

## RESEARCH PAPER

## Virodhamine relaxes the human pulmonary artery through the endothelial cannabinoid receptor and indirectly through a COX product

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**Background and purpose:** The endocannabinoid virodhamine is a partial agonist at the cannabinoid CB<sub>1</sub> receptor and a full agonist at the CB<sub>2</sub> receptor, and relaxes rat mesenteric arteries through endothelial cannabinoid receptors. Its concentration in the periphery exceeds that of the endocannabinoid anandamide. Here, we examined the influence of virodhamine on the human pulmonary artery.

**Experimental approach:** Isolated human pulmonary arteries were obtained during resections for lung carcinoma. Vasorelaxant effects of virodhamine were examined on endothelium-intact vessels precontracted with 5-HT or KCl.

**Key results:** Virodhamine, unlike WIN 55,212-2, relaxed 5-HT-precontracted vessels concentration dependently. The effect of virodhamine was reduced by endothelium denudation, two antagonists of the endothelial cannabinoid receptor, cannabidiol and O-1918, and a high concentration of the CB<sub>1</sub> receptor antagonist rimonabant (5 μM), but only slightly attenuated by the NOS inhibitor L-NAME and not affected by a lower concentration of rimonabant (100 nM) or by the CB<sub>2</sub> and vanilloid receptor antagonists SR 144528 and capsazepine, respectively. The COX inhibitor indomethacin and the fatty acid amide hydrolase inhibitor URB597 and combined administration of selective blockers of small (apamin) and intermediate and large (charybdotoxin) conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels attenuated virodhamine-induced relaxation. The vasorelaxant potency of virodhamine was lower in KCl- than in 5-HT-precontracted preparations.

**Conclusions and implications:** Virodhamine relaxes the human pulmonary artery through the putative endothelial cannabinoid receptor and indirectly through a COX-derived vasorelaxant prostanoid formed from the virodhamine metabolite, arachidonic acid. One or both of these mechanisms may stimulate vasorelaxant Ca<sup>2+</sup>-activated K<sup>+</sup> channels.

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**Keywords:** endothelial cannabinoid receptor; virodhamine; cannabidiol; O-1918; K<sub>Ca</sub> channels; human pulmonary artery

**Abbreviations:** abn-cbd, abnormal cannabidiol; Apa, apamin; Chtx, charybdotoxin; DMSO, dimethyl sulphoxide; FAAH, fatty acid amide hydrolase; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; O-1918, 1,3-dimethoxy-5-methyl-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene; SR 144528, N-[(1S)-endo-1,3,3-trimethyl bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; URB597, 3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate; WIN 55,212-2, R(+) [2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)-methanone mesylate

## Introduction

Acute and chronic use of cannabis in human elicits complex cardiovascular effects (Jones, 2002). In addition, an increase in the levels of endogenously formed cannabinoids has been reported in the serum of patients with septic shock (Wang

*et al.*, 2001) and with liver cirrhosis (Fernández-Rodríguez *et al.*, 2004). In this respect, it is important to note that endogenous cannabinoids are involved in the hypotension occurring in animal models of the latter diseases (see Pacher *et al.*, 2006). The mechanisms underlying the cardiovascular effects of exo- and endocannabinoids have been examined mainly in experimental animals (see Pacher *et al.*, 2006), and the effects of endocannabinoids on human vessels have been investigated only in a few studies. Thus, the endocannabinoid anandamide failed to relax myometrial arteries isolated

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from pregnant women (Kenny *et al.*, 2002). The same compound increased human skin microcirculatory flow; however, this effect was obtained only if it was administered on the skin and not if it was injected intravascularly (Movahed *et al.*, 2005). In our own hands, anandamide relaxed isolated human pulmonary artery rings (Kozłowska *et al.*, 2007).

Anandamide exerts its cardiovascular effects in mice and rats through cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors and vanilloid TRPV<sub>1</sub> receptors (see Begg *et al.*, 2005; Pacher *et al.*, 2006). In addition, it relaxes isolated blood vessels of mice (Járai *et al.*, 1999) and rats (Járai *et al.*, 1999; Wagner *et al.*, 1999; Ho and Hiley, 2003; Offertáler *et al.*, 2003; Milman *et al.*, 2006), and decreases blood pressure in mice (Járai *et al.*, 1999; Offertáler *et al.*, 2003) through an as yet unidentified endothelial cannabinoid receptor, which is also activated by the cannabinoid, abn-cbd. The latter receptor appears to be involved in the effect of anandamide in the human pulmonary artery, as the vasorelaxation was mimicked by abn-cbd, and the effect of the latter was counteracted by two antagonists of the endothelial cannabinoid receptor (Kozłowska *et al.*, 2007). In recent years, anandamide and abn-cbd have been reported to activate the GPR55 receptor also, an orphan cannabinoid receptor (Pertwee, 2007; Ryberg *et al.*, 2007). However, no differences were noticed in hypotension and vasorelaxation of isolated mesenteric arteries elicited by abn-cbd between GPR55 knockout mice and their wild-type littermate controls (Johns *et al.*, 2007) arguing against the hypothesis that the GPR55 receptor is the endothelial cannabinoid receptor.

It is possible that the endothelial cannabinoid receptor in the human pulmonary artery could be a target for the treatment of pulmonary hypertension (Kozłowska *et al.*, 2007). To assess further the properties of this receptor in our experimental model, we studied the effect of another endocannabinoid, virodhamine. Virodhamine (*O*-arachidonoylethanolamine) is not only a partial agonist at the CB<sub>1</sub> and a full agonist at the CB<sub>2</sub> receptor (Porter *et al.*, 2002), but also a full agonist at the endothelial cannabinoid receptor (Ho and Hiley, 2004) with a potency even slightly higher than that of abn-cbd (Ho and Hiley, 2003). It also displays a markedly greater potency at GPR55 than at CB<sub>1</sub> or CB<sub>2</sub> receptors and a particularly high efficacy at GPR55 receptors (Pertwee, 2007; Ryberg *et al.*, 2007; McHugh *et al.*, 2008). The concentration of virodhamine in peripheral tissues is two- to ninefold higher than that of anandamide (Porter *et al.*, 2002). For comparison, the effect of WIN 55,212-2, a synthetic agonist at CB<sub>1</sub> and CB<sub>2</sub> receptors without effect on the endothelial cannabinoid receptor (Pacher *et al.*, 2006), was studied as well.

