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RESEARCH PAPER

Different β-adrenoceptor subtypes coupling to cAMP or NO/cGMP pathways: implications in the relaxant response of rat conductance and resistance vessels

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BACKGROUND AND PURPOSE

To analyse the relative contribution of β_1-, β_2- and β_3-adrenoceptors (*Adrb*) to vasodilatation in conductance and resistance vessels, assessing the role of cAMP and/or NO/cGMP signalling pathways.

EXPERIMENTAL APPROACH

Rat mesenteric resistance artery (MRA) and aorta were used to analyse the *Adrb* expression by real-time-PCR and immunohistochemistry, and for the pharmacological characterization of *Adrb*-mediated activity by wire myography and tissue nucleotide accumulation.

KEY RESULTS

The mRNAs and protein for all *Adrb* were identified in endothelium and/or smooth muscle cells (SMCs) in both vessels. In MRA, *Adrb1* signalled through cAMP, *Adrb3* through both cAMP and cGMP, but *Adrb2*, did not activate nucleotide formation; isoprenaline relaxation was inhibited by propranolol (β_1, β_2), CGP20712A (β_1), and SQ22536 (adenylyl cyclase inhibitor), but not by ICI118,551 (β_2), SR59230A (β_3), ODQ (soluble guanylyl cyclase inhibitor), L-NAME or endothelium removal. In aorta, *Adrb1* signalled through cAMP, while β_2- and β_3-subtypes through cGMP; isoprenaline relaxation was inhibited by propranolol, ICI118,551, ODQ, L-NAME, and to a lesser extent, by endothelium removal. CL316243 (β_3-agonist) relaxed aorta, but not MRA.

CONCLUSION AND IMPLICATION

Despite all three *Adrb* subtypes being found in both vessels, *Adrb1*, located in SMCs and acting through the adenylyl cyclase/cAMP pathway, are primarily responsible for vasodilatation in MRA. However, *Adrb*-mediated vasodilatation in aorta is driven by endothelial *Adrb2* and *Adrb3*, but also by the *Adrb2* present in SMCs, and is coupled to the NO/cGMP pathway. These results could help to understand the different physiological roles played by *Adrb* signalling in regulating conductance and resistance vessels.

Abbreviations

AC, adenylyl cyclase; CRCs, concentration-response curves; MRA, mesenteric resistance arteries; sGC, soluble guanylyl cyclase; SMCs, smooth muscle cells; *Adrb*, β-adrenoceptors

Introduction

One of the functions of vascular β-adrenoceptors (*Adrb*) is the regulation of blood pressure and vascular tone. To date, three different *Adrb* subtypes have been described: β_1, β_2 and β_3 (Alexander *et al.*, 2011). Although the three subtypes have been implicated in the vasodilator response, the role of each *Adrb* varies according to the vascular bed, the species and the precontracting stimulus used (Guimaraes and Moura, 2001). For a long time, it was thought that only *Adrb2* were responsible for catecholamine-mediated vasodilatation (Lands *et al.*, 1967). However, several studies have shown that *Adrb1* can also participate in blood vessel relaxation (O'Donnell and Wanstall, 1984; Graves and Poston, 1993). Interestingly, recent evidences suggest that *Adrb1* appear to be mainly responsible for vasorelaxation in mouse (Chruscinski *et al.*, 2001) and rat mesenteric resistance arteries (MRA) (Briones *et al.*, 2005), and mediate smooth muscle hyperpolarization in rat MRA (Garland *et al.*, 2011). In addition, the participation of *Adrb3* in the vasorelaxant response has also been documented in some blood vessels, such as the rat carotid artery (Oriowo, 1994), rat (Sooch and Marshall, 1997) and canine (Tagaya *et al.*, 1999) pulmonary artery, the human internal mammary (Rozec *et al.*, 2005) and placental (Rouget *et al.*, 2006) arteries, and also in coronary microarteries (Dessy *et al.*, 2004). Nevertheless, there have been conflicting reports on *Adrb3* involvement in vasodilator responses in rat aorta. Thus, although presence of the *Adrb3* gene, protein expression and functionality has been described (Trochu *et al.*, 1999; Rautureau *et al.*, 2002), lack of a functional *Adrb3* response has also been reported (Brahmadevara *et al.*, 2003; 2004).

Stimulation of adenylyl cyclase (AC) and, hence, cAMP formation in the vascular smooth muscle, is the prototypical signalling pathway of *Adrb* (Murray, 1990). The endothelial NO-cGMP pathway has also been implicated in *Adrb*-mediated vasodilatation and the presence of *Adrb* in endothelial cells was previously confirmed (Vanhoutte, 2001). Nevertheless, there is also evidence for and against the endothelium dependence of this response due to the variability between the species and the vascular bed; inconsistency was also found even in the same vessel. Thus, *Adrb*-mediated relaxation has been described as being completely or partially endothelium-dependent in aorta from rat (Gray and Marshall, 1992; Brawley *et al.*, 2000a; Ferro *et al.*, 2004) and mouse (Akimoto *et al.*, 2002), and as being totally endothelium-independent in rat aorta (Moncada *et al.*, 1991; Eckly *et al.*, 1994; Satake *et al.*, 1996). Conflicting results have also been reported in rat MRA, where we found that *Adrb*-mediated vasodilatation was independent of endothelial NO (Briones *et al.*, 2005), while other authors showed NO-dependent relaxation (Graves and Poston, 1993).

Based on former evidence, *Adrb*-induced relaxation seems to be mediated through the receptors located on smooth muscle and/or endothelial cells, but information on the cellular *Adrb* subtype distribution, the relative role of each subtype and their links to signalling pathways through which they exert their functional responses still remain under debate. Therefore, we studied the specific distribution and roles of each *Adrb* subtype in rat resistance (MRA) and conductance (aorta) arteries. We quantified the mRNA expression of the three *Adrb* subtypes in both whole tissue and isolated smooth muscle cells (SMCs), and we determined its distribution by immunohistochemistry. In addition, we characterized the cAMP/PKA and/or NO/cGMP signalling pathways underlying the activation of *Adrb* subtypes and evaluated the contribution of these two pathways to *Adrb*-mediated relaxation in both vessels.

Methods

Tissue preparation

Male Wistar rats (270–300 g) bred in our faculty's animal facility were anaesthetized with isoflurane and killed by decapitation. All the experimental procedures complied with guidelines established in Spanish legislation (Royal Decree RD 1201/2005) and were approved by the Experimental Animal Ethics Committee of the University of Valencia (Spain). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Segments of third-order branches of MRA and the thoracic aorta were removed, cleaned from adipose tissue and placed into Krebs solution (mM): NaCl 118; KCl, 4.75; $CaCl_2$, 1.8; $MgCl_2$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11; pH = 7.4. In some vessels, the endothelial layer was removed by passing a fine cannula through the lumen. For the immunofluorescence studies, vessels were fixed with 4% phosphate-buffered paraformaldehyde (pH = 7.4) for 1 h and washed in three changes of PBS solution (pH = 7.4). Arterial segments were placed in PBS containing 30% sucrose overnight, transferred to a cryomold containing Tissue Tek OCT embedding medium, and frozen in liquid nitrogen. Second- and third-order branches of the mesenteric artery and aorta segments to be used for real-time quantitative (RT-q)PCR were frozen in liquid nitrogen following dissection and were stored at −80°C.

