is  
61 involved in allergen-induced asthmatic responses by activation of TP receptors (Manning  
62 et al., 1991) and thereby induction of both airway and vascular smooth muscle  
63 constrictions (Larsson et al., 2011). TXA<sub>2</sub> may also cause airway hyperresponsiveness  
64 (Held and Uhlig, 2000) and contributes to cytokine-induced bronchoconstriction (Martin  
65 et al., 2001). PGD<sub>2</sub> is a pro-inflammatory mediator of allergic asthma (Matsuoka et al.,  
66 2000), a marker of mast cell activation (Dahlen and Kumlin, 2004) and induces airway  
67 and vascular smooth muscle contractions via the TP receptor (Armour et al., 1989;  
68 Beasley et al., 1989; Johnston et al., 1995; Larsson et al., 2011; McKenniff et al., 1991).  
69 PGE<sub>2</sub> is implicated to have a beneficial role in the lung (Pavord and Tattersfield, 1995;

70 Vancheri et al., 2004), since this prostanoid may maintain airway tone (Tilley et al.,  
71 2003) and attenuate allergic airway responses (Hartney et al., 2006; Martin et al., 2002).  
72 However, owing to the existence of various EP receptors the potential actions of PGE<sub>2</sub>  
73 are diverse (Coleman et al., 1994). Recent findings indicate that PGE<sub>2</sub> has its  
74 bronchodilatory effect mainly via the EP<sub>4</sub> receptor in man (Benyahia et al., 2012;  
75 Buckley et al., 2011). Prostanoids are implicated in the early allergic airway response in  
76 different species; rat (Dahlback et al., 1984), guinea pig (Larsson et al, 2005, 2011) and  
77 man (Benyahia et al., 2012; Buckley et al., 2011; Ressmeyer et al., 2006). However, in  
78 the rat lung, serotonin (5-hydroxytryptamine, 5-HT) is the major mediator released from  
79 mast cells granules during an allergen response. The release of serotonin induces potent  
80 contractions via the 5-HT<sub>2A</sub> receptor (Dahlback et al., 1984; Wohlson et al., 2001).  
81 Inhibition of the COX pathway enhanced the early allergic response in guinea pig lung  
82 (Larsson et al., 2005) and induced airway hyperresponsiveness in murine lung (Peebles et  
83 al., 2002). It is unknown whether this modulation is a general mechanism of the early  
84 allergic response. The purpose of this study was to examine the contribution of the COX  
85 pathway and prostanoids to the early allergic airway response in relation to the effects of  
86 serotonin in the rat lung.

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93 **2. Methods**

94 *2.1. Animals*

95 Precision-cut lung slices (PCLS) were prepared from 8-week-old Wistar rats ( $220 \pm 20$  g)  
96 obtained from Charles River (Sulzfeld, Germany) and kept under controlled conditions  
97 ( $22^{\circ}\text{C}$ , 55% humidity and 12-h day/night rhythm). Animal experiments were approved by  
98 the local ethics committee.

99

100 *2.2. Precision-cut lung slices*

101 Rat PCLS were prepared as previously described (Wohlsen et al., 2001). Rats were  
102 sacrificed by an overdose of pentobarbital i.p. (60 mg/kg). The trachea was prepared and  
103 fixed with a tracheal cannula. The lungs were filled with pre-warmed agarose solution  
104 (0.75%) via the trachea and subsequently chilled with ice. The heart-lung package was  
105 removed and put on ice to allow the agarose to further cool and solidify. The lung lobes  
106 were separated and cut into 5 to 10 mm thick tissue segments from which cores were  
107 made along the airways with a coring tool. The cores were cut into  $250 \pm 20$   $\mu\text{m}$  thick  
108 slices with a Krumdieck tissue slicer (Alabama Research and Development, Munford,  
109 AL). Slices were incubated in minimal essential medium, which was changed every half  
110 hour for the first two hours and then every hour for the next two hours to remove the  
111 agarose and inflammatory mediators from the airways. For the experiments, slices with  
112 airways that had intact surrounding epithelium were moved to 24-well plates and covered  
113 with 1 ml of medium. The airways were imaged and digitized using a digital video  
114 camera. For measurements, slices with comparable airway size ( $1.36 \pm 0.28$   $\text{mm}^2$ ) were  
115 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<sub>2</sub>, PGE<sub>2</sub>, the thromboxane receptor analogue  
136 u46619, serotonin and methacholine were studied in rat PCLS. Effects of selective COX  
137 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  
138 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).

145

#### 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%).

158

#### 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

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

191

## 192 2.7. Drugs

193 Indomethacin, ovalbumin (chicken egg albumin, grade V), serotonin, ketanserin and  
194 dimethylsulfoxid (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). 4-[5-(4-  
195 methylphenyl)-3-(trifluoromethyl) pyrazol-1-yl]benzenesulfonamide (Celecoxib;  
196 Celebrex<sup>®</sup>) was obtained from Pfizer (CA). 1-[4,5-bis(4-methoxyphenyl)-2-  
197 thiazoyl]carbonyl]-4-methylpiperazine hydrochloride (FR-122047), 3R-[[4-  
198 fluorophenyl)sulfonyl]amino]-1,2,3,4-tetrahydro-9H-carbazole-9-propanoic acid  
199 (BAYu3405, Ramatroban) was purchased from Bayer AG (Wuppertal, Germany). PGD<sub>2</sub>,  
200 PGE<sub>2</sub>, 9,11-dideoxy-9 $\alpha$ ,11 $\alpha$ -methanoepoxy PGF<sub>2 $\alpha$</sub>  (U46619), 9-oxo-6-propan-2-  
201 yloxyxanthene-2-carboxylic acid (AH6809) and N-[[4'-[[3-butyl-1,5-dihydro-5-oxo-1-[2  
202 (trifluoromethyl)-phenyl]-4H-1,2,4-triazol-4-yl]methyl][1,1'-biphenyl]-2-yl]sulfonyl]-3-  
203 methyl-2-thiophenecarboxamide (L-161,982) were bought from Cayman Chemical (Ann  
204 Arbor, MI, USA). (E)-3-[4-[[2-(furan-2-ylsulfonyl)-(2-methylpropyl)amino]-5-  
205 (trifluoromethyl)phenoxy]methyl]phenyl]prop-2-enoic acid (ONO-8713) (Norel et al.,  
206 2004) was a generous gift from ONO Pharmaceutical CO. LTD (Osaka, Japan). (E)-N-(5-  
207 bromo-2-methoxyphenyl)sulfonyl-3-[2-(naphthalen-2-ylmethyl)phenyl]prop-2-enamide

