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RESEARCH PAPER

Vasorelaxation induced by prostaglandin E_2 in human pulmonary vein: role of the EP_4 receptor subtype

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Background and purpose: PGE_2 has been shown to induce relaxations in precontracted human pulmonary venous preparations, while in pulmonary arteries this response was not observed. We investigated and characterized the prostanoid receptors which are activated by PGE_2 in the human pulmonary veins.
Experimental approach: Human pulmonary arteries and veins were cut as rings and set up in organ baths in presence of a TP antagonist. A pharmacological study was performed using selective EP_{1-4} ligands. The cellular localization of the EP_4 receptors by immunohistochemistry and their corresponding transcripts were also investigated in these vessels.
Key results: PGE_2 and the EP_4 agonists (L-902688, ONO-AE1-329) induced potent vasodilatation of the human pulmonary vein, pEC_{50} values: $<7.22 \pm 0.20$, 8.06 ± 0.12 and 7.80 ± 0.09, respectively. These relaxations were inhibited by the EP_4 antagonist GW627368X and not modified in presence of the DP antagonist L-877499. Higher concentrations ($\geqslant 1\ \mu M$) of the EP_2 agonist ONO-AE1-259 induced relaxations of the veins. The EP_4 agonists had no effect on the precontracted arteries. Finally, the EP_1 antagonists ONO-8713 and SC-51322 potentiated the relaxation of the veins induced by PGE_2. EP_4 and EP_1 receptors were detected by immunohistochemistry in the veins but not in the arteries. EP_4 mRNA accumulation was also greater in the veins when compared with the arterial preparations.
Conclusions and implications: Of the 4 EP receptor subtypes, smooth muscle cells in the human pulmonary vein express the EP_4 and EP_1 receptor subtypes. The relaxations induced by PGE_2 in this vessel result from the activation of the EP_4 receptor.
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Keywords: prostaglandin E_2; EP_4 receptor; EP_2 receptor; human pulmonary vein; human pulmonary artery; vasorelaxation

Abbreviations: HPA, human pulmonary artery; HPV, human pulmonary vein; L-NOARG, N^G-nitro-L-arginine; PG, prostaglandin

Introduction

The prostanoids (prostaglandin (PG) and thromboxane (Tx)) derive from arachidonic acid metabolism via COX activities (COX-1 and COX-2, the constitutive and inducible isoforms, respectively). In the vascular system, these metabolites have different physiological roles, they are involved in vascular wall inflammation, angiogenesis and the regulation of vascular smooth muscle tone depending of the receptor subtypes activated. These subtypes include the DP, EP_{1-4}, FP, IP and TP receptors that preferentially respond to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2, respectively.

PGE_2 as well as PGI_2 are the two prostanoids preferentially synthesized by human vascular cells under inflammatory, hypoxic or shear stress conditions (Okahara *et al.*, 1998; Pichiule *et al.*, 2004; Camacho *et al.*, 2007). This increased production is mainly due to the COX-2 and microsomal PG E synthase enzymic activities (Caughey *et al.*, 2001; Uracz *et al.*, 2002).

The prostanoids and PGE_2 are involved in the control of vascular tone in mammals. Activation of the TP, EP_1, EP_3, FP receptor subtypes present on the smooth muscle cells induces vasoconstriction whereas the IP, EP_2, EP_4, DP receptor subtypes are responsible for vasodilatation (Norel, 2007). Although TxA_2 and PGI_2 are well known for their opposing effect on human vascular tone (Norel, 2007), PGE_2 is also responsible either for vasodilatation or vasoconstriction, depending of the PGE_2 receptor subtype (EP_{1-4}) stimulated. Furthermore, PGE_2 may activate the thromboxane receptor (TP) and induce vasoconstriction as reported in human umbilical and cerebral arteries (Boersma *et al.*, 1999; Davis *et al.*, 2004).

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In human pulmonary vessels, the contraction induced by a TP agonist (U46619) and the relaxation produced by the PGI_2 analogues (iloprost, cicaprost) are in agreement with a role of the TP and IP receptor subtypes (Haye-Legrand *et al*., 1987; Norel *et al*., 1991; Walch *et al*., 1999, 2001). Furthermore, in human pulmonary arteries and veins, the stimulation by PGE_2 of the EP_3 and EP_1 receptor subtypes, respectively produces vasoconstriction (Qian *et al*., 1994; Walch *et al*., 2001; Norel *et al*., 2004a). In contrast, in a previous report (Walch *et al*., 1999), relaxant effects of PGD_2 and PGE_2 have been shown in human pulmonary venous preparations, suggesting the presence of DP and EP_2/EP_4 receptor subtypes in this vessel. In this latter report, an attempt was made to pharmacologically determine the EP receptor responsible for the relaxation induced by PGE_2. However, the EP_2/EP_4 agonists and antagonists available at that time did not allow the discrimination between an EP_2 or EP_4 receptor subtype activation (Walch *et al*., 1999). A similar problem was described for the determination of the EP_2/EP_4 receptor responsible for the rabbit jugular vein relaxations induced by PGE_2 (Milne *et al*., 1995). In these studies, the use of AH28848, a low-affinity EP_4 antagonist, and the absence of potent EP_4 selective agonist was a limiting factor. Today, the new agonists (ONO-AE1-329, L-902688; Maruyama and Ohuchida, 2000; Suzawa *et al*., 2000; Billot *et al*., 2003; Young *et al*., 2004) and antagonist (GW627368X; Wilson *et al*., 2006) available for the EP_4 receptor subtype and the EP_2 agonist (ONO-AE1-259; Suzawa *et al*., 2000) are more selective and potent ligands useful for the determination of the EP_2/EP_4 receptor subtype(s) involved in the physiological responses. Therefore, the aim of this study was to identify and characterize the EP receptor subtype(s) involved in the PGE_2-induced vasorelaxation of human pulmonary vein.