SMCs isolated from rat aorta

Endothelium was scraped off the aorta and the vessel was ground and incubated for 90 min in a collagenase (Sigma-Aldrich, St Louis, MO, USA) solution (2.5 $mg \cdot mL^{-1}$) dissolved in DMEM F-12 Ham's medium (Sigma-Aldrich) supplemented with 180 $\mu g \cdot mL^{-1}$ streptomycin, 180 $U \cdot mL^{-1}$ penicillin, L-glutamin 20 nM, fungizone 2.5 $U \cdot mL^{-1}$, gentamicin 4 $mg \cdot mL^{-1}$, rotated at 37°C and mechanically disaggregated every 30 min. Two consecutive centrifugations (390× *g*, 10 min) were performed to wash the pellet, which was frozen and stored at −80°C.

RT-qPCR

The total RNA from frozen MRA, aorta and SMCs isolated from aorta was obtained and the RT reaction was performed as described previously (Martí *et al.*, 2005). The mRNAs encoding the three *Adrb*, *Nos3* and *Gapdh* as an internal standard were quantified by TaqMan RT-PCR in a Gene-Amp 5700 sequence-detection system (Applied Biosystems, Foster City, CA, USA). We analysed (in duplicate reactions) a 10-fold

dilution of the RT reaction of each sample using the TaqMan Gene Expression Assays (Applied Biosystems).

The specific primer-probes were: *Adrb1* (Rn00824536_s1), *Adrb2* (Rn00560650_s1), *Adrb3* (Rn00565393_m1), *Nos3* (Rn02132634_s1) and *Gapdh* (Rn99999916_s1) (Applied Biosystems). RT-PCR reactions were done in 25 µL Taq-Man Universal PCR Master Mix (Applied Biosystems), including 5 µL of diluted RT reaction and 1.25 µL of the 20X TaqMan Gene Expression Assay Mix (250 nM for the probe and 900 nM for each primer). cDNA was amplified following the manufacturer's conditions: one initial hold step at 95°C for 10 min, a second step with 40 cycles, 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension). The targets and reference (*Gapdh*) were amplified in parallel reactions. A minimum of three samples from three different animals were analysed for each condition.

The threshold cycle values obtained for each gene were referenced to *Gapdh* and converted into the linear form using the term $2^{-\Delta Ct}$ as a value directly proportional to the copy number of mRNA.

Immunofluorescence

Frozen aorta and MRA sections (14 µm thick) were incubated with a rabbit polyclonal antibody against *Adrb1* and *Adrb2* (1:30; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or a goat polyclonal antibody against *Adrb3* (1:30; Santa Cruz Biotechnology Inc.). After washing, rings were incubated with the secondary antibody, donkey anti-rabbit (1:200) or donkey anti-goat (1:200) IgG conjugated to Cy™3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), and sections were processed essentially as previously described (Martínez-Revelles *et al.*, 2012). Sections were stained with nuclear dye Hoechst 33342 (0.01 mg·mL^{-1}; Sigma-Aldrich). Natural autofluorescence elastin images were also taken.

Tissue cAMP and cGMP accumulation assay

Segments from MRA and aorta were incubated in Krebs solution at 37°C and gassed with 95% O_2 and 5% CO_2 (pH 7.4). After a 30-min equilibration period, vessels were incubated for 30 min in the absence and presence of *Adrb* selective agonists (1 µM): isoprenaline, dobutamine (β_1), salbutamol (β_2) or CL316243 (β_3). The concentration of agonists used was selected taking into account their ability to relax around 70–100% of the maximal response. In another group of experiments, isoprenaline-induced cyclic nucleotide formation was studied in either the absence or presence of selective *Adrb* antagonists (1 µM) [propranolol, CGP20712A (β_1), ICI118,551 (β_2) and SR59230A (β_3)], NOS formation inhibitor [N_w-Nitro-L-Arginine Methyl Ester (L-NAME), 100 µM] or AC inhibitor (SQ22536, 100 µM). The inhibitors were added 15 min prior to isoprenaline addition and were further incubated for 30 min. All the experiments were performed in the presence of the non-selective phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 0.5 mM) to prevent the degradation of cyclic nucleotides (Bender and Beavo, 2006).

Afterwards, tissues were frozen in liquid nitrogen, ground to powder in a ceramic mortar, suspended in HCl 0.1 N and 0.5 mM IBMX, homogenized and centrifuged at 600× *g* for 5 min. The supernatant was decanted and kept frozen at –80°C until nucleotide determination using a specific cAMP or cGMP immunoassay kit (Assay Designs, Ann Arbor, MI, USA). All the experiments were performed in triplicate. Cyclic nucleotide content was expressed as pmol·mg^{-1} protein of tissue or as a percentage of basal accumulation. The protein content of each sample was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

Functional experiments

Aorta (4 mm) and MRA (2 mm) rings were set-up in an isometric organ bath or a wire myograph (model 610 M, J.P. Trading, Denmark) respectively, filled with Krebs solution at 37°C and gassed with 95% O_2 and 5% CO_2 as previously described (Martí *et al.*, 2005). An initial load of 1 g was applied to the aorta, while the internal diameter of MRA was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure (l_{100} = 90–180 µm) according to the standard procedure of Mulvany and Halpern (1977).

After a 1-h stabilization period, the vessels were contracted with a depolarizing solution (80 mM KCl-Krebs obtained by an isotonic replacement of NaCl by KCl) to check the vessels' functionality. After the washout and returning to the stable baseline, vessels were contracted with the α_1-adrenoceptor agonist phenylephrine (1 µM) and endothelium integrity was tested with acetylcholine (ACh, 10 µM). Only the rings that relaxed more than 70% to ACh were considered endothelium intact; those that failed to relax (0%) to ACh were considered to have the endothelium removed.

To investigate the AC and the soluble guanylyl cyclase (sGC) pathways, concentration-response curves (CRCs) of relaxation to isoprenaline were performed in maximal phenylephrine (1 µM in aorta and 10 µM in MRA) precontracted vessels in the absence and presence of AC (SQ22536, 10–100 µM), protein kinase A (H89, 0.1 µM), sGC (ODQ, 10 µM) or NOS (L-NAME, 100 µM) inhibitors. The inhibitors were added 30 min prior to phenylephrine addition.

To determine the functional *Adrb* subtypes, cumulative CRCs of relaxation to the selective agonists, isoprenaline, dobutamine, salbutamol and CL316243 were performed in the absence or presence of *Adrb* antagonists: propranolol (1 µM), CGP20712A (1 µM), ICI118,551 (1 µM) or SR59230A (0.1 µM) which were added 30 min before the maximal phenylephrine-induced contraction. The concentration of antagonists used was selected based on preliminary experiments and literature data taking into account their affinities values for the different *Adrb* subtypes (Bilski *et al.*, 1983; Lemoine and Kaumann, 1991; Kaumann and Molenaar, 1996; Manara *et al.*, 1996; Hutchinson *et al.*, 2001; Baker, 2005; Alexander *et al.*, 2011; Bond *et al.*, 2012). Cumulative CRCs were constructed with sequential increments of 0.5 log units until a stable state was observed. The contact time for every single concentration of agonist was 8 min for the aorta and 5 min for the MRA. In each arterial segment, only one CRC was performed. Antagonist affinity (pK_B value) was estimated by a single concentration-ratio method (Furchgott, 1972).

To analyse the participation of endothelium and NO in *Adrb*-mediated vasodilatation, L-NAME (100 µM) was incubated for 30 min prior to performing CRCs to *Adrb* agonists in aorta rings with and without endothelium. In addition, the effect of *Adrb* selective antagonists was evaluated on the CRCs

to isoprenaline performed in endothelium denuded aorta vessels or after incubation (30 min) with L-NAME (100 μM). This concentration of L-NAME largely decreased ACh-induced relaxation (11.8 ± 1.9% of phenylephrine-induced contraction at 10 μM) suggesting an inhibition of NOS in these conditions.

