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Correlation between vasoconstrictor roles and mRNA expression of α_1-adrenoceptor subtypes in blood vessels of genetically engineered mice

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1 We examined the contribution of each α_1-adrenoceptor (AR) subtype in noradrenaline (NAd)-evoked contraction in the thoracic aortas and mesenteric arteries of mice. Compared with the concentration–response curves (CRCs) for NAd in the thoracic aortas of wild-type (WT) mice, the CRCs of mutant mice showed a significantly lower sensitivity. The pD_2 value in rank order is as follows: WT mice (8.21) > α_{1B}-adrenoceptor knockout (α_{1B}-KO) (7.77) > α_{1D}-AR knockout (α_{1D}-KO) (6.44) > α_{1B}- and α_{1D}-AR double knockout (α_{1BD}-KO) (5.15). In the mesenteric artery, CRCs for NAd did not differ significantly between either WT (6.52) and α_{1B}-KO mice (7.12) or α_{1D}-KO (6.19) and α_{1BD}-KO (6.29) mice. However, the CRC maximum responses to NAd in α_{1D}- and α_{1BD}-KO mice were significantly lower than those in WT and α_{1B}-KO mice.

2 Except in the thoracic aortas of α_{1BD}-KO mice, the competitive antagonist prazosin inhibited the contraction response to NAd with high affinity. However, prazosin produced shallow Schild slopes in the vessels of mice lacking the α_{1D}-AR gene. In the thoracic aorta, pA_2 values in WT mice for KMD-3213 and BMY7378 were 8.25 and 8.46, respectively, and in α_{1B}-KO mice they were 8.49 and 9.13, respectively. In the mesenteric artery, pA_2 values in WT mice for KMD-3213 and BMY7378 were 8.34 and 7.47, respectively, and in α_{1B}-KO mice they were 8.11 and 7.82, respectively. These pharmacological findings were in fairly good agreement with findings from comparison of CRCs, with the exception of the mesenteric arteries of WT and α_{1B}-KO mice, which showed low affinities to BMY7378.

3 We performed a quantitative analysis of the mRNA expression of each α_1-AR subtype in these vessels in order to examine the correlation between mRNA expression level and the predominance of each α_1-AR subtype in mediating vascular contraction.

4 The rank order of each α_1-AR subtype in terms of its vasoconstrictor role was in fairly good agreement with the level of expression of mRNA of each subtype, that is, α_{1D}-AR > α_{1B}-AR > α_{1A}-AR in the thoracic aorta and α_{1D}-AR > α_{1A}-AR > α_{1B}-AR in the mesenteric artery. No dramatic compensatory change of α_1-AR subtype in mutant mice was observed in pharmacological or quantitative mRNA expression analysis.
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Abbreviations: α_1-AR, α_1-adrenoceptor; BMY7378, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]-ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride; CRCs, concentration–response curves; EC_{50}, 50% effective concentration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KMD-3213, (−)-1-(3-hydroxypropyl)-5-((2*R*)-2-{[2-({2-[(2,2,2-trifluoroethyl) propyl)-2,3-dihydro-1H-indole-7-carboxamide; NAd, noradrenaline; pA_2 value, negative logarithm of dissociation constant, obtained from mechanical response; pK_B, negative logarithm of dissociation constant, obtained from Scatchard plot; pK_i value, negative logarithm of dissociation constant, obtained from Hill plot of displacement curve

Introduction

The sympathetic nervous system plays an important role in vasoconstriction and blood pressure regulation. Catecholamines cause vascular smooth muscle contraction, primarily by activating α_1-adrenoceptor (α_1-ARs) (Hoffman, 2001). Currently, α_1-AR can be characterized as three subtypes, α_{1A}-, α_{1B}-, and α_{1D}-AR, by molecular cloning (Cotecchia *et al*., 1988;