208 (L-798,106) were bought from Tocris Bioscience, Bristol, UK. The EIA kits for TXB<sub>2</sub>,  
209 PGD<sub>2</sub>-mox and PGE<sub>2</sub> were obtained from Cayman Chemicals (Ann Arbor, MI). ELISA  
210 kit for serotonin was purchased from IBL-Hamburg (Hamburg, Germany). FR-122047,  
211 ONO-8713, BAYu3405 and celecoxib were dissolved in DMSO. The final concentration  
212 of DMSO or ethanol never exceeded 0.3% (v/v) and did not influence the induced  
213 contractions. The other drugs were dissolved and diluted in distilled water. Dilutions of  
214 drugs were freshly made from the stocks for each experiment. The drugs were present in  
215 the medium fluid during the remaining experiment. Pretreatments were given 20 minutes  
216 before the addition of ovalbumin, serotonin or methacholine.

217

## 218 *2.8. Calculations and Statistics*

219 Airway area before addition of any drug was defined as 100%. Airway contractions were  
220 expressed as the percentage decrease in airway area compared to the initial airway area.  
221 All data are presented as mean  $\pm$  standard error of the mean (S.E.M.). In all experiments  
222 the numbers of n represent animals and not the number of slices. Time courses were  
223 analyzed by the area under the curve (AUC). Enzyme immune assay measurements and  
224 allergen-induced bronchoconstriction curves were analyzed by Student t-test (two curves  
225 or bars) or by analyses of variances (ANOVA, more than two curves or bars) followed by  
226 Bonferroni's post-hoc test. Concentration-response curves to methacholine and serotonin  
227 were analyzed by comparison of sigmoid curves with Prism 5 (Graphpad, San Diego,  
228 USA). A p-value of less than 0.05 was considered significant. The Statistic program JMP  
229 5.1 (Cary, NC, USA) was used to calculate the power of the experiments.

230

231 **3. Results**

232 *3.1. Effect of the COX pathway on ovalbumin-induced contractions*

233 Ovalbumin (10 µg/ml) generated a stable and reproducible airway bronchoconstriction  
234 (Fig 1A). The antigen-induced contractions to ovalbumin (10 µg/ml) were significantly  
235 attenuated by the unselective COX inhibitor indomethacin (10 µM; P=0.012; Fig 2A), the  
236 selective COX-1 inhibitor FR-122047 (5 µM; P=0.016; Fig 2B) and the selective COX-2  
237 inhibitor celecoxib (10 µM; P=0.044; Fig 2C). PGD<sub>2</sub>, PGE<sub>2</sub> and TXA<sub>2</sub> analogue u46619  
238 were tested on non-sensitized slices to evaluate the attenuated bronchoconstriction  
239 induced by the different COX-inhibitors. PGD<sub>2</sub> (10 µM; n=3) and PGE<sub>2</sub> (10 µM; n=3)  
240 did not induce any bronchoconstriction or dilatatory effects in the rat PCLS (*Table 1*), nor  
241 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  
242 TP receptor analogue u46619 (50 µM; n=3) induced airway contractions that were  
243 completely blocked by the TP receptor antagonist BAYu3405 (10 µM; n=3; P<0.05;  
244 *Table 1*). In line, only the TP receptor agonist u46619 induced contractions in the lung  
245 slices. However, both EP and TP receptor activation has been shown to be involved in  
246 allergen-mediated airway contractions. PGE<sub>2</sub> may have diverse roles acting on both  
247 contractile EP receptors (mainly EP<sub>1</sub>, but also EP<sub>3</sub>) and relaxant EP receptors (EP<sub>2</sub> and  
248 EP<sub>4</sub>) (Buckley et al., 2011). EP<sub>1</sub> receptor antagonist ONO 8713 (Norel et al., 1999), EP<sub>2</sub>  
249 receptor antagonist AH6809, EP<sub>3</sub> receptor antagonist L-798,106 and EP<sub>4</sub> receptor  
250 antagonist L-161,982 and the TP receptor antagonist BAYu3405 were therefore tested on  
251 ovalbumin-induced contractions to further characterise the contractile response after  
252 COX inhibition. The EP<sub>1</sub> receptor antagonist ONO-8713 (10 µM) significantly reduced  
253 the ovalbumin-induced contractions (P=0.004; Fig 3A), whereas the EP<sub>4</sub> receptor

254 antagonist L-161,982 (0.5  $\mu$ M) significantly increased the ovalbumin-induced  
255 contractions ( $P=0.042$ ; Fig 3D) whereas pre-treatment with EP<sub>2</sub> receptor antagonist  
256 AH6809 (5  $\mu$ M) or EP<sub>3</sub> receptor antagonist L-798,106 (0.5  $\mu$ M) or TP receptor  
257 antagonist BAYu3405 (10  $\mu$ M), had no significant effect (Fig 3B, C, E). Since serotonin  
258 is known as a major mediator of this particular ovalbumin model, the inhibitory effect of  
259 the COX inhibitors and the EP receptor antagonists was compared with the effect of the  
260 5-HT<sub>2A</sub> receptor antagonist ketanserin. Ketanserin (0.1  $\mu$ M;  $P=0.005$ ) completely  
261 inhibited the contractile response to ovalbumin (Fig 3F).