## Methods

All protocols were approved by the Human Ethics Committee of the Medical University of Białystok.

### Tissue preparation

Human lung tissue was obtained from 54 patients (46 men and 8 women, mean age 58.2 ± 0.9 years; including 78.4%

smokers) undergoing lobectomy or pneumonectomy during resection of lung carcinoma. Preoperative echocardiography revealed normal left and right ventricular function in each case. Patients did not have any clinical evidence of pulmonary hypertension nor did they receive β-blockers. Before the operation, all patients received cephalosporins and low-molecular-weight heparin as anti-infection and antithrombotic prophylaxis, respectively. The tissue was transported to the laboratory within half an hour in cold (4 °C), pregassed Tyrode's bicarbonate solution (for composition, see below). Lobar and segmental pulmonary artery branches were cleaned from lung parenchyma and cut into rings (from the middle portion of each artery; 3–5 mm length and 2–4 mm outer diameter).

### Organ bath technique

The arterial rings were suspended on stainless steel wires in 10 mL organ baths containing Tyrode's solution (concentration in mM: NaCl, 139.2; KCl, 2.7; CaCl<sub>2</sub>, 1.8; MgCl<sub>2</sub>, 0.49; NaHCO<sub>3</sub>, 11.9; NaH<sub>2</sub>PO<sub>4</sub>, 0.4; glucose, 5.5 and pH 7.4), gassed continuously with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, at 37 °C. Pulmonary artery rings were allowed to equilibrate for 90 min; during this time period, the bath fluid was changed every 10 min with fresh Tyrode's solution. The optimal resting tension was 2–2.5 g (depending on the rings' internal diameter), which ensured that responses to agonists were maximal. Muscle tension was recorded by a force displacement transducer (PIM 100RE, BIO-SYS-TECH, Białystok, Poland) and displayed on a computer.

### Concentration–response curves

After the equilibration period, all rings were constricted submaximally with phenylephrine (10 μM) to prime the tissues and to check the functionality of the endothelium (at least 80% relaxation in response to acetylcholine 1 μM). When the endothelium was not required, vessels were denuded by rubbing the intimal surface mechanically, and successful endothelial removal was confirmed by a lack of vasorelaxant response to acetylcholine. To examine the vasorelaxant effect of virodhamine, WIN 55,212-2 and iloprost, arteries were constricted submaximally with 5-HT (creatinine sulphate complex; 0.5–1.2 μM; for details see Table 1) or high K<sup>+</sup> (60 mM) Tyrode's solution, which was prepared by equimolar substitution of NaCl by KCl. Then cumulative concentration–response curves to virodhamine were constructed.

To examine the mechanisms involved in the vasorelaxant effect of virodhamine, rings were treated for 30 min with the following antagonists and enzyme or ion channel inhibitors: cannabidiol 3 μM and O-1918 10 μM (putative endothelial cannabinoid receptor; Kozłowska *et al.*, 2007); and rimona-bant 100 nM and 5 μM (CB<sub>1</sub> receptor and putative endothelial cannabinoid receptor, respectively; Járai *et al.*, 1999; O'Sullivan *et al.*, 2004); SR 144528 1 μM (CB<sub>2</sub> receptor; Zoratti *et al.*, 2003), capsazepine 5 μM (vanilloid TRPV<sub>1</sub> receptor; Járai *et al.*, 1999); indomethacin 10 μM (COX; Kozłowska *et al.*, 2007); L-NAME 300 μM (NOS; Kozłowska *et al.*, 2007); URB597 1 μM (FAAH; Di Marzo *et al.*, 2005) and apamin

**Table 1** Contractile responses induced by 5-HT or KCl and pEC<sub>25</sub> values for the vasorelaxant effects of virodhamine in isolated human pulmonary arteries

Vasoconstrictor agents	Concentration	Group	Endothelium	n	Tension (mN)	pEC <sub>25</sub>	
5-HT	1 μM	Control rings	Intact	37	7.85 ± 0.49	5.67 ± 0.08	
		Rimonabant (100 nM)		5	7.55 ± 0.68	5.35 ± 0.10	
		Rimonabant (5 μM)		5	8.63 ± 1.17	4.93 ± 0.22***	
		O-1918 (10 μM)		5	6.87 ± 1.47	4.58 ± 0.11***	
		SR 144528 (1 μM)		6	7.45 ± 0.68	5.32 ± 0.05	
		Capsazepine (5 μM)		6	7.65 ± 0.98	5.68 ± 0.17	
		URB597 (1 μM)		7	9.81 ± 0.86	4.87 ± 0.21***	
		Cannabidiol (3 μM)		5	7.84 ± 0.69	4.50 ± 0.15***	
		Apa (0.3 μM) + Chtx (0.3 μM)		5	9.81 ± 1.56	4.88 ± 0.13***	
		L-NAME (300 μM)		7	9.81 ± 1.37	5.67 ± 0.16	
		Indomethacin (10 μM)		7	9.50 ± 1.08	4.60 ± 0.15***	
		Control rings		Denuded	11	7.85 ± 0.59	4.51 ± 0.13***
		O-1918 (10 μM) + indomethacin (10 μM) + URB597 (1 μM)			5	8.33 ± 0.9	4.50 ± 0.14***
		KCl		60 mM	Control rings	Intact	4

Abbreviations: Apa, apamin; Chtx, charybdotoxin; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester, ND, not determined because the extent of vasorelaxant effect of virodhamine is very low under this condition.

Data are expressed as means ± s.e.mean.

\*\*\**P* < 0.001, compared with control rings (with intact endothelium; as determined by one-way ANOVA followed by Dunnett's *post hoc* test) precontracted with 5-HT (1 μM).

0.3 μM given together with charybdotoxin 0.3 μM (blockers of Ca<sup>2+</sup>-sensitive K<sup>+</sup> channels; Koziłowska *et al.*, 2007). In control tissues, the respective vehicles were used instead. In each individual preparation, only one experimental curve was determined.