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Schwinn *et al*., 1990; Perez *et al*., 1991; Hirasawa *et al*., 1993; Esbenshade *et al*., 1995; Hieble *et al*., 1995b) and by pharmacological analysis (McGrath, 1982; Han *et al*., 1987; Foglar *et al*., 1995; Guarino *et al*., 1996). The functional roles of each α_1-AR subtype, in particular in relation to vasoconstriction and blood pressure regulation, have been elucidated through the recent development of α_1-AR subtype-selective agonists and antagonists (Goetz *et al*., 1995; Hieble *et al*., 1995a, b; Ford *et al*., 1996; Saussy *et al*., 1996; Yamamoto & Koike, 2001a, b). The thoracic aorta and mesenteric artery, vessels with great potential as experimental models, have often been used to analyze the vasoconstrictor role of each α_1-AR subtype in noradrenaline (NAd)-evoked vasoconstriction (Shi *et al*., 1989; Han *et al*., 1990; Kenny *et al*., 1995; Hussain & Marshall, 2000; Yamamoto & Koike, 2001a, b). For example, α_{1D}-AR-mediated vasoconstriction has been demonstrated to predominate in the rat thoracic aorta (Ford *et al*., 1996) and mouse thoracic aorta (Yamamoto & Koike, 2001b). In contrast, in the mouse mesenteric artery, it is still uncertain whether α_{1D}-AR-mediated vasoconstriction predominates (Yamamoto & Koike, 2001a), although α_{1D}-AR has been shown pharmacologically to be present in this artery (Goetz *et al*., 1995; Saussy *et al*., 1996). In addition, the gene knockout approach is increasingly being used to elucidate the functional roles of individual α_1-AR subtypes in vasoconstriction and blood pressure regulation (Cavalli *et al*., 1997; Rokosh & Simpson, 2001; Daly *et al*., 2002; Tanoue *et al*., 2002). This gene knockout approach has revealed that all three α_1-AR subtypes might be involved in vasoconstriction and blood pressure regulation (Cavalli *et al*., 1997; Rokosh & Simpson, 2001; Daly *et al*., 2002; Tanoue *et al*., 2002). However, we could not simply compare these results, because of the different genetic backgrounds and potential compensatory effects. Hence, the exact roles of the α_1-AR subtypes in vasoconstriction need further investigation, both from a pharmacological and a gene expression viewpoint. To date, we have obtained limited information on the mRNA expression profiles of each α_1-AR subtype in the cardiovascular system (Guarino *et al*., 1996; Miller *et al*., 1996). Real-time PCR techniques (Heid *et al*., 1996; Harrison *et al*., 2000; Medhurst *et al*., 2000) have enabled us to analyze the mRNA expression of each α_1-AR subtype quantitatively in a variety of tissues (Volgin *et al*., 2001; Tanoue *et al*., 2002; Nomiya & Yamaguchi, 2003). Here, we performed a pharmacological characterization of the thoracic aorta and mesenteric arteries of wild-type (WT) and mutant mice with the same genetic background, in order to compare the expression of each α_1-AR subtype in these vessels, as monitored by real-time PCR assay.

Methods

Generation of mice lacking both the α_{1B}-AR and α_{1D}-AR subtypes

α_{1B}-KO and α_{1D}-KO mice had already been generated and their viability confirmed (Cavalli *et al*., 1997; Tanoue *et al*., 2002). α_{1B}-KO mice with the genetic background of 129Sv and a mixture of C57Black/6J strains (Cavalli *et al*., 1997) were mated with α_{1D}-KO mice with the genetic background of 129Sv and a mixture of C57Black/6J strains (Tanoue *et al*., 2002) to produce F1 mice heterozygous for both traits. F1 heterozygous mice were mated to produce F2 WT and α_{1B}- and α_{1D}-AR double knockout (α_{1BD}-KO) mice. Breeding pairs from these two lines produced offspring, which were used in our experiments. Thus, the genetic backgrounds of the WT, α_{1B}-KO, α_{1D}-KO, and α_{1BD}-KO mice were the same. The genotypes of each α_1-AR subtype were determined from DNA isolated from the tail (Cavalli *et al*., 1997; Tanoue *et al*., 2002). Four groups of male mice, WT, α_{1B}-KO, α_{1D}-KO, and α_{1BD}-KO, with body weights of about 20–30 g, were used. All mice were housed in animal quarters with a 12-h light–dark cycle and were given food and distilled water *ad libitum*.