Adrb-mediated relaxations were expressed as a percentage of phenylephrine-mediated contraction. Data were plotted using the Graph Pad Software version 4.0 (San Diego, CA, USA), with sigmoid curve fitting performed by non-linear regression; these curves were used to derive E_{max} (the maximal relaxant response) and pEC_{50} (-log of the agonist concentration needed to produce 50% of E_{max}).

Drugs

The following drugs were obtained from Sigma-Aldrich: acetylcholine chloride, (R)-(-) phenylephrine hydrochloride, (±)-isoprenaline hemisulphate salt, dobutamine hydrochloride, salbutamol hemisulphate salt, CL316243 (disodium 5-[(2R)-2-[[(2R)-2-(3-Chlorophenyl)-2-hydroxyethyl]amino] propyl]-1,3-benzodioxole-2,2-dicarboxylate hydrate), (±)-propranolol hydrochloride, CGP20712A ((±)-2-Hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl] amino]ethoxy]-benzamide methanesulphonate salt), ICI118,551 ((±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride), SR59230A (3-(2-Ethylphenoxy)-1-[[(1S)-1,2,3,4-tetrahydronaphth-1-yl]amino]-(2S)-2-propanol oxalate salt), L-NAME, SQ22536 (9-(tetrahydro-2-furanyl)-9H-purin-6-amine), ODQ (1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one), H-89 (N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulphonamide dihydrochloride) and IBMX. All the drugs were prepared in distilled water, except isoprenaline which was dissolved in 0.01% ascorbic acid, SR59230A in 20% ethylene glycol and ODQ in 20% ethanol.

Data analysis

The analysis was performed using the Graph Pad Software. Data are presented as the mean ± SEM of *n* experiments obtained from different animals. Statistically significant differences in mean values were tested by the Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

Expression of the Adrb subtypes in MRA and aorta

RT-qPCR was carried out to determine the expression of the *Adrb* subtypes in MRA (Figure 1A) and aorta (Figure 1B). The analysis of the mRNA levels showed that *Adrb1*, *Adrb2*, *Adrb3* were present, although differences in the relative amount of the mRNA levels of each subtype were observed. *Adrb3* mRNAs were the most and least abundant in aorta and MRA respectively (Figure 1).

By immunofluorescence labelling, we studied the cellular location of the *Adrb* subtypes in MRA (Figure 2A) and aorta (Figure 2B). All three subtypes were expressed in both tissues, but with a different cellular distribution. In MRA, *Adrb1* appeared to be located mainly in smooth muscle, but also in endothelial cells and in the adventitia, β_3-subtypes were exclusively located in endothelium and adventitia, whereas the *Adrb2* were observed in the endothelial cells and in the adventitial/medial border. In aorta, *Adrb1* and *Adrb2* were expressed in the three layers of the vessel wall, where β_1 and β_2 were predominantly expressed in the media or the endothelium respectively. *Adrb3* were scarcely expressed in endothelial cells and were predominantly detected along elastic lamina.

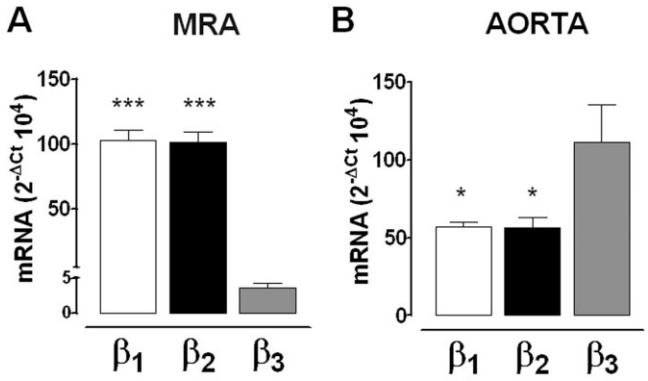


Figure 1

mRNA levels of the *Adrb1* (β_1), *Adrb2* (β_2), *Adrb3* (β_3) in the mesenteric resistance artery (MRA; A) or aorta (B) with endothelium. Values are expressed as $2^{-\Delta Ct}$ 10^4 using *Gapdh* as a housekeeping gene and are the mean ± SEM of $n = 4$–6 different animals. *$P < 0.05$, ***$P < 0.001$ versus *Adrb3*.

To confirm the cellular distribution of *Adrb* in aorta, mRNA levels were evaluated in arteries with and without endothelium, and in the SMCs freshly isolated from aorta (Figure 2C). As expected, the *Adrb1* and *Adrb2* were detected in aorta, irrespectively of the presence or absence of endothelium, and in isolated SMCs. However, the *Adrb3* subtype was absent in isolated SMCs, but was expressed in whole aorta with and without endothelium, thus confirming the presence of this subtype in the media layer in a different place to SMCs.

Adrb subtypes involved in cAMP and cGMP accumulation

Coupling to cAMP and cGMP formation was tested using *Adrb* agonists (Figure 3) with different subtype-selective profiles: isoprenaline (non-selective), dobutamine (β_1), salbutamol (β_2) and CL316243 (β_3).

In MRA, isoprenaline, dobutamine, CL316243, but not salbutamol, increased the cAMP levels (Figure 3A), suggesting that only *Adrb1* and *Adrb3* are coupled to cAMP production in this artery. Isoprenaline and CL316243, but not salbutamol or dobutamine, also induced cGMP formation (Figure 3A), indicating that only *Adrb3* activate cGMP production. *Adrb2* were apparently uncoupled to the nucleotides accumulation in this vessel.

In aorta, cAMP formation increased only with isoprenaline and dobutamine (Figure 3B), showing that *Adrb1* promote cAMP formation. cGMP accumulation was induced by isoprenaline, salbutamol and CL316243 (Figure 3B), and these results indicate that *Adrb2* and *Adrb3* couple to the cGMP pathway.