To examine the antagonistic properties of virodhamine against 5-HT-induced contraction, some rings were treated for 30 min with one concentration (10 μM) of virodhamine or its vehicle (control tissues), and then a concentration–response curve for 5-HT (0.01–100 μM) was constructed.

#### Calculations and statistical analysis

The relaxation produced with virodhamine, WIN 55,212-2 and iloprost or their solvents was expressed as the percentage relaxation of the precontraction induced by 5-HT or KCl. To determine the potency of agonists, EC<sub>50</sub> values (that is, the concentrations causing the half maximum effect) were determined from the individual concentration–response curves. A series of experimental procedures markedly attenuated the vasodilator effect of virodhamine. To have a rough measure for its potency under such conditions, the concentration causing a relaxation of 25% of the precontracted vessel was determined (EC<sub>25</sub>). The EC<sub>50</sub> and EC<sub>25</sub> values were transformed into pEC<sub>50</sub> and pEC<sub>25</sub> values, that is, the negative logarithms of EC<sub>50</sub> and EC<sub>25</sub> values, respectively.

The antagonistic potency (estimated pK<sub>b</sub>) of cannabidiol, O-1918 and rimonabant against virodhamine (vasorelaxation) was calculated from the equation:

$$\text{Estimated pK}_b = \log(\text{CR} - 1) - \log(\text{B}),$$

where (B) is the molar concentration of the antagonist and CR is the concentration ratio of the EC<sub>25</sub> values of virodhamine in the presence and absence of the antagonist.

Results are expressed as means ± s.e.mean of *n* experiments. Statistical analyses were performed using the *t*-test for unpaired data. When two or more treatment groups were compared with the same control, ANOVA followed by Dunnett's test was used. Differences were considered as significant when *P* < 0.05.

#### Drugs used

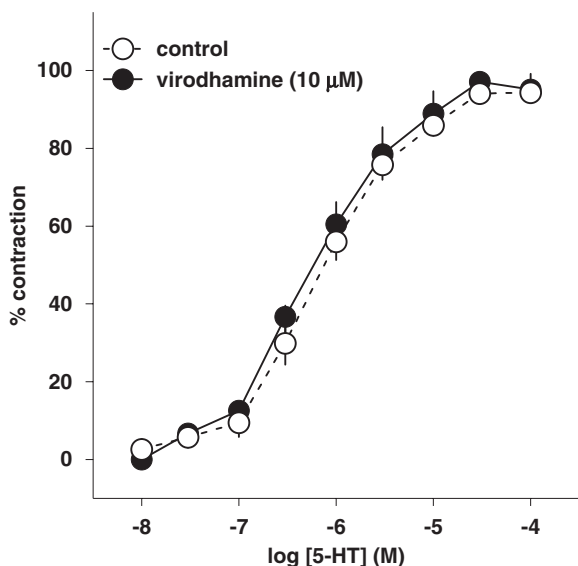
(–)-Phenylephrine hydrochloride, acetylcholine chloride, 5-HT, apamin, charybdotoxin, L-NAME, indomethacin (Sigma, Munich, Germany) were dissolved in deionized water with the exception of 5-HT (dissolved in 0.1 N HCl) and indomethacin (dissolved in 0.5 M NaHCO<sub>3</sub>). Iloprost (Schering AG, Berlin, Germany) was dissolved in 0.9% NaCl. Virodhamine, (–)-cannabidiol, capsazepine (Tocris, Bristol, UK), O-1918 (1,3-dimethoxy-5-methyl-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-benzene), URB597 (3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate; Cayman Chemicals, Ann Arbor, MI, USA); rimonabant and SR 144528 (Sanofi-Aventis, Montpellier, France) were supplied in 100% ethanol. WIN 55,212-2 (R(+)) [2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)-methanone mesylate (RBI, Natick, MA, USA) was dissolved in a mixture of water and DMSO (4:6).

## Results

#### General

5-HT (0.01–100 μM) induced a concentration-dependent contraction of human pulmonary artery rings (pEC<sub>50</sub> = 5.98 ± 0.26; Figure 1). Virodhamine (10 μM) failed to modify the concentration–response curve for 5-HT (pEC<sub>50</sub> = 6.24 ± 0.21; Figure 1).

The vasorelaxant effects of virodhamine, WIN 55,212-2 and iloprost on the human pulmonary artery were examined

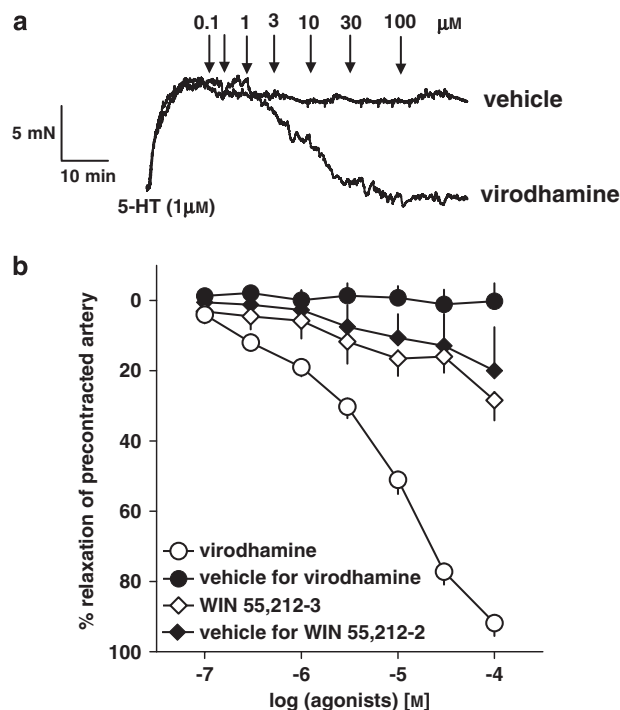


**Figure 1** Concentration–response curve of 5-HT for its contractile effect on endothelium-intact human pulmonary arteries and its interaction with virodhamine. Results are expressed as percentage of maximal contraction. Means  $\pm$  s.e.mean of five arteries for each curve. In many cases, s.e.mean is smaller than or equal to the size of symbols.

in vascular rings precontracted with 5-HT using a concentration of 1  $\mu$ M (that is, approximately equivalent to its EC<sub>60</sub>) or with 60 mM KCl (virodhamine only); the majority of the experiments was carried out on preparations precontracted with 5-HT. As L-NAME, indomethacin and K<sup>+</sup> channel blockers slightly increased and cannabidiol slightly decreased the resting tension of pulmonary rings by themselves, a reduced (0.5–1  $\mu$ M) and higher (1–1.2  $\mu$ M) concentration of 5-HT was used, respectively (for details, see Table 1). Virodhamine (0.1–100  $\mu$ M) and the other drugs under study did not affect the tone of vessels not precontracted with 5-HT, when given alone (results not shown).