Mechanical responses

All experiments were conducted in accordance with the guidelines for the care and use of animals, as approved by the ethical committee of the National Research Institute for Child Health and Development. Each mouse was killed by a blow on the head, and then the thoracic aorta and mesenteric artery were isolated and dissected free of excess fat and connective tissue. The intimal surface of each artery was gently rubbed with a polyethylene tube to remove the endothelium, and functional loss of endothelial cells was confirmed by loss of the relaxation response to acetylcholine (1 μM). Each artery was cut into 4-mm ring segments. Ring segments were suspended in a 20-ml organ bath filled with Ringer–Locke solution (in mM: NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 2.1, $NaHCO_3$ 5.9, and glucose 2.8), kept at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. To prevent oxidation of NAd, ascorbic acid (1 mg ml^{-1}) was added to the solution. The tension was monitored continuously and recorded isometrically by a force displacement transducer. Experiments were conducted in the presence of propranolol (10 μM), yohimbine (0.3 μM), desmethylimipramine (0.1 μM), and normetanephrine (1 μM) to block β-adrenoceptors and α_2-adrenoceptors and to inhibit neural and non-neural uptake of NAd, respectively. The strips were allowed to equilibrate for 90 min and then contracted with NAd and allowed to equilibrate for 30 min after washing. This was repeated until two successive contractions of approximately equal size had been obtained. The competitive antagonistic activities were expressed as pA_2 values (negative logarithms of the dissociation constant). The concentration–response curves (CRCs) of NAd were obtained cumulatively. A contraction was expressed as grams force of the maximum contraction produced by NAd. After determination of a control CRC, the strips were equilibrated with a competitive antagonist for 30 min. CRCs were then determined in the presence of the antagonist and the procedure repeated with two further concentrations of the antagonist in the same preparation. After determination of the control CRCs, three successive cumulative CRCs for the antagonists were recorded. For each tissue, pD_2 values and maximum tensions for the first, second, third, and fourth CRCs for NAd were not significantly different in preliminary experiments. We used the nonselective α_1-antagonist prazosin ($1–30 \times 10^{-9}$ M), the α_{1D}-selective antagonist 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]-ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride (BMY7378) ($10–3000 \times 10^{-9}$ M), and the α_{1A}-selective antagonist (−)-1-(3-hydroxypropyl)-5-((2*R*)-2-{[2-({2-[(2,2,2-trifluoroethyl) propyl)-2,3-dihydro-1H-indole-7-carboxamide (KMD-3213) ($10–100 \times 10^{-9}$ M) to determine the pA_2 values. The pA_2 values were calculated according to the method of Tallarida

et al. (1979), which was originally reported by Arunlakshana & Schild (1959). Antagonist pA_2 values were obtained from the *X*-intercept of the plot of log(agonist DR−1) against the log antagonist concentration using regression analysis. pA_2 provides an estimate of the pK_B, when the antagonism has been shown to meet all the criteria of competition. In cases where prazosin produced shallow Schild slopes, which were significantly different from unity, further pharmacological analyses using KMD-3213 or BMY7378 were not carried out.

RNA isolation and cDNA synthesis

The thoracic aorta and mesenteric artery were isolated and dissected free of excess fat and connective tissue. These materials were then immediately pooled in RNAlater RNA stabilization solution (Ambion Inc., Austin, TX, U.S.A.) for 1 day at room temperature to preserve as much RNA as possible. After that, total RNA was extracted from each sample using Isogen (Nippon Gene Co., Ltd, Tokyo, Japan). Total RNA ($<5\,\mu$g) was treated with RNase-free DNase (Takara Shuzo Co., Tokyo, Japan) and reverse-transcribed using random hexamers (Tanoue *et al*., 2002). One-tenth of each cDNA sample was amplified by PCR with a receptor-specific primer set and a primer set specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sabath *et al*., 1990). Each sample contained the upstream and downstream primers (10 pmol of each), 0.25 mM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.6), 1.5 mM $MgCl_2$, and 2.5 U of Taq DNA polymerase (TaKaRa Shuzo Co., Tokyo, Japan). Thermal cycling was performed for 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C for 27 cycles. Control PCR reactions were also performed on non-reverse-transcribed RNA to exclude any contamination by genomic DNA. Amplified DNA was analyzed on a 1.5% agarose gel with 100-bp DNA markers (New England Biolabs Inc., Beverly, MA, U.S.A.).