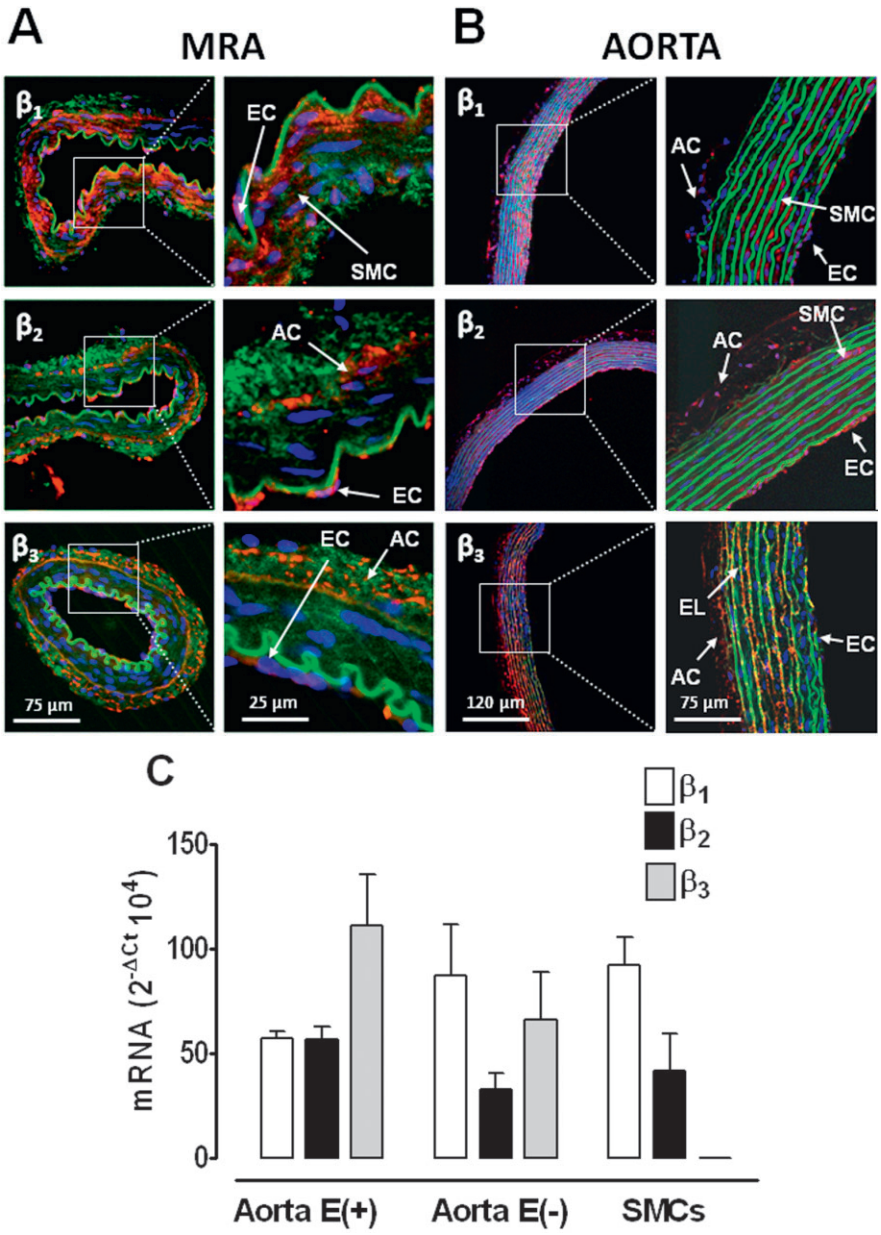


Figure 2

Representative immunofluorescence photomicrographs of the confocal microscopic sections of the *Adrb1 (β_1)*, *Adrb2 (β_2)*, *Adrb3 (β_3)* adrenoceptors (red) in the mesenteric resistance artery (MRA; A) or aorta (B). Natural autofluorescence of elastin (green) and nuclear staining (blue) are also shown. AC, adventitial cell; EC, endothelial cell; SMC, smooth muscle cell; EL, elastic lamina. $n = 4$–6 different animals. (C) Comparative analysis of the mRNA levels of the *Adrb1 (β_1)*, *Adrb2 (β_2)*, *Adrb3 (β_3)* in aorta with (E+) or without (E–) endothelium and freshly isolated smooth muscle cells (SMCs) from aorta. Values are expressed as $2^{-\Delta Ct}\ 10^4$ using *Gapdh* as a housekeeping gene and are the mean ± SEM of $n = 4$–6 different animals.

To confirm the pattern of the *Adrb* subtypes in nucleotide formation, the effect of selective *Adrb* antagonists [propranolol (β_1, β_2), CGP20712A (β_1), ICI118,551 (β_2) and SR59230A (β_3)] was tested on isoprenaline-mediated cAMP and cGMP formation. We also tested the effect of the AC (SQ22536) and the NOS (L-NAME) inhibitors.

In MRA, propranolol, CGP20712A, SR59230A, but not ICI118,551, inhibited the isoprenaline-induced increase in cAMP (Figure 3C), thus confirming the role of *Adrb1* and *Adrb3*. SR59230A, but not the other antagonists used, inhibited cGMP formation, (Figure 3C), suggesting that only *Adrb3* were implicated in this signal pathway.

In aorta, propranolol and CGP20712A diminished cAMP formation, whereas cGMP accumulation was decreased by propranolol, ICI118,551 and SR59230A (Figure 3D), which confirms the coupling of *Adrb1* to cAMP and *Adrb2* and *Adrb3* to the cGMP pathway.

In addition, the AC inhibitor SQ22536 significantly decreased the isoprenaline-induced formation of cAMP in MRA and aorta. The NOS inhibitor, L-NAME, completely

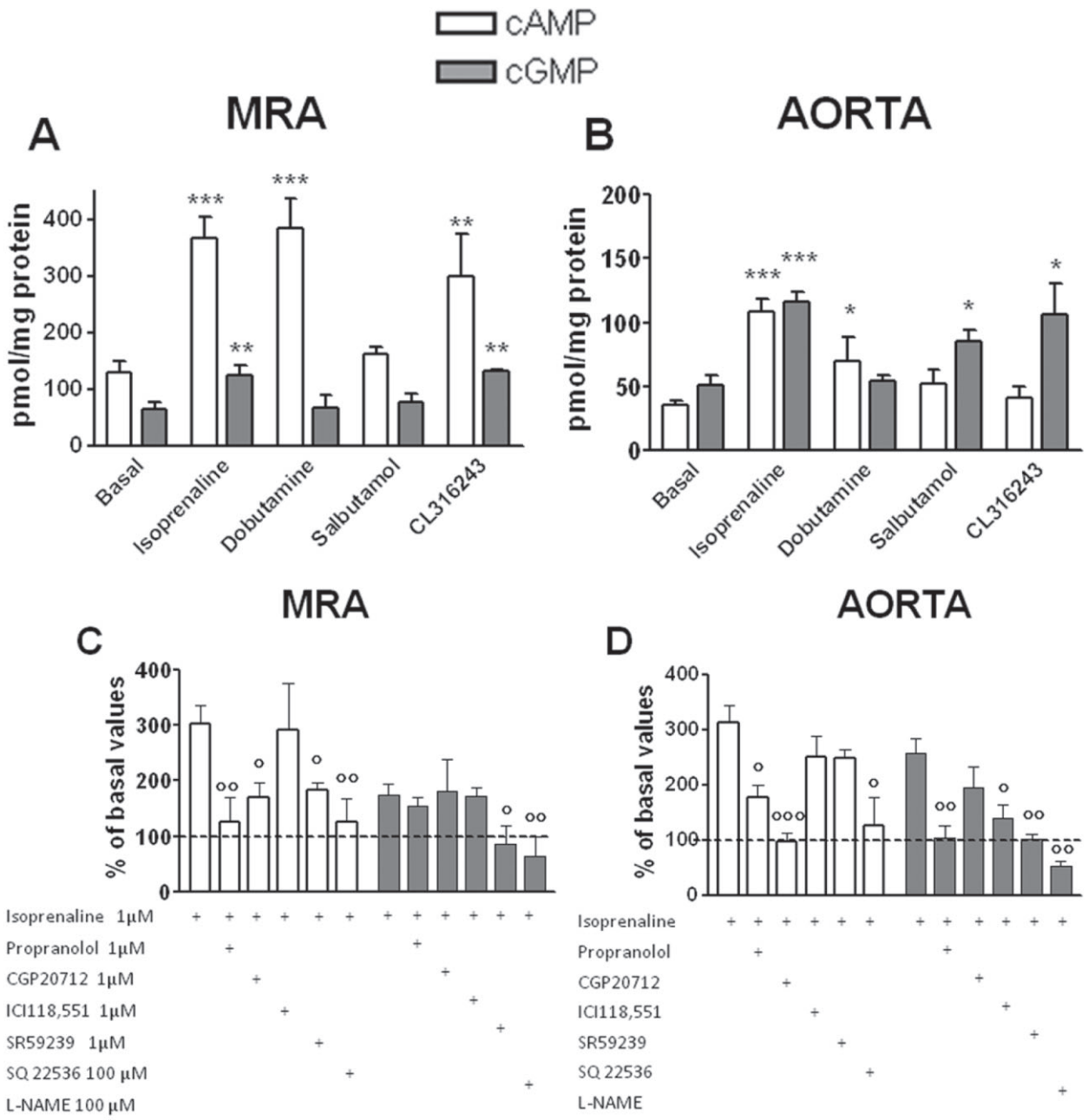


Figure 3

Agonists-induced cAMP and cGMP accumulation in mesenteric resistance arteries (MRA; A) or aorta (B). Effect of selective *Adrb* antagonists, SQ22536 and L-NAME on isoprenaline-induced cAMP or cGMP accumulations in MRA (C) or aorta (D). Values are expressed as pmol·mg^{-1} protein (A, B) or % of basal values (C, D) and represent the mean ± SEM of $n = 4$–5 different animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus Basal; $^{o}P < 0.05$, $^{oo}P < 0.01$, $^{ooo}P < 0.001$ versus isoprenaline.