262

### 263 *3.2. Synthesis of serotonin and prostanoids after challenge with ovalbumin*

264 The supernatant was analysed to verify if there were any changes in generation of  
265 serotonin and prostaglandins after ovalbumin stimulation and COX inhibition. The  
266 medium contained relatively high levels of PGE<sub>2</sub>, TXB<sub>2</sub> and PGD<sub>2</sub> that were not changed  
267 after stimulation by ovalbumin. The COX inhibitors indomethacin (10  $\mu$ M) and celecoxib  
268 (10  $\mu$ M) significantly decreased the formation of PGE<sub>2</sub>, TXB<sub>2</sub> and PGD<sub>2</sub>, whereas the  
269 COX-1 inhibitor FR-122047 (5  $\mu$ M) significantly reduced the synthesis of TXB<sub>2</sub> (Fig 4C)  
270 and PGD<sub>2</sub> (Fig 4D), but not PGE<sub>2</sub> (Fig 4B). The formation of serotonin was significantly  
271 increased after addition of ovalbumin 10  $\mu$ g/ml ( $P=0.032$ ). Notably, its synthesis or  
272 release was not affected by the different COX inhibitors (Fig 4A). In addition, pre-  
273 incubation with COX inhibitors or EP receptor antagonists did not change the initial  
274 airway size in PCLS, indicating no direct effect on airway tone.

275

### 276 *3.3. Effect of the COX pathway on serotonin-induced contractions*

277 Since serotonin was the major contractile mediator of the antigen-induced response, the  
278 effect of COX and prostanoids on serotonin-induced contractions was also evaluated to  
279 investigate potential downstream modulations. Indomethacin (10  $\mu$ M) significantly  
280 attenuated the airway contraction induced by serotonin (0.01-10  $\mu$ M;  $P=0.02$ ; Fig 5A)  
281 and shifted the concentration-response to the right ( $pEC_{50}$ :  $6.22 \pm 0.01$  vs control  $pEC_{50}$ :  
282  $6.40 \pm 0.05$ ;  $P=0.008$ ). FR-122047 (5  $\mu$ M) had no effect on serotonin-induced  
283 bronchoconstriction (ns; Fig 5B), whereas celecoxib (10  $\mu$ M) decreased the response to  
284 serotonin ( $P=0.001$ ; Fig 5C) and shifted the concentration-response curve to the right  
285 ( $pEC_{50}$ :  $5.89 \pm 0.08$  vs control  $pEC_{50}$ :  $6.12 \pm 0.07$ ;  $P=0.03$ ). Pretreatment with the EP<sub>1</sub>  
286 receptor antagonist ONO-8713 (10  $\mu$ M) attenuated the contractile response to cumulative  
287 doses of serotonin ( $P=0.01$ ; Fig 5D) and shifted the concentration-response to the right  
288 ( $pEC_{50}$ :  $5.66 \pm 0.07$  vs control  $pEC_{50}$ :  $6.27 \pm 0.11$ ;  $P=0.003$ ). The EP<sub>2</sub> receptor antagonist  
289 AH6809 (5  $\mu$ M) also attenuated the contractile response to cumulative doses of serotonin  
290 and shifted the concentration-response to the right, and the bottom of the concentration-  
291 response curves was unequal ( $P=0.02$ ; Fig 5E), showing differences in potency and  
292 efficacy. Also the EP<sub>4</sub> receptor antagonist L-161982 (0.5  $\mu$ M) shifted the concentration-  
293 response curve to the right ( $pEC_{50}$ :  $5.89 \pm 0.07$  vs control  $pEC_{50}$ :  $6.15 \pm 0.06$ ;  $P=0.004$ ;  
294 Fig 5G). Neither the EP<sub>3</sub> receptor antagonist L-798,106 (0.5  $\mu$ M; Fig 5F) nor the TP  
295 receptor antagonist BAYu3405 (10  $\mu$ M; Fig 5H) had any significant effects on the  
296 contractions induced by serotonin. Analysis of the 5-HT<sub>2A</sub> receptor expression indicated  
297 that COX-inhibition with indomethacin or celecoxib enhanced the expression of the 5-  
298 HT<sub>2A</sub> receptor after 4h (Fig 6).

299

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).