Real-time PCR assay

For accurate quantification of RT-PCR products, a TaqMan 5′ nuclease fluorogenic quantitative PCR assay (Applied Biosystems Japan Ltd, Tokyo, Japan) was conducted in accordance with the manufacturer's instructions, using total RNA from the thoracic aortas and mesenteric arteries of WT, α_{1B}-KO, α_{1D}-KO, and α_{1BD}-KO mice. cDNAs were synthesized from total RNA (0.5–1.0 μg), as described above. Real-time PCR assays with the ABI Prism 7700 Sequence Detection System (Applied Biosystems Japan Ltd) were then carried out using the following oligonucleotides (5′–3′): 1A reverse primer, TCACACCAATGTATCGGTCGA; 1A probe, 6FAM-CCA TCATGGGCCCTGCATCATCT-TAMRA; 1B forward primer, CCTGGTCAT-GTACTGCCGA; 1B reverse primer, GA CTCCCGCCTCCAGATTC; 1B probe, 6FAM-TCTACATC GTGGCAAAGAGGACCACC-TAMRA; 1D forward primer, CGCTGTGGTGGGAACCGGCAG; 1D reverse primer, AGTTGGTGACCGTCTGCAAGT; 1D probe, 6FAM-CGG GCAACCT-TCTCGTCATCCTCTC-TAMRA; 1A forward primer, GCGGTGGACGTCTTATGCT. Commercially available TaqMan rodent GAPDH control reagents containing primers and probe for GAPDH were also used (Applied Biosystems Japan Ltd). All primers used for real-time PCR assays were derived from the nucleotide sequences within the first exon of each gene. In this assay, we added 5 pmol of each primer, 10 pmol of the TaqMan probe, 25 μl of Universal Master Mix (Applied Biosystems Japan Ltd), and 1 μl of cDNA in a total reaction volume of 50 μl. After enzyme inactivation for 10 min at 95°C, 50 cycles were performed (15 s at 95°C, 60 s at 60°C). The level of GAPDH expression was measured in all samples for normalization of sample-to-sample differences in RNA input, RNA quality, and reverse transcription efficiency.

Data analysis

Numerical results were expressed as mean ± s.e. CRCs to NAd in wild-type (WT) and mutant mice were compared by two-way analysis of variance (ANOVA) for repeated measures. The CRC maximum responses, the pD_2 values, pA_2 values, and the number of α_1-AR subtype copies were compared by a one-way ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons. A *P*-value of <0.05 was considered to denote a significant difference.

Drugs

The following drugs were used: (−)-NAd bitartrate (Wako-Junyaku, Osaka, Japan); BMY7378 (Research Biochemicals, Natick, MA, U.S.A.); prazosin hydrochloride, desmethylimipramine hydrochloride, (±)-propranolol hydrochloride, and yohimbine hydrochloride (Sigma, St Louis, MO, U.S.A.); and KMD-3213 (Kissei Pharmaceutical Co. Ltd, Fukuoka, Japan).