#### Vasorelaxant effect of virodhamine and WIN 55,212-2

As shown in Figure 2, the endocannabinoid virodhamine (0.1–100  $\mu$ M), but not its vehicle (0.01–1% v/v; ethanol), produced a concentration-dependent relaxation of endothelium-intact isolated human pulmonary arteries precontracted with 5-HT (for original traces, see Figure 2a). A full relaxation (96.2  $\pm$  3.1%,  $n$  = 37; expressed as percentage of relaxation of the tone induced by 5-HT 1  $\mu$ M) was induced by the highest concentration of the agonist. The pEC<sub>50</sub> value for the vasorelaxant effect of virodhamine in vessels precontracted with 5-HT was 5.07  $\pm$  0.07 ( $n$  = 37). We did not observe any differences in virodhamine-elicited relaxation of 5-HT-precontracted human pulmonary arteries isolated from smokers and non-smokers; this refers to the potency (pEC<sub>50</sub> of 5.08  $\pm$  0.09 ( $n$  = 29) and 5.14  $\pm$  0.10 ( $n$  = 8), respectively) and the maximum effect (results not shown). The vasorelaxant effect of virodhamine was gradual and it took 46.5  $\pm$  4.1 min ( $n$  = 37) to construct complete concentration–response curves.



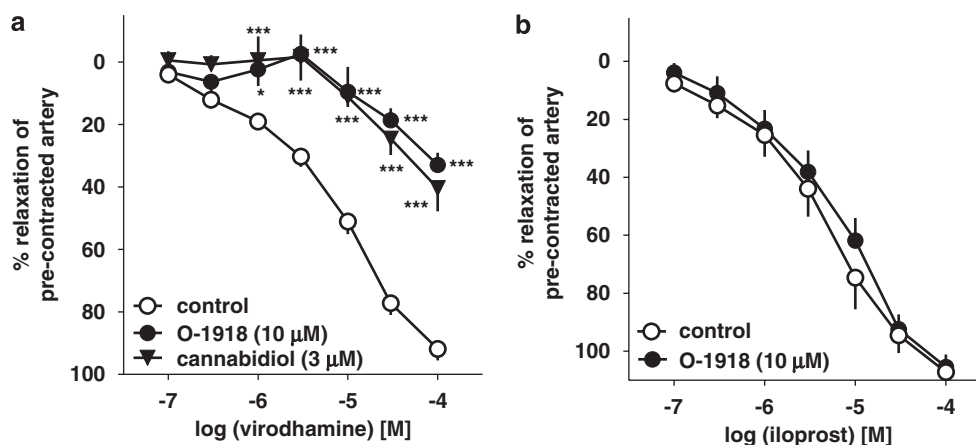
**Figure 2** Representative original traces of vasorelaxant effects of virodhamine and its vehicle (0.01–1% v/v; ethanol; a) and concentration–response curves of virodhamine and WIN 55,212-2 for their effects on endothelium-intact rings of human pulmonary artery, precontracted with 5-HT (b). (a) Arrows show the moment of application of the particular concentrations of virodhamine or of vehicle. (b) Results are expressed as percentage relaxation of the isometric contraction induced by 5-HT. Means  $\pm$  s.e.mean of 5–37 tissues for each curve. In few cases, s.e.mean is smaller than or equal to the size of symbols.

Figure 2b also shows the effect of the synthetic cannabinoid receptor agonist WIN 55,212-2 (0.1–100  $\mu$ M) and of its vehicle DMSO (0.06–0.92% v/v) on the rings of human pulmonary arteries precontracted with 5-HT. Under both experimental conditions, a slight and virtually identical vasorelaxant effect was obtained (Figure 2b).

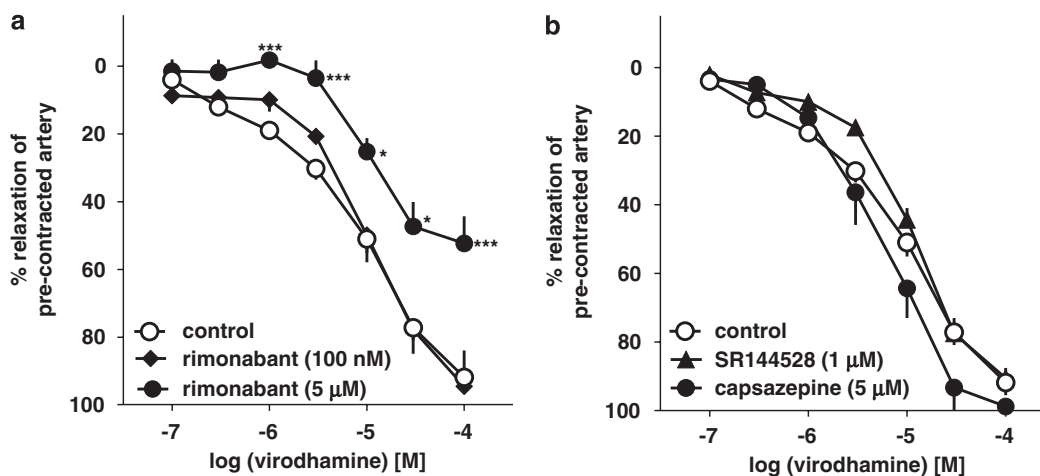
#### Influence of cannabinoid and TRPV<sub>1</sub> receptor antagonists on the virodhamine-induced relaxation