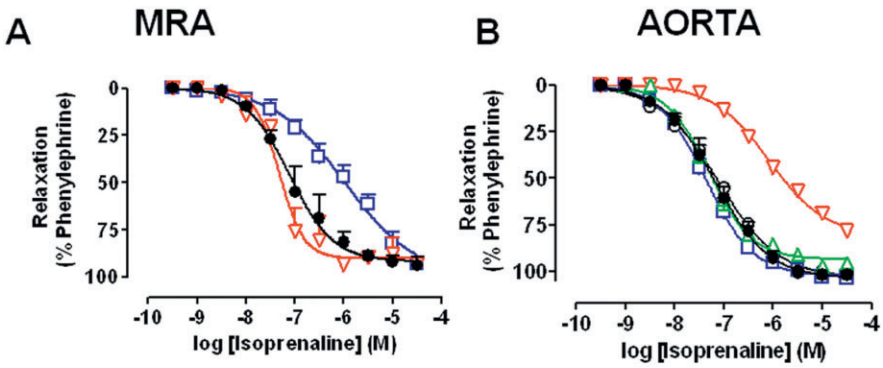
abolished the cGMP accumulation induced by isoprenaline in both vessels, evidencing the NO dependence of this signal (Figure 3C,D).

Role of cAMP and cGMP on Adrb-mediated vasorelaxation

Vasodilator CRCs to isoprenaline in maximal phenylephrine precontracted arteries were performed in the absence or presence of either the AC (SQ22536) or sGC (ODQ) inhibitors.

In MRA, the CRC to isoprenaline was right-shifted by 10 μM SQ22536, but not by 10 μM ODQ (Figure 4A), which suggests a role for cAMP, but not for cGMP, in the *Adrb*-induced vasorelaxant response.

A different scenario was observed in aorta (Figure 4B), where the CRC to isoprenaline was not affected by SQ22536 at 10 μM, not even at a higher concentration (100 μM). In addition, protein kinase A inhibitor H-89 (0.1 μM) showed no effect. However, ODQ inhibited the CRC to isoprenaline.



C

		pEC_{50}	
		MRA	AORTA
●	Isoprenaline (Control)	7.10 ± 0.09	7.17 ± 0.07
□	+ SQ22536 10 μM	5.99 ± 0.09***	7.35 ± 0.04
○	+ SQ22536 100 μM		7.10 ± 0.06
△	+ H89 0.1 μM		7.29 ± 0.05
▽	+ ODQ 10 μM	7.31 ± 0.08	6.07 ± 0.06***

Figure 4

Concentration response curves (CRC) to isoprenaline in phenylephrine precontracted mesenteric resistance arteries (MRA; A) or aorta (B). The relaxant response to isoprenaline was evaluated in the absence or presence of adenylyl cyclase (SQ22536), PKA (H-89) or soluble guanylyl cyclase (ODQ) inhibitors at the indicated concentrations. Parameters of the CRC (C). Values are the mean ± SEM of $n = 4–6$ different animals. ***$P < 0.001$ versus control.

Table 1

Parameters of the concentration-response curves of relaxation of the β-adrenoceptors agonists in MRA or aorta precontracted with phenylephrine

	MRA			AORTA		
	Emax	pEC_{50}	pK_B	Emax	pEC_{50}	pK_B
Isoprenaline (control)	95.9 ± 2.4	7.00 ± 0.10		98.7 ± 1.3	7.38 ± 0.18	
+ propranolol 1 μM	90.6 ± 8.1	5.47 ± 0.26***	7.52	98.0 ± 2.0	6.38 ± 0.10***	6.95
+ CGP20712A 1 μM	98.1 ± 1.9	5.20 ± 0.09***	7.80	95.8 ± 2.8	6.96 ± 0.15	
+ ICI118,551 1 μM	96.7 ± 1.3	6.65 ± 0.19		95.8 ± 1.9	6.20 ± 0.11***	7.15
+ SR59230A 0.1 μM	98.0 ± 2.0	6.75 ± 0.13		98.3 ± 0.8	6.95 ± 0.11	
Dobutamine (control)	99.6 ± 0.4	6.95 ± 0.04		96.8 ± 5.4	6.03 ± 0.11††	
+ CGP20712A 1 μM				83.8 ± 3.6	5.46 ± 0.08*	6.43
Salbutamol (control)	96.0 ± 4.0	4.87 ± 0.10		98.0 ± 1.4	6.81 ± 0.08††	
+ ICI118,551 1 μM				86.8 ± 3.9	4.80 ± 0.28**	8.00
CL316243 (control)	12.0 ± 6.2			76.7 ± 4.4	6.48 ± 0.09	
+ SR59230A 0.1 μM				68.8 ± 7.5	5.80 ± 0.25*	7.58

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control; ††$P < 0.01$, †††$P < 0.0001$ between aorta and MRA. Values are the mean ± SEM. of 4–8 experiments.

Adrb subtypes involved in modulation of the vascular tone

All the *Adrb* agonists tested, except β_3-agonist CL316243 in MRA, relaxed the phenylephrine-induced tone in the MRA and the aorta in a concentration-dependent manner, while the potency of each agonist differed between vessels (Table 1). Dobutamine showed higher potency in MRA than aorta, whereas salbutamol was more potent in aorta than in MRA, suggesting a major implication of *Adrb1* in the vasorelaxant response in MRA and a predominance of *Adrb2* in aorta. The participation of *Adrb3* in MRA was excluded given the lack of vasodilatation noted after the addition of CL316243.

The effect of selective *Adrb* antagonists on isoprenaline-induced vasorelaxation was also analysed. In MRA, the CRC

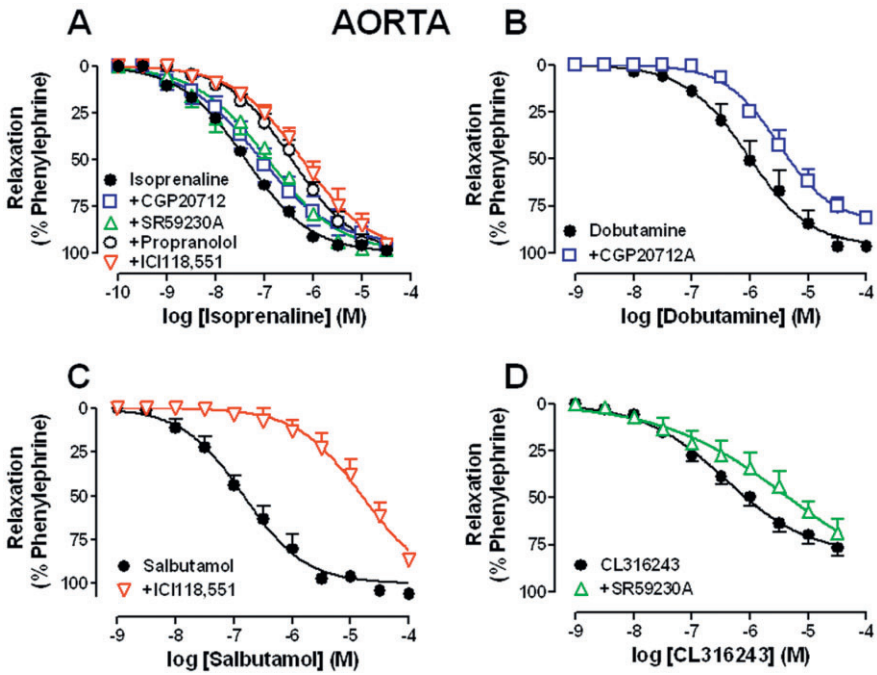


Figure 5

CRC to isoprenaline (A), dobutamine (B), salbutamol (C) and CL316243 (D) in the phenylephrine precontracted aorta. The relaxant response to *Adrb* agonists was evaluated in the absence or presence of the appropriate *Adrb* antagonist (propranolol, CGP20712A, ICI118,551 at 1 μM or SR59230A at 0.1 μM). Values are the mean ± SEM of $n = 4–8$ different animals.