Methods

Isolated vascular preparations

All research programs involving the use of human tissue were approved and supported by the INSERM Ethics Committee, and these tissues are considered as surgical waste in accordance with French ethical laws (L. 1211-3–L.1211-9). Human lung tissues were obtained from consenting patients (18 male and 3 female) who had undergone surgery for lung carcinoma. The mean age was 65 ± 02 years. Pulmonary arteries and veins (3- to 6-mm internal diameter) were cut as rings (219 preparations) and set up in 10 mL organ baths containing Tyrode's solution (concentration mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5, gassed with 5% CO_2 and 95% O_2, at 37 °C and pH 7.4. Each ring was initially stretched to an optimal load (1.5–2 g). Changes in force were recorded by isometric force displacement transducer (Narco F-60, Houston, TX, USA) and physiographs (Linseis, Paris, France). Subsequently, preparations were equilibrated (90 min) with bath fluid changes taking place every 10 min.

Contraction/relaxation studies

After the equilibration period, first maximal contraction was induced with noradrenaline (NA, 10 μM); when the preparations reached a plateau, the bath fluid was exchanged at 10-min intervals until the preparations returned passively to their initial resting tone. Subsequently, most of the preparations were incubated (30 min) with Tyrode's solution containing BAY u3405, 10 μM; Norel *et al*., 1991), indomethacin (1.7 μM) and (L-NOARG) (0.1 mM) to prevent TP receptor activation and to inhibit endogenous synthesis of prostanoid and nitric oxide, respectively. In addition to these treatments, the preparations were incubated in the presence or the absence of one of the following prostanoid receptor antagonists: GW627368X (EP_4), ONO-8713 (EP_1; Maruyama and Ohuchida, 2000), AH-6809 (EP_1, EP_2, DP; Keery and Lumley, 1988; Norel *et al*., 1999; Walch *et al*., 2001), L-877499 (DP; Campos *et al*., 2003) and CAY10441 (IP; Clark *et al*., 2004). In some protocols, the human pulmonary veins were incubated only with Tyrode's solution containing BAY u3405 (1 μM) and indomethacin (1.7 μM). After the 30-min incubation period, a second contraction was induced with noradrenaline (10 μM) and when the contraction reached a plateau, cumulative concentrations of PGE_2 or EP selective agonists were added to the baths every 2–4 min during about 20 min. With ONO-AE1-259, the induced relaxation was similar to an increased spontaneous relaxation, as there was no inflexion point after addition of a new dose (excepted for the last two concentrations).

RT-PCR and Southern blot analysis

The human pulmonary vascular preparations were disrupted in guanidinium isothiocyanate, using a Polytron apparatus. RNAs were isolated using ultracentrifugation on CsCl. The identification of the EP_4 receptor and glyceraldehyde 3-phosphate dehydrogenase transcripts were performed by reverse transcriptase-polymerase chain reaction (RT-PCR) using 32 and 22 cycles, respectively and confirmed by Southern blot analysis after hybridization with ^{32}P-end-labelled internal probes of RT-PCR and autoradiography. The primers and probes used to amplify the EP_4 receptor were forward primer, 5′-tggtatgtgggctggctg-3′; reverse primer, 5′-gaggacggtggcgagaat-3′; probe, 5′-cctcacgctctttgcagtct-3′.

Immunohistochemistry

Transverse slices (5 μM) of human pulmonary vessels were obtained from paraffin-embedded preparations. The sections were submitted to high temperature (80 °C) antigen-unmasking technique using Vector's solution (H-3300). Cayman rabbit antibodies directed against the human EP_1, EP_2 or EP_4 (C-Term) receptor subtypes were used as primary antibodies (1/100; overnight 4 °C). In addition, some slices were incubated with a rabbit non-immune antibody (Dako Cytomation, Trappes, France) or with the Cayman anti-EP_4 primary antibody and the respective blocking peptide. Biotinylated anti-rabbit was the secondary antibody, and peroxidase Vectastain Elite ABC kits were used for detection followed by haematoxylin treatment for cell nuclei staining.

Data analysis

Contraction/relaxation studies: The effects induced by the different agonists were expressed in gram (g) or normalized

(%) with respect to the second noradrenaline precontraction. The data are positive for the contractions and negative for the relaxations. Where possible, a four-parameter logistic equation of the form: $E = \frac{E_{max}[A]^{nH}}{EC_{50}^{nH}+[A]^{nH}}$ was fitted to data obtained from each organ bath protocols to provide estimates of the maximal relaxation (E_{max}) produced by 10 μM of the EP receptor ligands (A), the half-maximum effective concentration values (EC_{50}), as well as Hill slope (nH) parameters. All results were analysed using SigmaPlot Jandel software (version 7.101). The pEC_{50} values were calculated as the negative log of EC_{50} values. The equilibrium dissociation constant for the antagonist (K_B) was calculated using the following equation: $K_B = [B]/(DR-1)$, where [B] is the concentration of the antagonist and DR (dose ratio) is the ratio of EC_{50} of agonist in the presence and absence of antagonist. The affinity of the antagonist (pK_B) was calculated as the negative log of the K_B value.