Two antagonists of the putative endothelial cannabinoid receptor, cannabidiol (3  $\mu$ M) and O-1918 (10  $\mu$ M), shifted the concentration–response curve for virodhamine to the right by factors of 13.8 and 11.5 (Figure 3a), yielding estimated pK<sub>b</sub> values of 6.65  $\pm$  0.17 ( $n$  = 5) and 6.26  $\pm$  0.17 ( $n$  = 5), respectively (for the respective pEC<sub>25</sub> values, see Table 1). In contrast, O-1918 (10  $\mu$ M) did not affect the relaxant effect of the stable analogue of prostacyclin (PGI<sub>2</sub>), iloprost (0.1–100  $\mu$ M; mean pEC<sub>50</sub> values for iloprost were 5.43  $\pm$  0.14 ( $n$  = 5) and 5.24  $\pm$  0.14 ( $n$  = 5) in control and O-1918-treated rings (Figure 3b)).

The vasorelaxant effect of virodhamine was not modified by the CB<sub>1</sub> receptor antagonist rimonabant given at a concentration of 100 nM, known to block CB<sub>1</sub> receptors. However, a higher concentration of this antagonist (5  $\mu$ M), which also antagonizes endothelial cannabinoid receptors, produced a 5.1-fold shift to the right of the concentration–response curve of virodhamine (Figure 4a). Its estimated pK<sub>b</sub>



**Figure 3** Influence of cannabidiol and O-1918 on the concentration–response curve of virodhamine (a) or iloprost (b) for their relaxant effects on 5-HT-precontracted endothelium-intact human pulmonary arteries. Results are expressed as percentage relaxation of the isometric contraction induced by 5-HT. Means  $\pm$  s.e.mean of 5–37 tissues for each curve. \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with the respective control (virodhamine alone; as determined by one-way ANOVA followed by Dunnett's *post hoc* test). In few cases, s.e.mean is smaller than or equal to the size of symbols.



**Figure 4** Influence of rimonabant (a), SR144528 and capsazepine (b) on the concentration–response curve of virodhamine for its relaxant effect on 5-HT-precontracted endothelium-intact human pulmonary arteries. Results are expressed as percentage relaxation of the isometric contraction induced by 5-HT. Means  $\pm$  s.e.mean of 5–37 tissues for each curve. \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with the respective control (virodhamine alone; as determined by one-way ANOVA followed by Dunnett's *post hoc* test). In few cases, s.e.mean is smaller than or equal to the size of symbols.

value was  $5.87 \pm 0.28$  ( $n = 5$ ; for the respective  $pEC_{25}$  value, see Table 1). The CB<sub>2</sub> receptor antagonist SR144528 (1 μM) and the TRPV<sub>1</sub> receptor antagonist capsazepine (5 μM) failed to affect virodhamine-induced relaxations (Figure 4b).

#### *Influence of endothelium removal, L-NAME, indomethacin and URB597 on the virodhamine-induced relaxation*

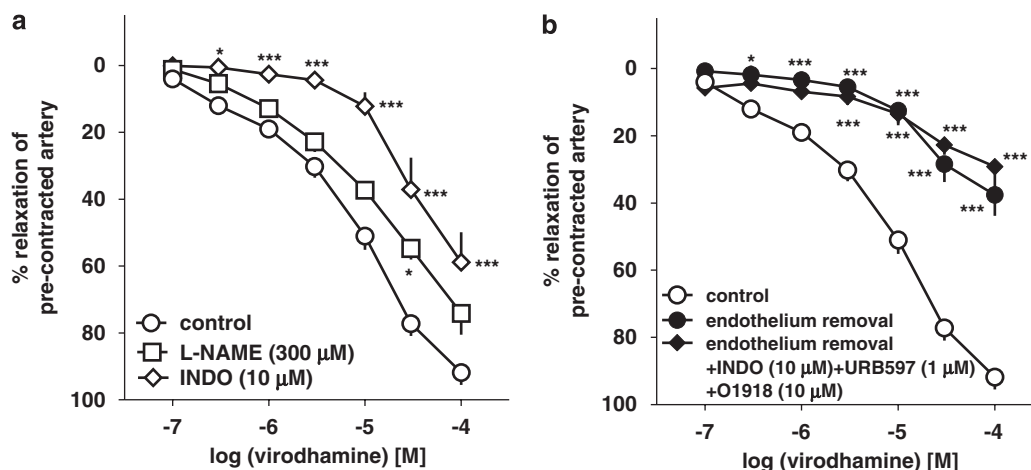
The virodhamine-induced vasorelaxation of endothelium-intact human pulmonary arteries was attenuated by the COX inhibitor indomethacin (10 μM), which shifted its concentration–response curve to the right by a factor of 9.1 (Figure 5a). The NOS inhibitor L-NAME (300 μM) only slightly attenuated the vasorelaxant effect of the three highest concentrations of virodhamine; a statistically significant influence was noticed for virodhamine 30 μM only (Figure 5a; see Table 1 for  $pEC_{25}$  values). The FAAH inhibitor URB597 (1 μM) produced a 5.9-fold rightward shift of the concentra-

tion–response curve for virodhamine (Figure 6; for  $pEC_{25}$  values, see Table 1).