308

309 **4. Discussion**

310 In this study, we present evidence that in the early allergic airway response in rat;  
311 especially PGE<sub>2</sub> may act both as a primary mediator of antigen-induced airway  
312 contraction via COX and the EP<sub>1</sub> and EP<sub>4</sub> receptors and as a downstream modulator of  
313 serotonin-induced bronchoconstriction via COX-2 and the EP<sub>1</sub> receptor after antigen  
314 challenge. Previous studies have indicated that prostanoids and serotonin are involved in  
315 the early allergic airway response in the rat (Dahlback et al., 1984; Hele et al., 2001;  
316 Nagase et al., 1996; Wohlsen et al., 2001). The relative contributions of these mediators,  
317 however, remained uncertain. In the rat PCLS, the antigen-induced contractions to  
318 ovalbumin were significantly attenuated by selective COX-1 and COX-2 inhibitors,  
319 suggesting a role for both isoenzymes in the peripheral rat lung during the early allergic  
320 airway response. Both COX-1 and COX-2 have been shown to be constitutively  
321 expressed in the normal rat lung (Ermert et al., 1998b) with high enzyme activity (Baber  
322 et al., 2003; Ermert et al., 1998a), suggesting a crucial role for COX isoenzymes in the  
323 regulation of pulmonary responses. The beneficial effect of COX inhibition during the  
324 antigen response in the rat PCLS was somewhat surprising and opposite to other studies,  
325 since in other models COX inhibition resulted in airway hyperresponsiveness and  
326 increased contractility (Larsson et al., 2005; Peebles et al., 2002; Watts and Cohen,  
327 1993). On the other hand, leukotrienes, the major mediators in COX-sensitive asthma,  
328 play only a minor role in rat PCLS (Wohlsen et al., 2001), which may explain the  
329 influence on the AHR and contractility. In addition, passively sensitized PCLS represents  
330 a mast-cell dependent model to study mainly early allergic airway responses (Ressmeyer  
331 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

355 al, who found different EP<sub>3</sub>-receptor subtypes, which can either be coupled to the G-  
356 protein G<sub>s</sub> or G<sub>i</sub> (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<sub>2</sub> and PGD<sub>2</sub> are known to  
359 mediate airway contractions via the TP receptor (Larsson et al., 2011; McKenniff et al.,  
360 1991), whereas PGD<sub>2</sub> may also cause bronchodilation via the DP<sub>1</sub> 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<sub>2</sub>, PGD<sub>2</sub> or  
363 PGE<sub>2</sub> 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<sub>1</sub> antagonism,  
372 whereas in contrast to the antigen-induced response, COX-1 inhibition had no effect. The  
373 EP<sub>2</sub> and EP<sub>4</sub> receptor antagonist may also affect the serotonin-induced constriction. This  
374 may result is probably depending on the fact that the EP<sub>2</sub> receptor antagonist has similar  
375 affinity to the EP<sub>1</sub> receptor (Buckley et al., 2011). However, apart from the EP<sub>3</sub> 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<sub>2</sub> and EP<sub>4</sub> on smooth muscle

378 cells. For EP<sub>1</sub> antagonism, the strongest effect was found, when both EP<sub>2</sub> and EP<sub>4</sub>  
379 receptors were triggered by endogenous PGE<sub>2</sub> produced by the PCLS during challenge.  
380 In cases where either the EP<sub>2</sub> or EP<sub>4</sub> receptor was blocked this relaxation was reduced.  
381 Again the EP<sub>3</sub> receptor seems to play a minor role also in smooth muscle cells.  
382 Interestingly, the EP<sub>2,4</sub> receptors have been found on human smooth muscle cells (Mori et  
383 al., 2011). From our data, we would assume that the EP<sub>1</sub> receptor must have a role on  
384 smooth muscle cells or mast cells, maybe only in the rat species.

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

401 Thus, while there is some evidence that the COX-2-derived prostanoids might at  
402 least to some extent have been produced in epithelial and smooth muscle cells, it is  
403 tempting to speculate that the effect of the COX-1 inhibitor was occurring in mast cells  
404 that contain both COX-1 and COX-2 (Ermert et al., 1998b). This speculation is based on  
405 our observation that the COX-1 inhibitor had no effect on serotonin-induced  
406 bronchoconstriction or 5-HT<sub>2</sub>AR expression in the present study, and on the finding that  
407 COX-2 inhibitors had no effect on antigen-induced release of PGD<sub>2</sub> from rat mast cells  
408 (Lau and Stenton, 1998). Unfortunately, high basal levels of PGE<sub>2</sub>, TXB<sub>2</sub> and PGD<sub>2</sub> in  
409 the supernatant of the lung slices, made it difficult to interpret the findings in Fig 4,  
410 although the reduced levels of TXB<sub>2</sub> and PGD<sub>2</sub> in FR122047-treated slices could be  
411 explained by the inhibition of COX-1 in mast cells. By note, high levels of PGE<sub>2</sub> are  
412 typical in asthmatic situation, where increased levels of PGE<sub>2</sub> have been measured in  
413 lung tissue and bronchoalveolar lavage fluid (Aggarwal et al., 2010; Krawiec et al.,  
414 2001), representing the pathophysiological situation of asthma.

415

## 416 **5. Conclusions**

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

424 response appears to be modulated by locally formed prostanoids, in particular by PGE<sub>2</sub>,  
425 derived from COX-2 and to some extent from COX-1. Apparently, the mechanisms by  
426 which prostanoids contribute to the early allergic airway response differ among species.  
427 In guinea pigs and humans, prostanoids are primary mediators of the antigen-induced  
428 bronchoconstriction (Larsson et al., 2005; Ressmeyer et al., 2006; Wohlsen et al., 2003),  
429 whereas in the rat lung prostanoids, and especially PGE<sub>2</sub>, act both as primary mediators  
430 of the antigen-induced airway contraction and modulate the serotonin-induced  
431 bronchoconstriction. Interestingly, the EP<sub>4</sub> receptor has a bronchoprotective role during  
432 antigen exposure in this model which correlate with the bronchodilatory results obtained  
433 in man (Benyahia et al., 2012; Buckley et al., 2011), suggesting that the rat may be a  
434 promising test model for asthma therapy with EP<sub>4</sub> agonists.