to isoprenaline significantly shifted to the right only by propranolol and CGP20712A (Table 1). Once again, and as previously described (Briones *et al.*, 2005), this suggests a major role for the *Adrb1* subtype.

In aorta, propranolol and ICI118,551 significantly right-shifted the CRC to isoprenaline (Figure 5A) and salbutamol (Figure 5C), thus confirming a main role for *Adrb2* in this vessel (Table 1). As expected, the CRC to dobutamine was slightly inhibited by CGP20712A with a low pK_B value (Figure 5B, Table 1), which rules out any major involvement of *Adrb1* in vasodilatation. The selective β_3-antagonist, SR59230A, displaced the relaxant curve to CL316243 (Figure 5D, Table 1) with a pK_B value (7.58) that agrees with a *Adrb3*-mediated response (Kaumann and Molenaar, 1996).

Adrb subtypes coupled to the endothelial NO pathway

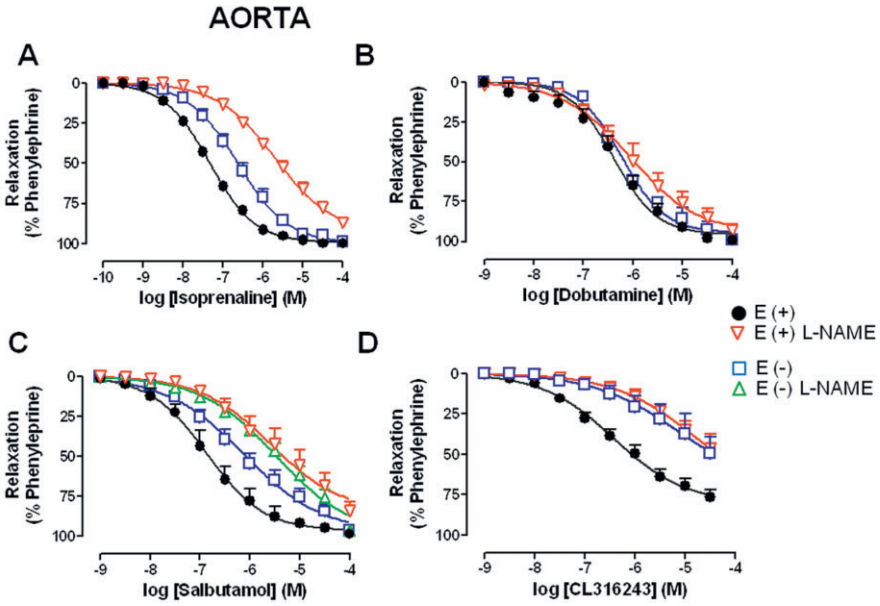
In MRA, endothelium removal or preincubation with a concentration of L-NAME that almost abolishes NO synthesis in aorta had no effect on the vasodilator responses induced by isoprenaline, dobutamine or salbutamol (results not shown). This is consistent with the lack of a significant participation of the NO/cGMP pathway in the *Adrb*-induced vasodilatation in MRA.

In aorta, the CRC to isoprenaline, salbutamol and CL316243, but not to dobutamine, shifted rightwards by preincubation with L-NAME (Figure 6A–E), confirming that the effect of *Adrb2* and *Adrb3* depends on NO. The CRC to *Adrb3* selective agonist CL316243 (Figure 6D) in endothelium denuded arteries was similar to that observed in the presence of L-NAME. Endothelium removal inhibited the relaxant response to isoprenaline (Figure 6A) and salbutamol (Figure 6C) to a lesser extent than preincubation with L-NAME. In addition in endothelial denuded aortic rings, L-NAME inhibited the relaxation induced by salbutamol, suggesting that the *Adrb2* present in SMCs stimulate NO production. Furthermore, we detected *Nos3* mRNA not only in endothelium denuded aortas, but also in isolated SMCs (Figure 6F).

To further analyse the *Adrb* subtypes involved in both the endothelium-independent and L-NAME insensitive relaxation in aorta, the effect of selective antagonists on isoprenaline-induced response was evaluated. In denuded aortas, propranolol, CGP20712A and ICI118,551, but not SR59230A, shifted the CRC to isoprenaline to the right (Figure 7A), whereas only propranolol and CGP20712A inhibited isoprenaline-mediated relaxation in the presence of L-NAME (Figure 7B). These results demonstrate that both *Adrb1* and *Adrb2* are involved in isoprenaline-induced relaxation in endothelium denuded vessels, and that only the β_1-subtype is responsible for the L-NAME-insensitive relaxant component to isoprenaline. Estimation of the apparent affinity of CGP201712 yielded a low pK_B value (Figure 7), suggesting the implication of the low-affinity state of *Adrb1* (Mallem *et al.*, 2004; Kaumann and Molenaar, 2008).

Discussion and conclusions

The present study highlights that although the mRNA and protein expressions of the three *Adrb* subtypes are present in



E

	E (+)	E (+)/ L-NAME	E (-)	E (-)/ L-NAME
Isoprenaline pEC_{50}	7.33 ± 0.03	5.68 ± 0.11*****††††	6.62 ± 0.09***	
E_{max}	99.7 ± 1.8	86.9 ± 2.3	98.8 ± 1.3	
Dobutamine pEC_{50}	6.39 ± 0.06	6.19 ± 0.08	6.22 ± 0.05	
E_{max}	99.3 ± 0.4	93.4 ± 4.1	99.0 ± 1.0	
Salbutamol pEC_{50}	6.86 ± 0.09	5.48± 0.19***†	6.08 ± 0.12**	5.47 ± 0.16†
E_{max}	98.4 ± 0.8	89.5 ± 7.1	98.4 ± 2.1	96.5 ± 3.0
CL316243 pEC_{50}	6.48 ± 0.09	5.52 ± 0.18	5.62 ± 0.22	
E_{max}	76.7 ± 4.4	46.4 ± 8.7*	49.4 ± 10.1*	