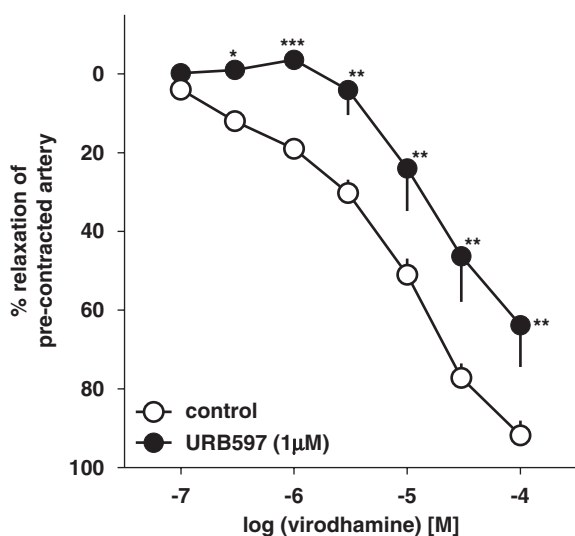
Removal of endothelium (which completely abolished the relaxation induced by acetylcholine 1 μM; data not shown) strongly reduced the vasorelaxant effect of virodhamine (Figure 5b), which amounted to 37.6  $\pm$  6.2% of relaxation of the precontracted vessels at 100 μM ( $n = 11$ ). Pretreatment of endothelium-denuded rings with the combination of O-1918 (10 μM), indomethacin (10 μM) and the FAAH inhibitor URB597 (1 μM) did not further amplify the inhibitory effect of endothelium removal on the vasorelaxant response to virodhamine (Figure 5b).

#### *Influence of KCl (60 mM) and of K<sup>+</sup> channel blockers on the virodhamine-induced relaxation*

Next, the vasodilator effect of virodhamine was studied in endothelium-intact human pulmonary arteries precon-



**Figure 5** Influence of *N*<sup>C</sup>-nitro-L-arginine methyl ester (L-NAME) and indomethacin (INDO; a), endothelium removal and endothelium removal plus combined administration of O-1918, INDO and URB597 (b) on the concentration–response curve of virodhamine for its relaxant effect on human pulmonary arteries. Results are expressed as percentage relaxation of the isometric contraction elicited by 5-HT. Means ± s.e.mean of 5–37 arteries for each curve. \**P* < 0.05, \*\*\**P* < 0.001 compared with the respective control (virodhamine alone in endothelium-intact rings; as determined by one-way ANOVA followed by Dunnett’s *post hoc* test). In few cases, s.e.mean is smaller than or equal to the size of symbols.



**Figure 6** Influence of URB597 on the concentration–response curve of virodhamine for its relaxant effect on 5-HT-precontracted endothelium-intact rings of human pulmonary arteries. Results are expressed as percentage relaxation of the isometric contraction induced by 5-HT. Means ± s.e.mean of 7–37 arteries for each curve. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 compared with the respective control (virodhamine alone; as determined by the *t*-test for unpaired data). In many cases, s.e.mean is smaller than or equal to the size of symbols.

tracted with KCl (60 mM) and its effect in 5-HT-precontracted vessels was compared. KCl (60 mM) induced an increase in vessel tone comparable to that elicited by 5-HT (1 μM; see Table 1). However, it markedly attenuated the virodhamine-induced relaxation, reducing its effect obtained for the highest dose of the agonist to 15.9 ± 2.2% (*n* = 4; Figure 7). For this reason, we were not able to determine the pEC<sub>25</sub> value for the vasorelaxant effect of virodhamine in KCl-precontracted vessels. Figure 7 also shows that combination of apamin (selective blocker of small conductance Ca<sup>2+</sup>-

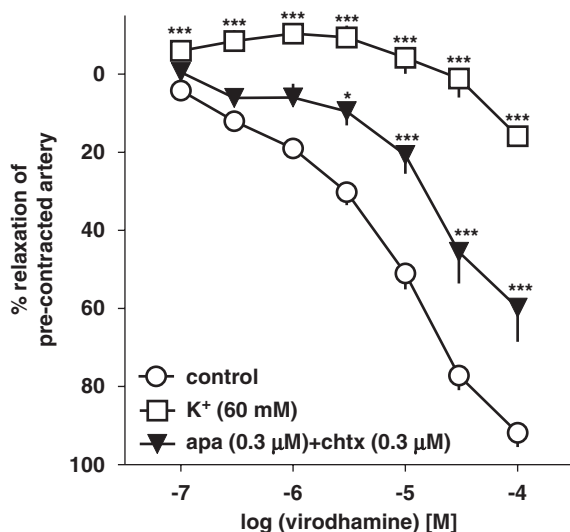
activated K<sup>+</sup> channels SK<sub>Ca</sub>; 0.3 μM) and charybdotoxin (selective blocker of intermediate conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels IK<sub>Ca</sub> and large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels BK<sub>Ca</sub>; 0.3 μM) diminished the virodhamine-induced relaxation by shifting its concentration–response curve to the right by a factor of 5.8 (for pEC<sub>25</sub> values, see Table 1).

## Discussion

This study was carried out to further characterize the cannabinoid-induced vasorelaxation of the human pulmonary artery described by Kozłowska *et al.* (2007) and is based on experiments with virodhamine, an endocannabinoid and WIN 55,212, a synthetic cannabinoid receptor agonist. To precontract the human pulmonary artery rings, we used 5-HT because of the extensive physiological function of endothelium in this circulatory bed and also because of the fact that 5-HT has a major function as vasoconstrictor in the pulmonary circulation (Dempsie and MacLean, 2008). The 5-HT-induced contraction of the human pulmonary artery is predominantly, if not exclusively, related to the activation of 5-HT<sub>2A</sub> receptors (Kozłowska *et al.*, 2007).

### Receptor involved in the virodhamine-induced relaxation

Virodhamine caused full relaxation of the endothelium-intact isolated human pulmonary artery. The majority (80%) of the pulmonary arteries used in our study were obtained from smokers and this might mean that the effect of virodhamine represents an abnormal rather than the real physiological response. This possibility, however, is very unlikely as there was no difference in the response to this agonist in tissue taken from smoking and non-smoking patients. As observed in abn-cbd (Kozłowska *et al.*, 2007), the relaxation elicited by virodhamine developed slowly and, for both of these agonists, the construction of the complete



**Figure 7** Concentration–response curves for virodhamine-induced relaxation of 5-HT and of KCl (60 mM) precontracted endothelium-intact human pulmonary arteries and interaction of virodhamine with a combination of the K<sup>+</sup> channel blockers, apamin (apa) and charybdotoxin (chtx). Results are expressed as percentage relaxation of the isometric contraction induced by KCl (open squares) or 5-HT (other experiments). Means  $\pm$  s.e. mean of 4–37 arteries for each curve. \* $P < 0.05$ , \*\*\* $P < 0.001$  compared with the respective control (that is, 5-HT-precontracted arteries not exposed to K<sup>+</sup> channel blockers; as determined by one-way ANOVA followed by Dunnett's *post hoc* test). In many cases, s.e. mean is smaller than or equal to the size of symbols.