435

#### 436 **Competing interests**

437 The authors declare that they have no competing interests.

438

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606 **Figure legends**

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

611

612 **Fig 2.** Effect of COX inhibition on ovalbumin-induced bronchoconstriction. Before  
613 airway contractions were induced by ovalbumin (10  $\mu\text{g/ml}$ ), the lung slices were  
614 pretreated with A) the unselective COX inhibitor indomethacin (10  $\mu\text{M}$ , n=15; control  
615 n=16), B) the selective COX-1 inhibitor FR-122047 (5  $\mu\text{M}$ , n=7; control n=7), C) the  
616 selective COX-2 inhibitor celecoxib (10  $\mu\text{M}$ , 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  $\pm$  S.E.M. \*, P<0.05.

620

621 **Fig 3.** Effect of EP, TP and 5-HT<sub>2A</sub> receptor antagonists on contractions to ovalbumin (10  
622  $\mu\text{g/ml}$ ). Before airway contractions were induced by ovalbumin (10  $\mu\text{g/ml}$ ), the lung  
623 slices were pretreated with A) the EP<sub>1</sub> receptor antagonist ONO-8713 (10  $\mu\text{M}$ , n=6;  
624 control n=6), B) the EP<sub>2</sub> receptor antagonist A6809 (5  $\mu\text{M}$ , n=6; control n=6) C) the EP<sub>3</sub>  
625 receptor antagonist L-798,106 (0.5  $\mu\text{M}$ , n=6; control n=6) D) the EP<sub>4</sub> receptor antagonist  
626 L-161,982 (0.5  $\mu\text{M}$ , n=6; control n=6), E) the TP receptor antagonist BAYu3405 (10  $\mu\text{M}$ ,  
627 n=5; control n=5) or F) the 5-HT<sub>2A</sub> receptor antagonist ketanserin (0.1  $\mu\text{M}$ , 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  $\pm$  S.E.M. \*\*, P<0.01.

631

632 **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  
633 compared with initial release in the medium supernatant after pretreatment with selective  
634 and unselective COX inhibitors and exposure to 10  $\mu$ g/ml ovalbumin. Control: Medium;  
635 Control: Ovalbumin 10  $\mu$ g/ml; Indomethacin: Indomethacin 10  $\mu$ M + ovalbumin 10  
636  $\mu$ g/ml; FR122047: FR-122047 5  $\mu$ M + ovalbumin 10  $\mu$ g/ml; Celecoxib: Celecoxib 10  
637  $\mu$ M + ovalbumin 10  $\mu$ g/ml. Data are expressed as the mean  $\pm$  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  $\mu$ M). Before airway contractions were induced by  
642 serotonin, the lung slices were pretreated with A) the COX inhibitor indomethacin (10  
643  $\mu$ M, n=9; control n=9), B) the COX-1 inhibitor FR-122047 (5  $\mu$ M, n=5; control n=5), C)  
644 the COX-2 inhibitor celecoxib (10  $\mu$ M, n=5; control n=5), D) the EP<sub>1</sub> antagonist ONO-  
645 8713 (10  $\mu$ M, n=5; control n=5), E) the EP<sub>2</sub> receptor antagonist A6809 (5  $\mu$ M, n=5;  
646 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  
647 EP<sub>4</sub> receptor antagonist L-161,982 (0.5  $\mu$ M, n=6; control n=6) or H) the TP antagonist  
648 BAYu3405 (10  $\mu$ M, 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  $\pm$  S.E.M. \*, P<0.05;  
651 \*\*, 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

<b>Agonists</b>	<b>Contractions (%)</b>	<b>SEM</b>
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

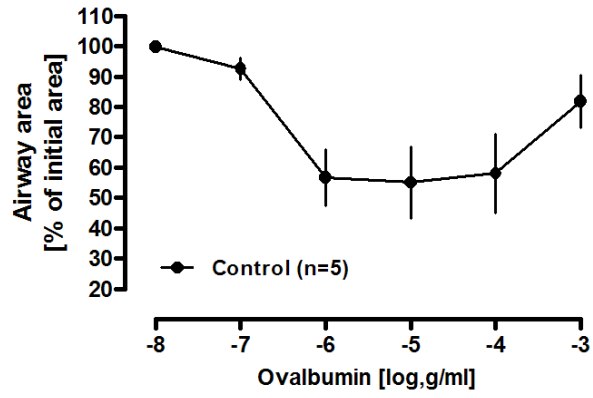
6 the decrease of airway area (%) compared to the initial airway area. Data are presented as

7 mean ± S.E.M.

8



Fig 1A



1B

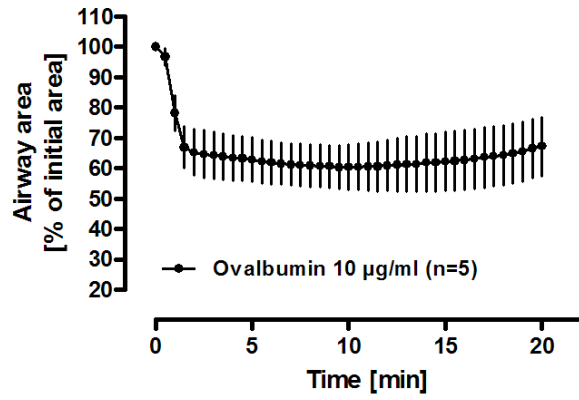
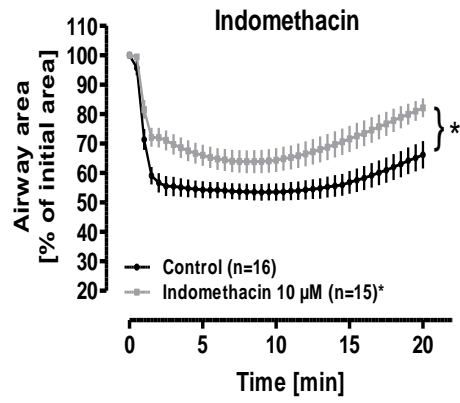
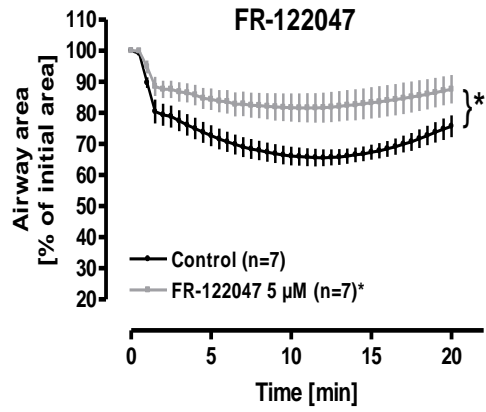