All data are means ± s.e.mean derived from (n) lung samples, and statistical analysis on the curves were performed using two-way ANOVA followed by Student–Newman–Keuls test or Student's t-test for the pEC_{50} values with a confidence level of 95%.

Compounds

L-902688 and L-877499 were gifts from Merck (Kirkland, Canada). ONO-AE1-259 ((16*S*)-9-deoxy-9β-chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-didehydro PGE_2), ONO-AE1-329 (16-(3-methoxymethyl)phenyl-ω-tetranor-3,7-dithia PGE_1) and ONO-8713 (4-[2-[*N*-isobutyl-*N*-(2-furylsulphonyl)amino]-5-trifluoromethylphenoxy-methyl] cinnamic acid) were gifts from Ono Pharmaceutical Co. (Osaka, Japan). GW627368X ((*N*-[2-[4-(4,9-diethoxy-1-oxo-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)phenyl]acetyl] benzene sulphonamide) was a gift from GlaxoSmithKline (Stevenage, UK). CAY10441 (4,5-dihydro-1*H*-imidazol-2-yl)-[4-(4-isopropoxy benzyl) phenyl] amine, AH-6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid), PGE_2 and primary antibodies were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). SC-51322 was provided by Biomol, Exeter, UK. BAY u3405 (3(*R*)-3-(4-fluorophenylsulphonamido)-1,2,3,4-tetrahydro-9-carbazole propanoic acid) was a gift from Bayer (Stokes Poges, UK). Molecular biology compounds and oligonucleotides were from Eurogentec (Angers, France). Noradrenaline, N^G-nitro-L-arginine (L-NOARG) and indomethacin were purchased from Sigma Chemical Co. (St Louis, MO, USA). All these compounds were dissolved in ethanol, dimethyl sulphoxide or Tyrode's solution at 0.1 mM. Finally, the drug/molecular target nomenclature (receptors) conforms with BJP's Guide to Receptors and Channels (Alexander *et al*., 2008).

Results

Contraction/relaxation studies

The first noradrenaline-induced contractions were 1.42 ± 0.15 g ($n = 20$) in human pulmonary veins and 1.22 ± 0.39 g ($n = 5$) in arteries. The second noradrenaline-induced contractions in the presence of indomethacin, L-NOARG and BAY u3405 were 1.94 ± 0.19 g ($n = 20$) in human pulmonary veins and 1.55 ± 0.35 g ($n = 5$) in pulmonary arteries, these second set of contractions being significantly increased in comparison with initial response. The incubations with BAY u3405 (10 μM), indomethacin (1.7 μM) and L-NOARG (0.1 mM) on the basal tone of human pulmonary vein and artery induced frequent, small but sustained contractions (< 0.5 g; 30 min) principally in the human pulmonary vein. The other additional treatments did not significantly modify the basal tone or the second contraction induced by noradrenaline (10 μM). The relaxations were not modified after incubation of the human pulmonary veins with either ethanol or dimethyl sulphoxide (1/1000; 30 min, data not shown).

The data presented in Figure 1a show potent and dose-dependent relaxations induced by PGE_2 and the two EP_4 selective agonists (L-902688, ONO-AE1-329) in isolated human pulmonary veins whereas the results presented in Figure 1b show the absence of relaxation induced by these synthetic agonists in arterial rings. In contrast, in the human pulmonary vein, relaxations were only observed with the highest doses (⩾ 1 μM) of ONO-AE1-259, the selective agonist for the EP_2 receptor subtype (Figure 2). When the venous preparations reached a plateau after the second noradrenaline-induced contraction, in the absence of EP agonist stimulation, a small spontaneous relaxation (−6 ± 4%; $n = 6$; Figure 2) was observed after 20 min (Figure 2). However, with ONO-AE1-259 (< 1 μM), an increased spontaneous relaxation was observed (−12%; Figure 2). The pEC_{50}, nH and E_{max} values derived from the dose-dependent relaxations induced by the different EP agonists in human pulmonary veins are presented in Table 1. With the exception of the PGE_2 concentration–response curves, the other EP agonist curves exhibit a Hill slope significantly not different from unity.

The human pulmonary vein sensitivities to ONO-AE1-329, L-902688 or PGE_2 were significantly reduced when the preparations were treated with GW627368X (1 and 10 μM; Figure 3 and Table 1). The E_{max} values obtained with ONO-AE1-329 and L-902688 in presence of GW627368X (1 μM) were not significantly different from the respective control values; in contrast, in the presence of GW627368X (10 μM), the E_{max} values were significantly increased (Table 1). For this reason, a pK_B value for GW627368X (1 μM) was calculated when the relaxations were induced by ONO-AE1-329 (pK_B value = 7.06 ± 0.21; $n = 6$) or by L-902688 (pK_B value = 6.58 ± 0.28; $n = 6$). The relaxation induced by ONO-AE1-329 was not affected by the presence of the DP antagonist L-877499. The treatments with 10 μM of the prostanoid receptor antagonists (L-877499 or AH-6809) blocked partially the relaxations induced by the highest concentrations of ONO-AE1-259 (10 μM) in human pulmonary veins (Figure 2 and Table 1). In contrast, CAY10441, the selective IP antagonist, significantly potentiated the relaxations induced by ONO-AE1-259 or ONO-AE1-329 (Table 1).