concentration–response curves took about 50 min. However, in comparison with abn-cbd, virodhamine exhibited a higher potency ( $pEC_{50}$   $4.80 \pm 0.06$ ;  $n = 23$ , and  $5.07 \pm 0.07$ ;  $n = 37$ , respectively;  $P < 0.01$ ). Similar differences in the potencies of abn-cbd and virodhamine were also observed in the rat mesenteric artery ( $pEC_{50}$  6.2 and 6.4; Ho and Hiley, 2003, 2004). Note that the level of potencies of the two agonists is higher in the rat mesenteric artery (Ho and Hiley, 2003, 2004) than in the human pulmonary artery (Koziowska *et al.*, 2007; this study). Importantly, the two endocannabinoids studied so far in the human pulmonary artery, anandamide (Koziowska *et al.*, 2007) and virodhamine (this study), exhibited higher potencies than the synthetic cannabinoid abn-cbd and, in contrast with abn-cbd, did not relax the basal tone by themselves (Koziowska *et al.*, 2007; this study).

Which type of receptor was involved in the vasorelaxant effect of virodhamine? First, the most trivial explanation, namely that this effect is because of antagonism of the 5-HT used to precontract the vessels, can be excluded as virodhamine (10  $\mu$ M) did not modify the contractor response of the pulmonary artery rings to 5-HT. Second, the lack of a vasorelaxant effect of WIN 55,212-2 argues against the involvement of CB<sub>1</sub> and CB<sub>2</sub> receptors in the effect of virodhamine. The involvement of CB<sub>1</sub> receptors can also be excluded as the selective CB<sub>1</sub> receptor antagonist rimonabant did not influence the effect of virodhamine at a concentration of 100 nM at which it antagonizes CB<sub>1</sub> receptor-mediated effects in rat resistance and conduit arteries (O'Sullivan *et al.*, 2004). Moreover, the CB<sub>2</sub> receptor antagonist SR 144528, used at a concentration of 1  $\mu$ M known

to block CB<sub>2</sub> receptor-mediated response in calf pulmonary endothelial cells (Zoratti *et al.*, 2003), failed to modify the response to virodhamine. Third, the possibility that virodhamine acts through TRPV<sub>1</sub> receptors (at which it exhibits a modest effect; Ho and Hiley, 2004) can be excluded because the selective TRPV<sub>1</sub> receptor antagonist capsazepine did not affect the virodhamine-induced relaxation in a concentration range at which it antagonizes TRPV<sub>1</sub> receptor-mediated effects, for instance, in the rat mesenteric artery (Járai *et al.*, 1999). Fourth, the involvement of the recently characterized GPR55 receptor in the effect of virodhamine had also to be considered, as it is an agonist at this receptor (Pertwee, 2007; Ryberg *et al.*, 2007; McHugh *et al.*, 2008). However, this possibility is very unlikely as recent experiments with a GPR55 receptor knockout mouse clearly showed that this receptor does not lead to a vasodilator response (Johns *et al.*, 2007). Nevertheless, the GPR55 receptor might occur in the pulmonary, as opposed to the systemic, circulation or in human, as opposed to rodent, blood vessels.

Experiments with three antagonists at the endothelial cannabinoid receptor suggest that this receptor was involved in the vasorelaxant effect of virodhamine. Cannabidiol and O-1918 antagonized the effect of virodhamine at concentrations at which they had been previously shown to antagonize the endothelial cannabinoid receptor-mediated vasodilatation in the rat mesenteric (Ho and Hiley, 2003) or human pulmonary artery (Koziowska *et al.*, 2007). Cannabidiol has a low affinity for cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors in binding studies, but a moderate potency as a non-competitive antagonist against CB<sub>1</sub> and CB<sub>2</sub> receptor agonists (Thomas *et al.*, 2007). O-1918 does not bind to CB<sub>1</sub> or CB<sub>2</sub> receptors and does not induce relaxation up to 30  $\mu$ M (Offertáler *et al.*, 2003) and may be superior to cannabidiol as an antagonist of endothelial cannabinoid receptors. Interestingly enough, both antagonists exhibited a higher potency in the human pulmonary artery towards virodhamine (this study) when compared with abn-cbd (Koziowska *et al.*, 2007). The respective  $pA_2$  values were 6.6 and 5.9 for cannabidiol and 6.3 and 5.1 for O-1918 (for the possible explanation of this discrepancy, see below). The inhibitory effect of the higher concentration of rimonabant (5  $\mu$ M) against the vasorelaxant effect of virodhamine in the human pulmonary artery is also compatible with the involvement of the endothelial cannabinoid receptor in this effect (Járai *et al.*, 1999; Wagner *et al.*, 1999; Ho and Hiley, 2003).

#### *Endothelial mechanisms involved in the virodhamine-induced relaxation*

The vasorelaxant effect of virodhamine (this study), like that of abn-cbd (Koziowska *et al.*, 2007), is endothelium-dependent, that is, removal of the endothelium reduces the vasorelaxant effect of both agonists by about 50% in the human pulmonary artery. In contrast, the vasorelaxant effect of virodhamine was reduced by about 80% in the endothelium-denuded rat mesenteric artery (Ho and Hiley, 2004). Thus, the vasorelaxant action of virodhamine, similar to that of abn-cbd, consists of an endothelium-dependent and endothelium-independent component. In this study, we focused on the endothelium-dependent action of virodhamine.