Fig 2A



2B



2C

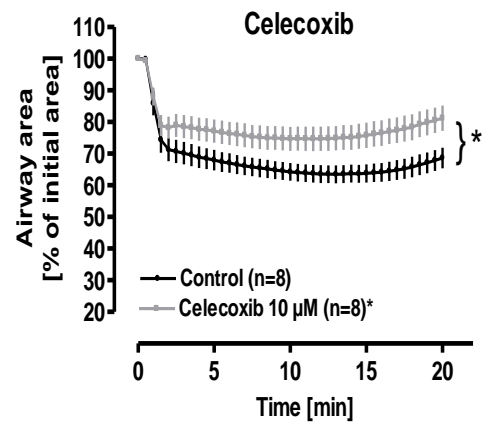


Figure 3

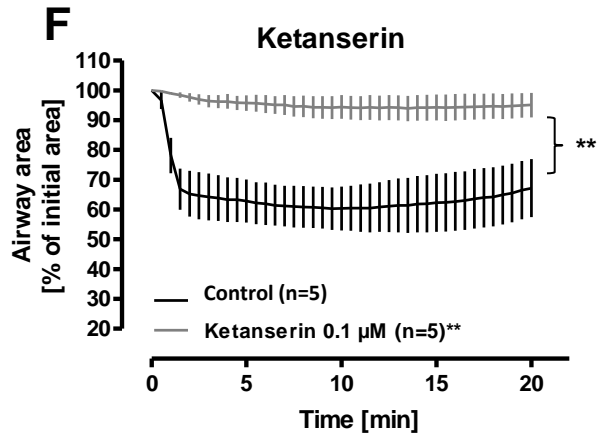
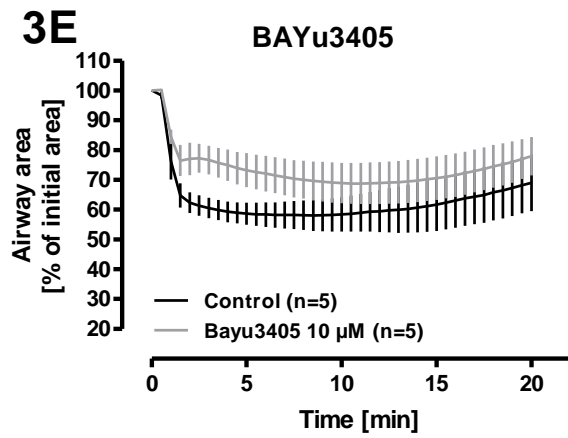
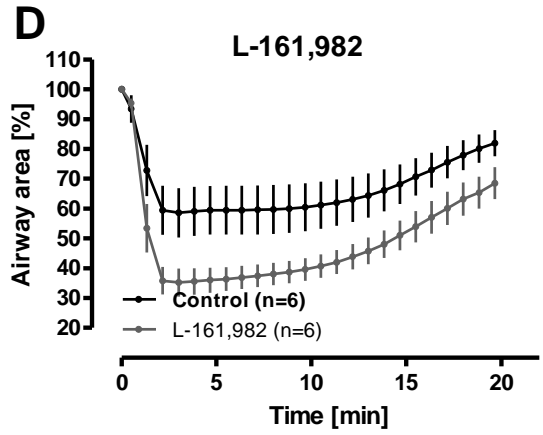
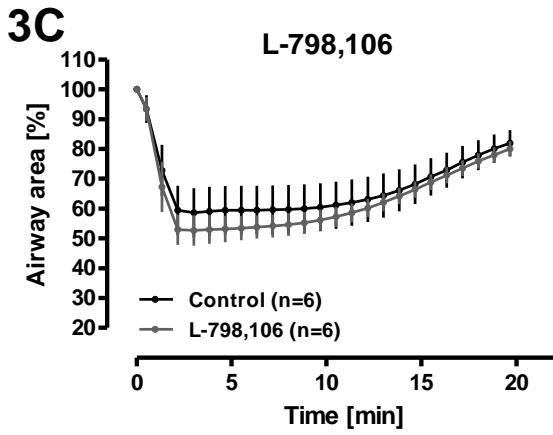
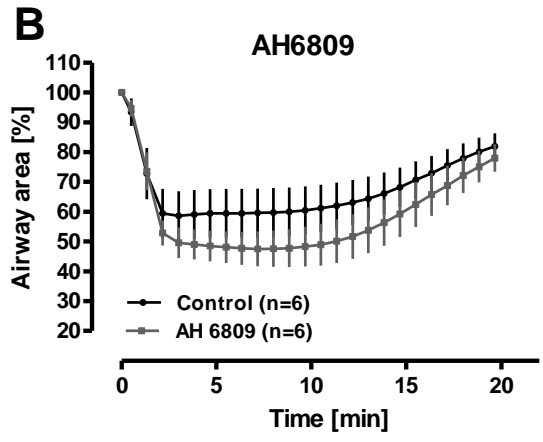
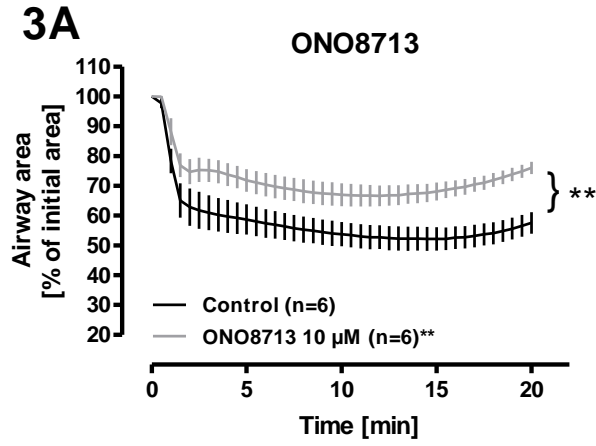