The PGE_2-induced relaxations in human pulmonary veins were significantly increased in the presence of the two EP_1 selective antagonists ONO-8713 or SC-51322 (Figure 4 and Table 1); similar significant results were obtained in absence of L-NOARG (0.1 mM; $E_{max} = -62 \pm 07\%$; $n = 5$). In contrast, when the noradrenaline-precontracted venous preparations

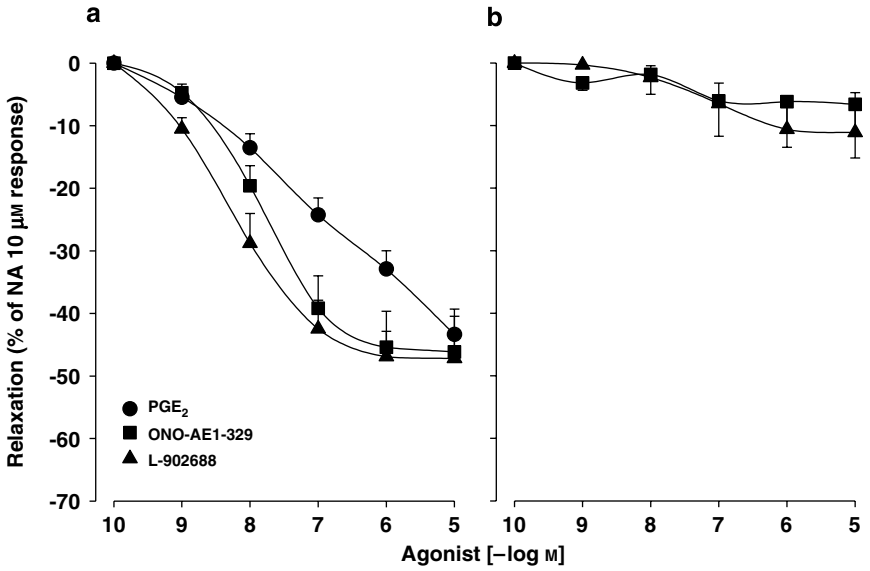

Figure 1 Cumulative concentration–response curves induced by EP agonists in human pulmonary veins (**a**) and arteries (**b**) precontracted with noradrenaline (NA, 10 μM; second contraction). All the preparations were treated (30 min) with BAY u3405 (10 μM), indomethacin (1.7 μM) and L-NOARG (0.1 mM). Responses are expressed in per cent of the precontraction; values are means ± s.e.mean derived from 13–17 and four different lung samples for veins and arteries, respectively.

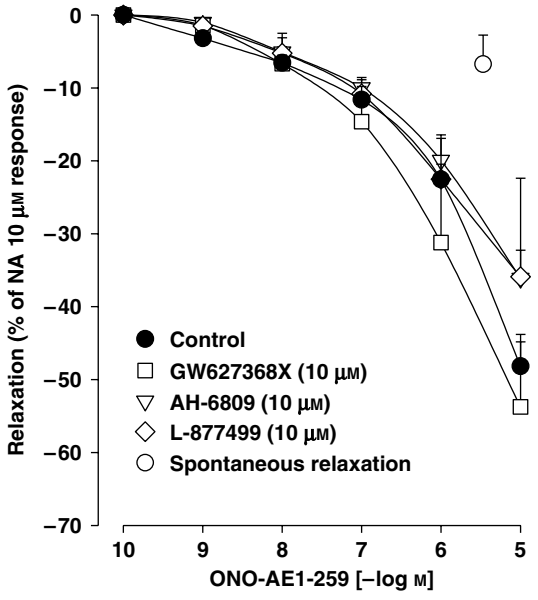

Figure 2 Cumulative concentration–response curves induced by ONO-AE1-259 in human pulmonary veins. The preparations were incubated (30 min) with Tyrode's solution containing (indomethacin, 1.7 μM; BAY u3405, 10 μM; L-NOARG, 0.1 mM): Control or in addition with GW627368X, AH-6809 or L-877499. Subsequently, the preparations were contracted with noradrenaline (NA, 10 μM; second contraction), and cumulative concentrations of ONO-AE1-259 were added into the baths. Spontaneous relaxation was measured 20 min after the contraction had reached a plateau (without EP agonist). Responses are expressed as per cent of the precontraction; values are means ± s.e.mean derived from 3–10 lung samples (see Table 1 for pEC_{50}, E_{max} values and statistics).

were not incubated with BAY u3405, indomethacin and L-NOARG, low concentration of PGE_2 induced relaxations whereas the higher doses ($\geqslant 0.1\,\mu M$) induced contractions ($E_{max} = +21 \pm 18\%$; $n = 4$).

RT-PCR and Southern blot analysis

Southern hybridization of RT-PCR products revealed higher levels of the transcript corresponding to the EP_4 receptor subtype in the human pulmonary vein in comparison to the artery whereas glyceraldehyde 3-phosphate dehydrogenase transcripts were the same in both the preparations (Figure 5).