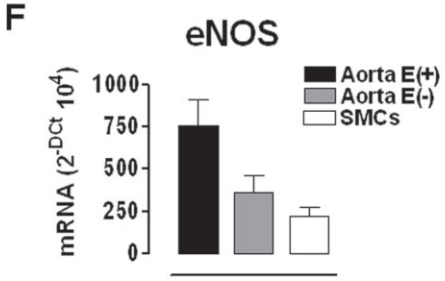


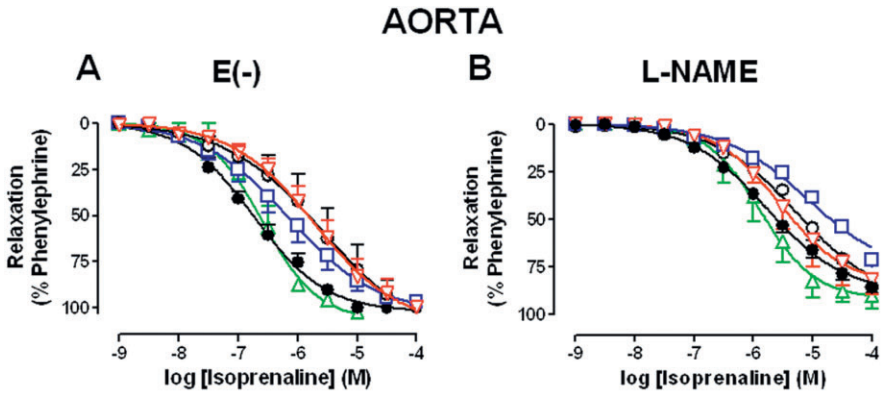
Figure 6

CRC to isoprenaline (A), dobutamine (B), salbutamol (C) and CL316243 (D) in the phenylephrine precontracted aorta with (E+) or without (E–) endothelium in the absence or presence of L-NAME (100 μM). Values are the mean ± SEM of $n = 4$–13 different animals. Parameters of the CRC (E). Comparative analysis of the mRNA levels of *Nos3* (eNOS) in aorta with (E+) or without (E–) endothelium and freshly isolated smooth muscle cells (SMCs) from aorta (F); values are expressed as $2^{-\Delta Ct}\ 10^4$ using *Gapdh* as a housekeeping gene and are the mean ± SEM of $n = 4$–5 different animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus E (+); †$P < 0.05$, †††$P < 0.001$ versus E (–).

MRA and aorta, the specific *Adrb* subtype contribution and signalling pathways involved in the vasodilator response differ between territories. Therefore, these findings suggest a different physiological role played by *Adrb* signalling in regulating conductance and resistance vessels.

In MRA, we previously described the presence of *Adrb* in smooth muscle, endothelial or adventitial cells using the fluorescent ligand BODIPY-TRM-CGP 12177 (Briones *et al.*, 2005). However, it was not possible to identify the specific subtype because this ligand has affinity for both *Adrb1* and *Adrb2* (Baker *et al.*, 2003). Although the use of *Adrb* antibodies proved controversial (Pradidarcheep *et al.*, 2009), according to our experience detection of *Adrb* subtypes using selective antibodies or the fluorescent ligand follows the same pattern.

We reveal that in MRA *Adrb1* are mainly, but not exclusively, localized in the smooth muscle layer, whereas *Adrb2* and *Adrb3* are expressed in the endothelium and adventitia. Nevertheless, the location of *Adrb2* in the adventitial/medial border could suggest its presence either in nerves or in pericytes as previously suggested for *Adrb* (Briones *et al.*, 2005). In our previous study using BODIPY-TMR-CGP 12177 (Briones



C

		E (-)		L-NAME	
		pEC_{50}	pK_B	pEC_{50}	pK_B
●	Isoprenaline (control)	6.72 ± 0.06		5.77 ± 0.06	
○	+ propranolol 1 μM	5.91 ± 0.19***	6.73	5.27 ± 0.10***	6.33
□	+ CGP20712 1 μM	6.14 ± 0.15**	6.45	5.08 ± 0.09***	6.59
▽	+ ICI118,551 1 μM	5.72 ± 0.17***	6.95	5.54 ± 0.15	
△	+ SR59230A 0.1 μM	6.60 ± 0.10		5.87 ± 0.09	

Figure 7

CRC to isoprenaline in phenylephrine precontracted aorta without endothelium (E–) (A) or in the presence of 100 μM L-NAME (B). Parameters of the CRC (C). The relaxant response to isoprenaline was evaluated in the absence or presence of propranolol, CGP20712A, ICI118,551 and SR59230A. Values are the mean ± SEM of $n = 4–5$ different animals. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control.

et al., 2005), *Adrb* where seen in the surface and inside the smooth muscle cells, particularly in the perinuclear region but this level of detail cannot be reached using antibodies in a whole tissue. Despite the presence of the three subtypes in the vessel wall, the vasodilator response appears to be mediated mainly by the *Adrb1* subtype, localized in the muscular layer which acts through the classical *Adrb* pathway; that is activation of AC and cAMP formation, without the participation of the cGMP pathway. Several evidences support this assumption: (i) isoprenaline and dobutamine, but not salbutamol and CL316246, increase the cAMP levels and relax the artery; (ii) isoprenaline-induced cAMP accumulation and relaxation are inhibited by propranolol and the selective *Adrb1* antagonist (CGP20712A), but not by the selective *Adrb2* or *Adrb3* antagonist (ICI118,551 or SR592310A respectively); (iii) vasodilatation to isoprenaline is inhibited by AC inhibitor SQ22536, but not by sGC inhibitor ODQ, which excludes the participation of the cGMP pathway in *Adrb1*-mediated relaxation; (iv) endothelium removal or L-NAME pretreatment have no effect on *Adrb* mediated relaxation excluding a major participation of NO. However, we cannot exclude the involvement of NO from stores not blocked by L-NAME (Kakuyama *et al.*, 1998) or a component of the NO vasodilatation response not mediated by sGC (Vanheel and Van de Voorde, 2000).

The major role of *Adrb1* in controlling the vascular tone of resistance arteries agrees with previous results (Graves and Poston, 1993; Briones *et al.*, 2005; Garland *et al.*, 2011) and reinforces the hypothesis that this mechanism may be disrupted in patients taking *Adrb* antagonists, as already suggested (Garland *et al.*, 2011). However, the link between *Adrb1* and the signalling pathway in MRA is far from being clear. The present study shows that the accumulation of cAMP, but not of cGMP, is related to *Adrb1* activation. While Graves and Poston (1993) reported in MRA that *Adrb1* stimulation releases NO, more recent studies (Briones *et al.*, 2005; Garland *et al.*, 2011), including the present one, do not support a significant participation of NO and point to cAMP being the predominant signalling pathway linked to *Adrb1*-mediated relaxation.

Even though the proportion of the mRNA expression of *Adrb2* is similar to that of *Adrb1* and, despite them being located in the endothelial and adventitial layers, as *Adrb3*, the agonists/antagonists profile excludes the participation of *Adrb2* in nucleotide formation and vasodilatation in MRA. Garland *et al.* (2011) reported in MRA endothelial *Adrb1* and *Adrb2*-mediated hyperpolarization, irrespectively of cAMP accumulation and NO formation, which is not essential for vasodilatation. These authors suggest that *Adrb*-linked hyperpolarization might initiate spreading dilatation passing along the artery wall via the endothelium. This proposal could help explain not only the presence, but also the function, of *Adrb2* in the endothelial layer of MRA.

The fact that CL316243 increases the cAMP and cGMP levels and that SR59230A causes a significant blockade of nucleotides accumulation without affecting the contractile tone, indicates that adventitial and/or endothelial *Adrb3* couple to cGMP and cAMP signals with no involvement in *Adrb*-mediated vasodilatation. Other studies (Briones *et al.*, 2005; Garland *et al.*, 2011) have also excluded the participa-

tion of *Adrb3* in the control of either the vascular tone or the hyperpolarization response in the MRA. Taken together, these results suggest that the accumulation of cyclic nucleotides induced by β_3-stimulation plays a functional role other than controlling the vascular tone.