Fig 4

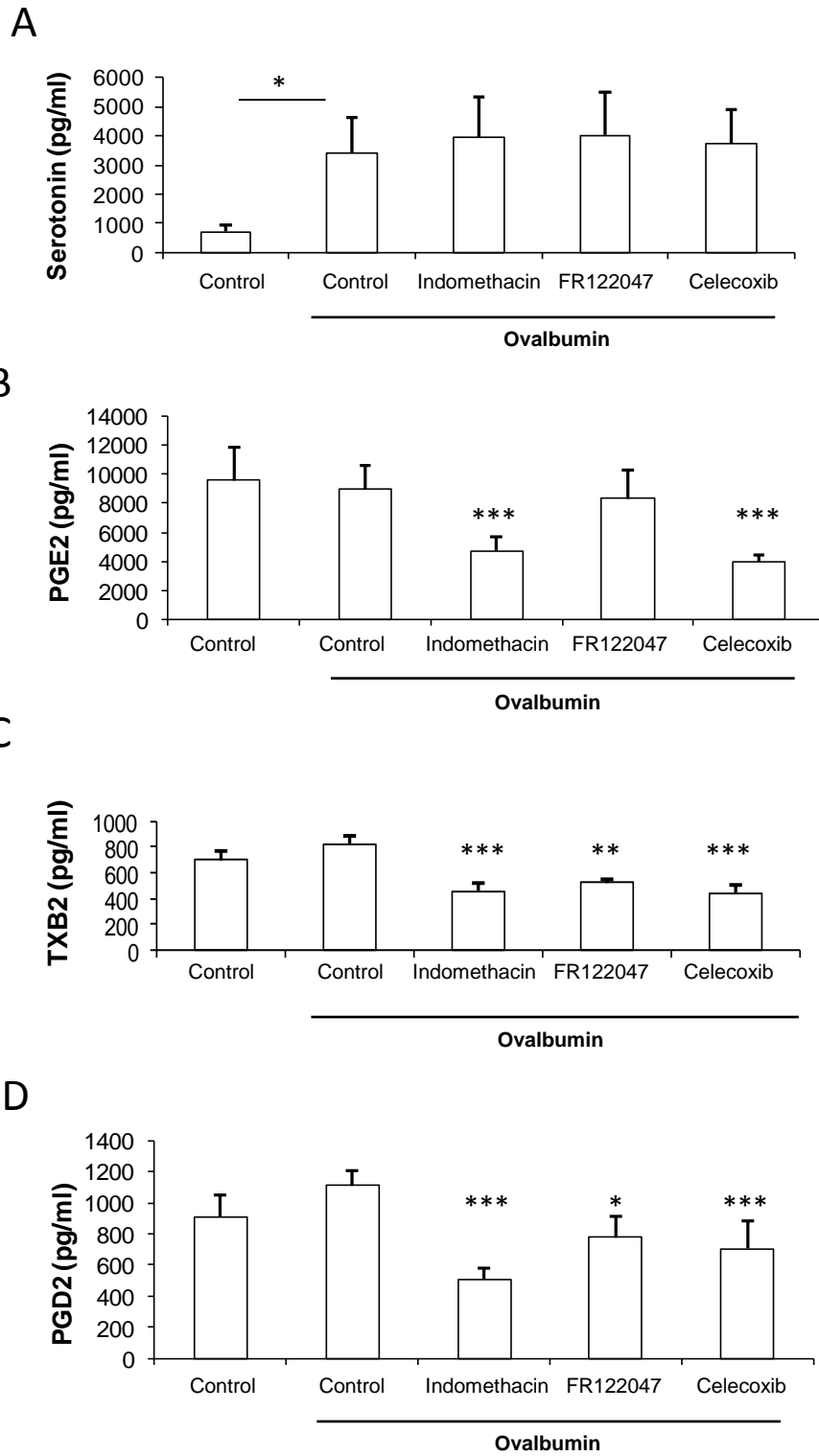


Figure 5