Immunohistochemistry

Representative immunohistochemical experiments derived from 25 paraffin sections, taken from five tissue samples, are presented in Figure 6. They were performed on human pulmonary vascular preparations using polyclonal antibodies for the EP_1, EP_2 or EP_4 receptor subtypes. In presence of the EP_4 receptor antibody incubated with the blocking peptide or in the absence of primary antibody (data not shown), the results were similar to those obtained with a non-immune antibody (Figure 6). There was strong staining for the EP_4 and EP_1 receptor subtypes in the human pulmonary veins (Figure 6). EP_4 and EP_1 receptor staining was mainly observed within the media on the smooth muscle cells whereas a very low staining was observed with the EP_2 antibody (Figure 6). In addition, in human pulmonary arteries, little or no staining was observed with the EP antibodies used (Figure 6).

Discussion

The present report suggests that the PGE_2-induced relaxations of human pulmonary venous preparations are due to the activation of the EP_4 receptor subtype. In addition, our data, in the presence of a TP antagonist, demonstrate a dual role for PGE_2 as the EP_4-mediated relaxations are reduced by the simultaneous activation of the EP_1 receptors, which are

Table 1 Effects of EP, DP and IP antagonists on the relaxations induced by EP agonists in human pulmonary veins precontracted with noradrenaline

EP agonist	*Antagonist treatment*	n	*nH*	pEC_{50}	E_{max} (%)
PGE_2	Control	17	0.69 ± 0.05*	< 7.22 ± 0.20	−43 ± 04
	ONO-8713 (10 μM)	7	0.89 ± 0.05	7.90 ± 0.09	−51 ± 07
	SC-51322 (10 μM)	5	0.76 ± 0.07*	7.75 ± 0.22	−45 ± 08
	GW627368X (1 μM)	5	1.19 ± 0.58	6.37 ± 0.24*	−32 ± 11
	GW627368X (10 μM)	3	3.99 ± 2.82	< 5.76 ± 0.14*	−51 ± 15*
ONO-AE1-329	Control	13	0.89 ± 0.05	7.80 ± 0.09	−46 ± 06
	GW627368X (1 μM)	6	0.96 ± 0.06	6.85 ± 0.30*	−56 ± 10
	GW627368X (10 μM)	5	0.71 ± 0.09*	6.01 ± 0.23*	−63 ± 13*
	L-877499 (10 μM)	3	0.94 ± 0.19	7.79 ± 0.21	−39 ± 07
	CAY10441 (10 μM)	4	0.84 ± 0.09	< 7.60 ± 0.20	−76 ± 07*
L-902688	Control	15	0.95 ± 0.11	8.06 ± 0.12	−47 ± 04
	GW627368X (1 μM)	6	0.81 ± 0.15	7.38 ± 0.20*	−39 ± 07
	GW627368X (10 μM)	4	0.60 ± 0.07	< 6.08 ± 0.22*	−59 ± 13*
ONO-AE1-259	Control	10	0.58 ± 0.07	< 6.07 ± 0.05	−48 ± 04
	GW627368X (10 μM)	3	0.79 ± 0.23	< 6.24 ± 0.23	−54 ± 09
	L-877499 (10 μM)	3	0.68 ± 0.13	< 6.52 ± 0.20	−36 ± 14*
	AH-6809 (10 μM)	3	0.60 ± 0.11	< 6.15 ± 0.03	−36 ± 04*
	CAY10441 (10 μM)	3	0.85 ± 0.24	< 6.51 ± 0.48	−83 ± 18*

The human venous preparations were treated for 30 min with indomethacin (1.7 μM), L-NOARG (0.1 mM), BAY u3405 (10 μM) and with one of the indicated antagonists. The maximal relaxations (E_{max}) induced by the EP agonists (10 μM) are expressed as % of the noradrenaline precontraction. The half-maximum effective concentration values (EC_{50}) as well as Hill slope (*nH*) parameters are presented. Values are means ± s.e.mean derived from (*n*) lung samples. *Data significantly different ($P < 0.05$) from respective control values (ANOVA).