In summary, we show for the first time to our knowledge that *Adrb1* (located in SMCs and endothelial cells) and *Adrb3* (located in adventitial and endothelial cells) are coupled to cAMP, and that only the β_1-subtype participates in controlling the vascular tone. In spite of the controversy about the role of NO (Graves and Poston, 1993; Briones *et al.*, 2005; Garland *et al.*, 2011), our results exclude its participation in the relaxant response to *Adrb* in MRA. The β_2-subtype, which is also present in the vessel, does not seem to play a relevant role in modulating the vascular tone, but may be involved in different functions, as previously suggested (Garland *et al.*, 2011).

A more complex scenario has been found in aorta where *Adrb*-induced relaxation has been extensively described. However, discrepancy persists in the literature as to the relative contribution of each subtype and their link to nucleotide signalling pathways. Previous reports have evidenced that β-adrenergic relaxation occurs through a mixed participation of *Adrb1*/*Adrb2* (Satake *et al.*, 1996) or entirely through the β_2 subtype (Ferro *et al.*, 2004). The functional participation of *Adrb3* (Trochu *et al.*, 1999; Oliver *et al.*, 2009), atypical *Adrb* like the low-affinity state of *Adrb1* (Mallem *et al.*, 2004; 2005a) or atypical *Adrb* that coexist with the β_1- and β_2-subtypes (Brawley *et al.*, 2000b) have also been proposed. More recently however, the latter authors ruled out the presence of functional *Adrb3* or low-affinity *Adrb1* (Brahmadevara *et al.*, 2003; 2004).

In our study, the use of selective *Adrb* agonists and antagonists in aorta shows that the activation of *Adrb1*, located in SMCs, endothelial and adventitial cells, increases the cAMP levels in the whole tissue, but, conversely to what occurs in MRA, the cAMP/PKA pathway does not seem to play an essential role in *Adrb*-mediated relaxation. In fact, the AC inhibitor (SQ22536), which blocks cAMP accumulation, does not modify the isoprenaline-induced relaxation in aorta, but significantly inhibits the vasorelaxation in MRA. We have also tested the effect of a PKA inhibitor, H-89, and although it has been reported to inhibit not only PKA but also other kinases (Davies *et al.*, 2000), the fact that in rat aorta H-89 was unable to modify the vascular tone reinforces the lack of participation of cAMP/PKA on *Adrb* mediated relaxation. This behaviour displayed by the *Adrb1* in aorta establishes a clear difference with that observed in MRA, where vasodilatation depends on the β_1-activation linked to cAMP. Once again, unlike MRA, *Adrb2* couple to cGMP in rat aorta. Several lines of evidence suggest the participation of the NO/sGC/cGMP pathway in the vasodilatation mediated by those adrenoceptors: (i) isoprenaline and salbutamol increase the cGMP levels and this increase is inhibited by ICI118,551; (ii) release of NO is essential for the promotion of cGMP accumulation since it is completely inhibited by L-NAME, (iii) the sGC inhibitor (ODQ) and L-NAME, but not the AC inhibitor (SQ22536), inhibit the relaxation induced by isoprenaline; (iv) only a *Adrb2* antagonist (ICI118,551) causes a significant blockade of isoprenaline-evoked relaxation. The implication of NO in *Adrb2*-induced relaxation in rat aorta has also been reported by other authors (Ferro *et al.*, 2004).

Two new findings of our study are relevant and suggest that the *Adrb2* localized in SMCs of aorta contribute to the relaxant response through the NO pathway. The first is that the CRCs to isoprenaline and salbutamol were right-forward displaced to a greater extent by L-NAME than by endothelium removal. The second is that ICI118,551 displaces the CRC to isoprenaline in the absence of the endothelium, and fails to modify these CRCs in L-NAME-treated rings. The involvement of NO released by SMCs as a complementary mechanism to that produced by endothelial *Adrb2* is also supported by the expression of *Nos3* mRNA found in the isolated aortic SMCs.

Moreover, in either endothelium denuded vessels or the presence of L-NAME, the *Adrb*-mediated relaxant response, which is significantly inhibited by CGP20712A, is observed, and might be attributed to the muscular β_1-subtype. However, the pharmacological profile of this subtype in aorta better fits a low-affinity state of *Adrb1* since, as the present results show, the apparent affinity for the *Adrb1* antagonist in aorta is lower than that estimated in MRA. These results agree with other authors, who have also suggested an implication of the low-affinity state of *Adrb1* in rat aorta vasodilatation (Mallem *et al.*, 2004; 2005a,b).

Regarding the role of the β_3-subtype in aorta, our results confirm and extend previous observations which have already assessed its presence and function in this vessel (Trochu *et al.*, 1999; Rautereau *et al.*, 2002). We detected a more prominent mRNA expression of β_3 in relation to *Adrb1* and *Adrb2*, which is mainly located along the elastic lamina, but scarcely in the endothelium. However, despite the observed abundant expression of this adrenoceptor, its role in relaxation does not seem to be as relevant as the β_2-subtype. The use of L-NAME and the selective *Adrb3* agonist (CL316243), as well as the antagonist (SR59230A) in *Adrb*-mediated cGMP accumulation and relaxation, demonstrates that this response is mediated through the NO/cGMP pathway. Yet unlike the β_2-subtype, the vasodilatation induced by *Adrb3* disappears in denuded rings, indicating the lack of participation of the receptors present along the elastic lamina in relaxation. The fact that in ligament fibroblasts a rise in cGMP stimulates elastin production (Mecham *et al.*, 1985) lead us to speculate that stimulation of *Adrb3* present along the elastic lamina may participate in elastin production. However, the assessment of the hypothetical role of these receptors on elastin production is beyond the scope of the present study.

In summary, we demonstrate that the three *Adrb* subtypes are expressed in aorta and MRA. The *Adrb1*, located in SMCs and acting through the canonical AC/cAMP pathway, is the subtype that is primarily responsible for the vasodilator response in MRA. The *Adrb* localized in endothelial cells do not participate in the relaxant response in this vessel. Conversely in aorta, the *Adrb2* and *Adrb3* localized in the endothelium, along with the *Adrb2* present in SMCs and coupled to the NO/cGMP pathway, play a prominent vasodilator role. The β_1-subtype, localized in SMCs, also contributes to vasodilatation, but not through the cAMP pathway. Its activity corresponds to a low-affinity state of this receptor and it becomes more evident in the absence of endothelial *Adrb*.

It is interesting to remark that endothelial *Adrb3* are coupled to cGMP in both vessels, but they only modulate vasodilatation in aorta, whereas the *Adrb2* uncoupled to cAMP in both arteries only play a vasodilator role through the NO/cGMP pathway in aorta.

These results indicate a role of endothelial *Adrb* in the control of the vasodilatation in a conductance vessel such as aorta, but not in a resistance artery such as MRA and highlight the different physiological role played by *Adrb* signalling in regulating the adrenergic contractile tone of conductance and resistance vessels. In aorta, a poorly innervated vessel, *Adrb2* located in the endothelium and having a greater affinity for circulating adrenaline (Westfall and Westfall, 2006), modulate the vessel tone and the blood flow distribution. In this context, endothelial cells are the first target for the vascular action of adrenaline. Importantly, recently it has been demonstrated that aortic endothelial cells are able to synthesize and release catecholamines (Sorriento *et al*., 2012). In addition, the *Adrb2* and *Adrb1* located in the SMCs also regulate the aortic tone. In contrast in highly innervated resistance vessels, the *Adrb1* located in SMCs and having a greater affinity for norepinephrine, which is released by the nerve endings, are those involved in the vasodilator response. These findings also suggest coordinated signalling through different *Adrb* subtypes located along different layers in regulating tone of conductance but not resistance vessels. Given the widespread clinical use of non-subtype and subtype-specific β-blockers, these findings are likely to be clinically relevant.

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Conflict of interest

The authors state no conflict of interest.

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