The next question addressed was which endothelium-derived vasodilator(s) is (are) involved in the virodhamine-induced vasodilator effect. The endothelial cannabinoid receptor is usually connected to the release of endothelium-derived hyperpolarizing factor, as shown in animal models (see Randall *et al.*, 2004) and in the human pulmonary artery (Kozłowska *et al.* 2007). Two sets of experiments show that this mechanism is also implicated in the vasorelaxant effect of virodhamine in this study. First, raising the extracellular concentration of  $K^+$  (60 mM KCl) markedly attenuated the virodhamine-induced relaxation compared with preparations precontracted with 5-HT. This indicates that the virodhamine-stimulated relaxation is partially mediated by the activation of  $K^+$  channels, as a high extracellular  $K^+$  concentration abolishes the electrochemical gradient for the  $K^+$  efflux. Second, the vasorelaxant effect in our model was markedly attenuated by a combination of apamin (a selective blocker of small conductance ( $SK_{Ca}$ )  $Ca^{2+}$ -activated  $K^+$  channels) and charybdotoxin (an inhibitor of intermediate ( $IK_{Ca}$ ) and large conductance ( $BK_{Ca}$ )  $Ca^{2+}$ -activated  $K^+$  channels).

The effect of virodhamine might also be related to nitric oxide, one of the main endothelial vasodilators (Moncada and Higgs, 2006). However, in our hands, L-NAME, an inhibitor of the biosynthesis of nitric oxide by endothelial cells, caused only a modest inhibition of the vasorelaxant effect of the highest concentrations of this endocannabinoid, suggesting that nitric oxide has only a minor function in the effect of virodhamine. Nitric oxide is not involved at all in the vasorelaxant effects of virodhamine in the rat mesenteric artery (Ho and Hiley, 2004) and of abn-cbd in the human pulmonary artery (Kozłowska *et al.* 2007).

#### *An additional mechanism involved in the virodhamine-induced relaxation*

The possibility that the effect of virodhamine is related to the increased formation of  $PGI_2$  (or another vasorelaxant product of COX activity) had also to be considered. This was likely as the virodhamine-induced vasorelaxation was attenuated by indomethacin, an inhibitor of COX. This effect might be because of an increase in COX activity following the activation of the endothelial cannabinoid receptor. There is, however, an entirely different possibility to account for this phenomenon, that is, that arachidonic acid formed from virodhamine is converted to  $PGI_2$ . This latter possibility is suggested by a series of our results.

The endocannabinoids known so far are derivatives of, and are degraded to, arachidonic acid from which vasorelaxant metabolites may be formed. The vasodilator effect of anandamide in bovine (Pratt *et al.*, 1998) and sheep coronary arteries (Grainger and Boachie-Ansah, 2001) or in rabbit mesenteric arteries (Fleming *et al.*, 1999) is related to this mechanism. Unfortunately, very little is known, at present, of the degradation pathways for virodhamine (Porter *et al.*, 2002; De Petrocellis *et al.*, 2004). It has been reported that, in human neocortex, this endocannabinoid can interact with FAAH (Steffens *et al.*, 2005), an enzyme known to hydrolyse anandamide to arachidonic acid. In our studies, we used the selective and potent FAAH inhibitor URB597 (Di Marzo *et al.*,

2005) at a concentration of  $1 \mu M$ , which has been shown to amplify the relaxation of the rat small mesenteric arteries to anandamide, but not that to 2-arachidonoylglycerol, another endocannabinoid (Ho and Randall, 2007). We found that blockade of FAAH by URB597 attenuated the vasorelaxant effect of virodhamine. This finding is in harmony with the concept of the involvement of this enzyme in the metabolism of virodhamine. However, the effect of URB597 was unexpected at first glance. We would have even expected an increase in the vasorelaxant effect of virodhamine because of its impaired degradation and increased availability in the biophase of the endothelial cannabinoid receptor.

The formation of a vasorelaxant COX product may also explain why the effect of virodhamine is attenuated by indomethacin, whereas that of abn-cbd is not. Abn-cbd, unlike virodhamine, is chemically not related to arachidonic acid. Moreover, the differential potency of the two antagonists, cannabidiol and O-1918, against virodhamine and abn-cbd may be caused by the fact that in the case of virodhamine, the antagonist under study is competing with a much lower concentration of the endocannabinoids, as a considerable amount of the compound has already been degraded. This phenomenon will lead to an erroneous increase of the estimated  $pK_b$  value. Finally, the fact that either enzyme inhibitor produced a similar effect on the virodhamine-related vasorelaxation may support the view that the two enzymes affect two subsequent steps of a metabolic pathway (although this phenomenon might be accidental as well). To our knowledge, this is the first report to demonstrate that the effects of virodhamine are sensitive to inhibition of FAAH or COX. We can exclude the possibility that virodhamine acts only through its vasorelaxant metabolite as O-1918, the antagonist of the endothelial cannabinoid receptor, inhibited the relaxation induced by virodhamine but not that elicited by iloprost, a stable  $PGI_2$  analogue. Interestingly, when endothelium-denuded human pulmonary arteries were exposed to O-1918, indomethacin plus URB597, the vasorelaxant effect of virodhamine was not attenuated suggesting that both its direct vasorelaxant effect through the endothelial cannabinoid receptor and its indirect effect through an endogenously formed prostanoid are connected with intact endothelium.

We have already suggested (Kozłowska *et al.* 2007) that the endothelial cannabinoid receptor may be a target for the treatment of pulmonary hypertension. A drug like virodhamine may offer a particular advantage over abn-cbd, as it has a dual action. It is of interest in this context that  $PGI_2$  receptor agonists are already in use for the treatment of this disorder in humans (Naeije and Huez, 2007).

## Conclusions

Virodhamine causes a full, slowly developing relaxation of the isolated human pulmonary artery. This effect is partially dependent on vascular endothelium and results from two mechanisms, such as the activation of the putative endothelial cannabinoid receptor (but not of cannabinoid  $CB_1$ ,  $CB_2$  or vanilloid receptors and probably not of the GPR55 receptor) and the hydrolysis of virodhamine to arachidonic



acid and subsequent production of a vasorelaxant prostanoid through COX. One or both of these mechanisms may stimulate vasorelaxant Ca<sup>2+</sup>-activated K<sup>+</sup> channels. In the periphery, the concentration of virodhamine is higher than that of anandamide. Thus, its ability to relax human pulmonary arteries suggests that it may be a candidate for controlling vascular tone under physiological or pathophysiological conditions or even for therapeutic purposes.

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## Conflict of interest

The authors state no conflict of interest.

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