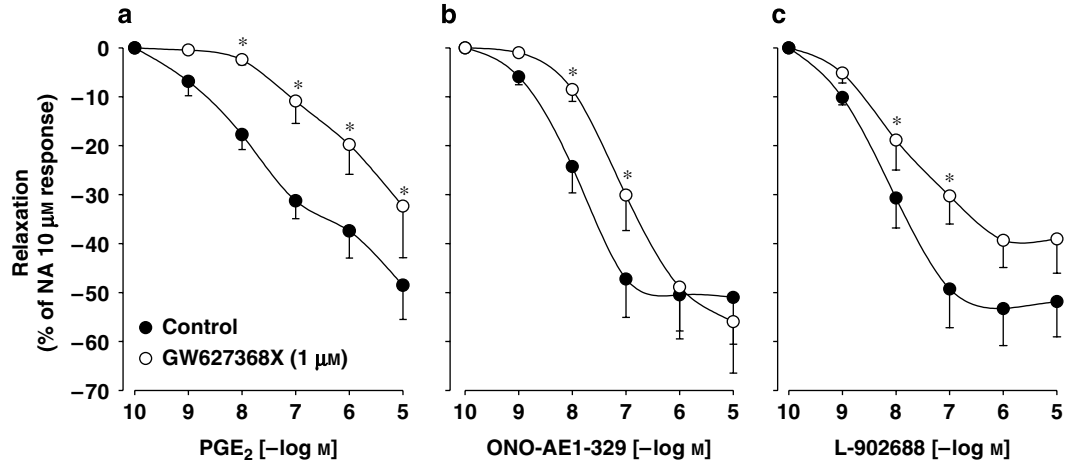

Figure 3 Effect of GW627368X (1 μM) on the relaxations induced by EP_4 agonists in human pulmonary veins. Paired preparations from each individual were incubated (30 min) with Tyrode's solution containing (indomethacin, 1.7 μM; BAY u3405, 10 μM; L-NOARG, 0.1 mM): Control or in addition with GW627368X (1 μM). Subsequently, the preparations were contracted with noradrenaline (NA, 10 μM; second contraction), and cumulative concentrations of PGE_2 (**a**; $n = 5$), ONO-AE1-329 (**b**; $n = 6$) or L-902688 (**c**; $n = 6$) were added into the baths. Responses are expressed as per cent of the precontraction; values are means ± s.e.mean derived from three to five lung samples. *Data significantly different from the respective control data (ANOVA; see Table 1 for pEC_{50} and E_{max} values).

responsible for vasoconstriction in the human pulmonary vein (Walch *et al*., 2001).

In the present study, the human pulmonary vein relaxations induced by the two EP_4 agonists (ONO-AE1-329, L-902688) and their inhibition by the EP_4 antagonist (GW627368X) are in agreement with the involvement of the EP_4 receptor subtype. Furthermore, the relaxations induced by PGE_2 were also inhibited by GW627368X. In this relaxation, the involvement of an EP_2 receptor can be excluded as the relaxations induced by the EP_2 agonist (ONO-AE1-259) were in part comparable to an increased spontaneous relaxation. Similarly, in a previous study (Walch *et al*., 1999), we had demonstrated that butaprost, another selective EP_2 agonist, induced relaxation curves with very-low potency (> 1 μM). These results obtained with butaprost are in accordance with an effect mediated via the EP_4 receptor subtype, as previously reported in human uterine and cerebral vessels (Baxter *et al*., 1995; Davis *et al*., 2004). In addition, the human pulmonary veins were at least 50 times less sensitive to the EP_2 agonist (ONO-AE1-259) than to the EP_4 agonist (ONO-AE1-329), whereas the two former agonists exhibit the same EC_{50} or K_i values for their

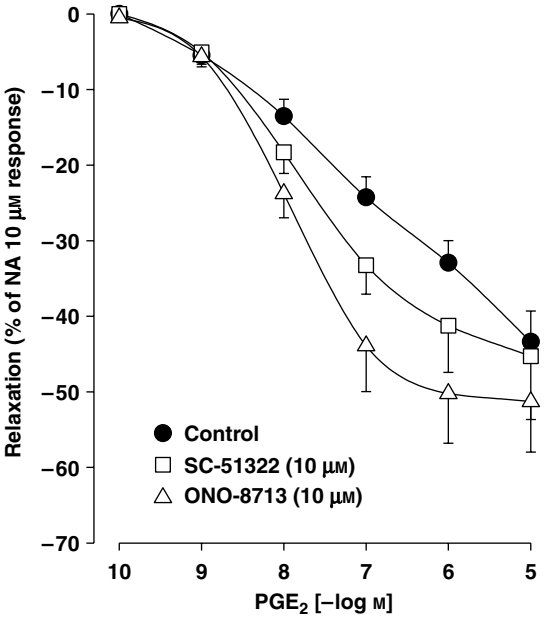

Figure 4 Effect of ONO-8713 or SC-51322 (10 μM) on the relaxations induced by PGE_2 in human pulmonary veins. The preparations were incubated (30 min) with Tyrode's solution containing (indomethacin, 1.7 μM; BAY u3405, 10 μM; L-NOARG 0.1 mM): Control or in addition with one of the EP_1 antagonists. Subsequent to these incubation periods, the preparations were contracted with noradrenaline (NA, 10 μM; second contraction), and cumulative concentrations of PGE_2 were added into the baths. Responses are expressed as per cent of the precontraction; values are means ± s.e.mean derived from paired preparations ($n = 5$–17; see Table 1 for pEC_{50} and E_{max} values and statistics).

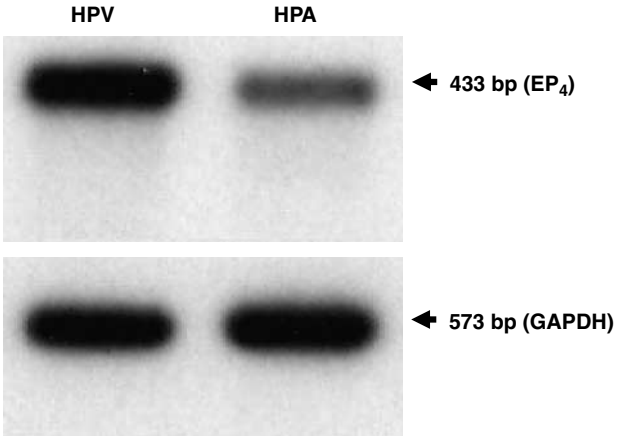

Figure 5 Southern hybridization of RT-PCR products showing the relative levels of expression of the EP_4 receptor subtype and GAPDH in human pulmonary vein (HPV) and artery (HPA). The arrows indicate the expected products of RT-PCR. The primers and probes used are described in the Methods section. The exposure time was 3 h with intensifying screen. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

respective recombinant mouse receptors expressed in CHO cells (Maruyama and Ohuchida, 2000; Suzawa *et al*., 2000). Finally, a contribution of the EP_2 and DP receptor subtypes could be excluded during the relaxations induced by PGE_2 or ONO-AE1-329 as these relaxations were unaffected by treatment with the DP/EP_2 antagonist AH-6809 (Walch *et al*., 1999) or with the DP antagonist L-877499 (Table 1).