Results

NAd-induced contraction of the thoracic aorta

In the thoracic aortas of WT mice, NAd evoked contraction in a concentration-dependent manner (Figure 1a). The maximum responses of the CRCs for NAd in the thoracic aorta were not significantly different between WT (155 ± 53 mg, $n=10$) and α_{1B}-KO mice (130 ± 43 mg, $n=10$) (Figure 1a). However, there was a significant difference in sensitivity to NAd between the CRCs for WT and α_{1B}-KO mice (pD_2 value in WT was 8.21 ± 0.07 and in α_{1B}-KO was 7.77 ± 0.07, $n=10$ ($P<0.05$)) (Figure 1a). The CRCs for NAd in α_{1D}-KO mice were shifted further to the right and were significantly different from those in WT mice ($P<0.05$). There was a significant difference in sensitivity to NAd ($P<0.05$) between the CRCs for α_{1D}-KO mice and WT mice (pD_2 value in α_{1D}-KO was 6.44 ± 0.05, $n=10$), without a significant depression in the maximum response (120 ± 21 mg in α_{1D}-KO, $n=10$) (Figure 1a). The CRC for NAd in α_{1BD}-KO mice was almost completely abolished (maximum response, 25 ± 1 mg; pD_2 value, 5.15 ± 0.05; $n=10$) (Figure 1a).

pA_2 values for antagonists in the thoracic aortas of WT mice

The response to NAd in the thoracic aortas of WT mice was antagonized in the presence of either prazosin, KMD-3213, or BMY7378 in a concentration-dependent manner. The CRCs for NAd were shifted right by prazosin, KMD-3213, or BMY7378. The pA_2 values of prazosin, KMD-3213, and BMY7378 were 9.99 ± 0.15 ($n=10$), 8.25 ± 0.04 ($n=7$), and

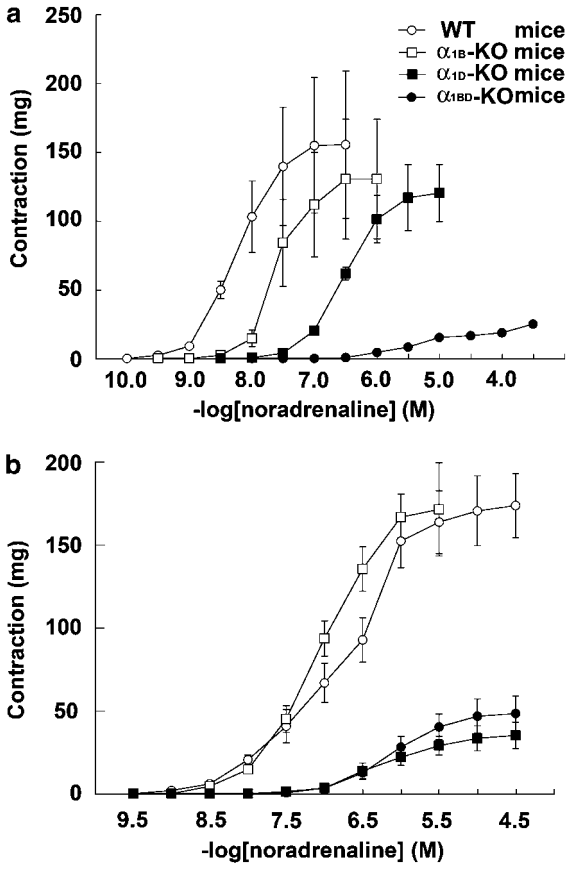

Figure 1 Comparison of CRCs for NAd in thoracic aortas (a) and mesenteric arteries (b) taken from each group of mice. Ordinate: contraction, expressed as milligrams force. Abscissa: negative log concentration (M) of NAd. Each value represents the mean ± s.e. of 10 experiments.

8.46 ± 0.22 ($n = 5$), respectively (Figures 2a–c and Table 1). The slopes of the Schild regression line were not significantly different from unity (Table 1).

pA_2 values for antagonists in the thoracic aortas of α_{1B}-KO mice

The response to NAd in the thoracic aortas of α_{1B}-KO mice was antagonized in the presence of prazosin, KMD-3213, or BMY7378 in a concentration-dependent manner. The CRCs for NAd were shifted rightwards by prazosin, KMD-3213, or BMY7378. The pA_2 values of prazosin, KMD-3213, and BMY7378 were 10.41 ± 0.04 ($n = 10$), 8.49 ± 0.11 ($n = 9$), and 9.13 ± 0.10 ($n = 7$), respectively (Figures 3a–c and Table 1). The slopes of the Schild regression line were not significantly different from unity (Table 1). The mean pA_2 value for BMY7378 against NAd-induced contraction in the thoracic aorta of α_{1B}-KO mice tended to be higher than in that of WT mice ($P = 0.062$).