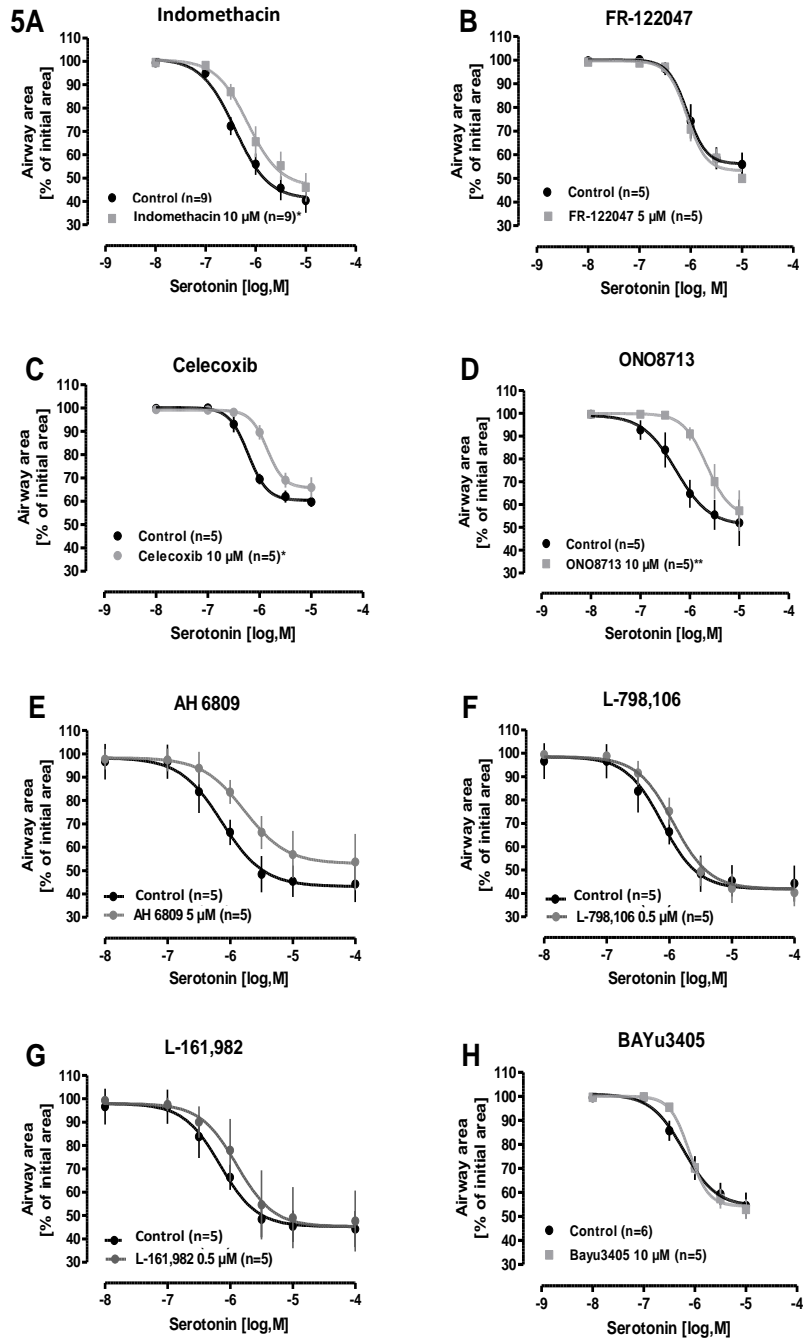


Fig 6

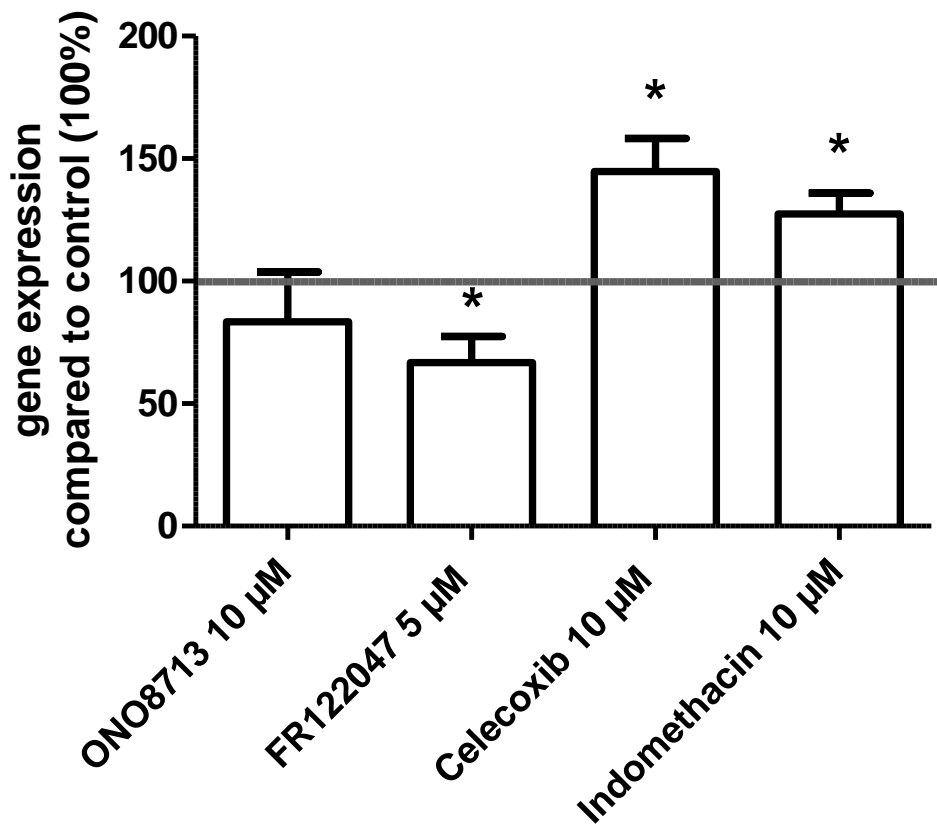


Fig 7