The sensitivity of human pulmonary veins during the relaxation induced by PGE_2 was significantly increased in the presence of the EP_1 antagonists ONO-8713 or SC-51322 (Figure 4 and Table 1). These results are in agreement with a contractile activity induced by PGE_2 via the EP_1 receptor in the human pulmonary vein as previously described (Walch *et al*., 2001). An EP_1 antagonism may also explain the potentiated maximal relaxation observed with ONO-AE1-329 and PGE_2 in the presence of a high concentration of GW627368X (10 μM). This EP_4 antagonist has some EP_1 affinity as indicated in functional assays using recombinant prostanoid receptors (Wilson *et al*., 2006). For this reason, the inhibition of a contractile component in the concentration–response curves induced by PGE_2 or ONO-AE1-329 will result in increased relaxations. The EP_1 antagonism observed with GW627368X (10 μM) also implicates an EP_1 agonist effect for the high concentrations (> 1 μM) of ONO-AE1-329 or L-902688. These results suggest that high concentrations of either of the EP_4 agonists may bind to the human EP_1 receptor subtype.

From the pharmacological protocols performed with L-902688 or ONO-AE1-329, the pK_B values calculated for GW627368X were about 10-fold lower when compared with the value obtained in functional assays at human recombinant prostanoid EP_4 receptors expressed in HEK293 cells (Wilson *et al*., 2006). This discrepancy could be explained by the use of vascular preparations containing EP_1 and EP_4 receptors instead of isolated cells expressing only one recombinant receptor subtype.

The increase in PGE_2-induced relaxations in the presence of L-NOARG suggests a partial involvement of nitric oxide in the relaxations of human pulmonary veins. This role for nitric oxide is not specifically related to the PGE_2-induced relaxation, as the ACh vasodilatations (Norel *et al*., 2004b), the second noradrenaline contraction and the basal tone described in the present report were also modified in the presence of L-NOARG. Finally, in the absence of treatment with BAY u3405, indomethacin and L-NOARG, high concentrations of PGE_2 induced vasoconstriction of the human pulmonary veins, suggesting an activation of the TP receptor and a predominance of the contractile effect versus the relaxant effect of PGE_2, as reported in the human cerebral arteries (Davis *et al*., 2004).

The maximal relaxations induced by ONO-AE1-259 were significantly inhibited by the DP/EP_2 antagonist AH-6809 or with the DP antagonist L-877499. These effects of the high concentrations of ONO-AE1-259 could be due to non-selective activation of the DP receptor subtype previously described in the human pulmonary veins (Walch *et al*., 1999). The significantly increased relaxations obtained with ONO-AE1-329 or ONO-AE1-259 in the presence of the IP antagonist (CAY10441) suggest the absence of any activation of the IP receptor by these agonists and perhaps an EP_1 antagonist effect of CAY10411.

The vasodilatations due to the activation of the EP_4 receptor subtype were observed *in vitro* in two other human vessels: in the uterine arteries (Baxter *et al*., 1995) and the

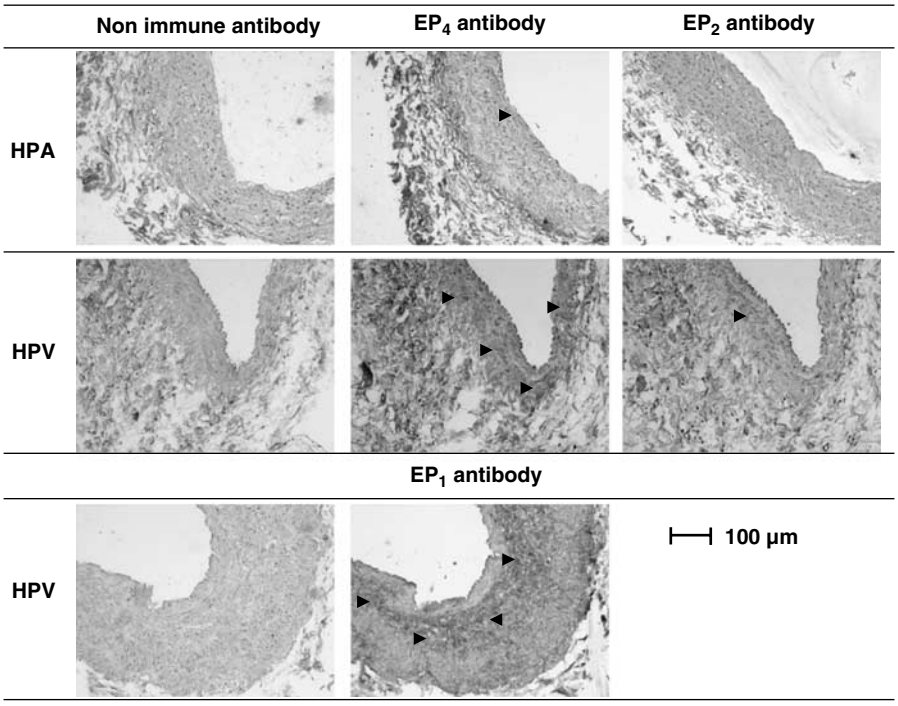

Figure 6 Immunohistochemistry was performed on paraffin serial sections using anti-EP_4, anti-EP_2, anti-EP_1 or non-immune antibodies. Significant staining was mainly detected in the smooth muscle layer (black arrowheads) of the human pulmonary vein (HPV) with the EP_4- or EP_1- and not EP_2-antibody. Low staining or nonspecific staining was observed in the human pulmonary artery (HPA). Representative results derived from $n=5$, 25 paraffin sections analysed.

middle cerebral arteries (Davis *et al*., 2004). However, in both vessels, in the presence of a TP antagonist, PGE_2 was a more potent relaxant agonist ($pEC_{50}=8.0$) than in human pulmonary vein ($pEC_{50}=7.2$; present report). In the uterine and cerebral arteries, only the EP_4 receptor subtype is stimulated whereas in human pulmonary vein, both EP_4 and EP_1 receptors are activated. This explanation is supported by the greater sensitivities to PGE_2 ($pEC_{50}=7.75$ and 7.90) observed in human pulmonary veins treated with the EP_1 antagonists. The results presented in the present report show an EP_4 receptor responsible for vasodilatation in the human pulmonary veins and there may be other human veins with similar EP_4 receptor control of the vascular tone. The human saphenous vein for example, may be an appropriate candidate as relaxations mediated via the EP_4 receptors have been described in this tissue isolated from piglet, rabbit and guinea pig (Coleman *et al*., 1994; Jones and Chan, 2005; Wilson and Giles, 2005; Wilson *et al*., 2006).

Some roles for the EP_4 receptor *in vivo* have been described in different blood vessels. In dogs, i.v. injection of $10\,ng\,kg^{-1}$ per minute of a selective EP_4 agonist (ONO-4819) produced an increase of the vessel diameter and the blood flow in the chronically compressed cauda equina (Sekiguchi *et al*., 2006). In isolated ductus arteriosus preparations (Smith *et al*., 1994; Smith, 1998) or in infants with certain cardiac malformations where ductal patency is maintained by i.v. injection of PGE_1, the vasodilatations are due to the activation of the EP_4 and IP receptor subtypes (Leonhardt *et al*., 2003b). In the treatment of the human pulmonary hypertension, iloprost (Wolff *et al*., 2007) and PGE_1 (von Scheidt *et al*., 2006) are the classically used IP/EP_1 agonists. However, recently, iloprost and cicaprost, another IP selective agonist, have been described as full agonists at human prostanoid EP_4 receptors (Abramovitz *et al*., 2000; Wilson *et al*., 2004; Wilson and Giles, 2005). Our results on human pulmonary veins that contain IP, EP_1 and EP_4 receptor subtypes suggest that *in vivo* dual selective agonists for the IP/EP_4 receptors such as cicaprost may be more useful as antihypertensive drugs in the lung, inducing exclusively vasodilatation and blood flow increase.

The involvement of the EP_4 receptor in the relaxation of human pulmonary vein induced by PGE_2 was confirmed by the detection of EP_4 mRNA transcript and protein in this preparation. The higher expression of EP_4 transcripts in the pulmonary venous preparations in comparison to the arterial preparations derived from the same patient is in accordance with the relaxations induced by PGE_2, ONO-AE1-329 or L-902688 observed in the veins and not in the arteries (present report; Walch *et al*., 1999). Furthermore, the strong expression of the EP_4 receptor and localization in the smooth muscle layer of the human pulmonary vein and not in the artery are also in agreement with the involvement of the EP_4 receptor during the venous relaxations induced by PGE_2. Similarly, the immunostaining obtained with the EP_1 antibody is in accordance with the EP_1 receptor-mediated contraction observed in the human pulmonary veins (present report; Walch *et al*., 2001). Finally, the EP_4 receptor has also been detected by immunohistochemistry in other human vascular smooth muscles such as myometrial vessels (Leonhardt *et al*., 2003a) or glomerular arteries (Therland

et al., 2004). In human kidney, EP_4 receptor labelling was colocalized with COX-2 in the smooth muscle cells of glomerular arteries where the EP_4 receptor activation may be associated with the control of renal blood flow, as reported in different animal models (Hao and Breyer, 2007). Finally, the EP_4 receptor subtype has been described in several processes related to vascular wall remodelling during ductus arteriosus closure (Yokoyama *et al*., 2006) and during different pathologies such as aneurysm and the late phase of atherosclerosis (Bayston *et al*., 2003; Cipollone *et al*., 2005).

In conclusion, our study provides strong evidence for the involvement of the EP_4 receptor subtype in the PGE_2-induced relaxation of the human pulmonary vein. Finally, in this preparation, the contraction and the relaxation induced by prostanoids are mediated by TP, EP_1 and IP, DP, EP_4 receptor subtypes, respectively. These findings may be relevant for the treatment of pulmonary hypertension where vasodilatations are induced by synthetic prostanoids. These compounds may be more efficient if they are exclusively specific for receptor subtypes involved in the vasodilatation (IP, DP and/or EP_4).

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Conflict of interest

The authors state no conflict of interest.

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