pA_2 values for antagonists in the thoracic aortas of α_{1D}-KO mice and α_{1BD}-KO mice

The response to NAd in the thoracic aortas of α_{1D}-KO mice was antagonized in the presence of prazosin in a concentration-dependent manner. The CRCs for NAd were shifted right by prazosin (Figure 4) and the pA_2 value was 9.30 ± 0.11 ($n = 10$). However, prazosin produced shallow Schild slopes, which were significantly different from unity (data not shown). There was almost complete abolition of the CRCs for NAd in the thoracic aortas of α_{1BD}-KO mice; therefore, the effects of α_1-selective antagonists on the thoracic aortas of α_{1BD}-KO mice could not be examined.

NAd-induced contraction of the mesenteric artery

In the mesenteric arteries of these groups of mice, NAd evoked contraction in a concentration-dependent manner (Figure 1b). The CRCs for NAd in the mesenteric artery were not significantly different between WT and α_{1B}-KO mice (Figure 1b). Also, CRCs for NAd in the mesenteric artery did not differ significantly between WT and α_{1B}-KO in either the maximum response (173 ± 19 mg in WT, $n = 10$, and 171 ± 28 mg in α_{1B}-KO, $n = 10$) or in sensitivity to NAd (pD_2 value, 6.52 ± 0.22 in WT, $n = 10$, and 7.12 ± 0.14 in α_{1B}-KO, $n = 10$) (Figure 1b). Similarly, there was no significant difference between CRCs for NAd in the mesenteric arteries of α_{1D}-KO mice and α_{1BD}-KO mice (Figure 1b). Furthermore, CRCs for NAd in the mesenteric artery did not differ significantly between α_{1D}-KO and α_{1BD}-KO mice in either maximum response (35 ± 8 mg in α_{1D}-KO, $n = 10$, and 48 ± 11 mg in α_{1BD}-KO, $n = 10$) or sensitivity (pD_2 value, 6.19 ± 0.07 in α_{1D}-KO, $n = 10$, and 6.29 ± 0.06 in α_{1BD}-KO, $n = 10$) (Figure 1b). The maximum responses of the mesenteric arteries from both α_{1D}-KO and α_{1BD}-KO mice were significantly lower (approximately 70% reduction, $P < 0.05$) than those from either WT or α_{1B}-KO mice (Figure 1b). There was a significant difference in sensitivity between the mesenteric arteries and thoracic aortas of WT mice ($P < 0.05$), but no significant difference in maximum response. Furthermore, NAd evoked contraction in the mesenteric arteries of α_{1D}-KO and α_{1BD}-KO mice with a significantly higher pD_2 value ($P < 0.05$) and maximum response than in the thoracic aorta of α_{1BD}-KO mice (Figure 1a and b).

pA_2 values for antagonists in the mesenteric arteries of WT mice

The response to NAd in the mesenteric arteries of WT mice was antagonized by the presence of prazosin, KMD-3213, or BMY7378 in a concentration-dependent manner. CRCs for NAd were shifted rightwards by prazosin, KMD-3213, or BMY7378. The pA_2 values of prazosin, KMD-3213, and BMY7378 were 9.92 ± 0.11 ($n = 10$), 8.34 ± 0.40 ($n = 8$), and 7.47 ± 0.18 ($n = 7$), respectively (Figures 5a–c and Table 2). The slopes of the Schild regression lines were not significantly different from unity (Table 1).

pA_2 values for antagonists in the mesenteric arteries of α_{1B}-KO mice

The response to NAd in the mesenteric arteries of WT mice was antagonized by the presence of prazosin, KMD-3213, or BMY7378 in a concentration-dependent manner. Prazosin, KMD-3213, or BMY7378 all shifted the CRCs for NAd to the right. The pA_2 values of prazosin, KMD-3213, and BMY7378 were 9.83 ± 0.06 ($n = 10$), 8.11 ± 0.05 ($n = 7$), and 7.82 ± 0.10 ($n = 6$), respectively (Figures 6a–c and Table 2). The slopes of

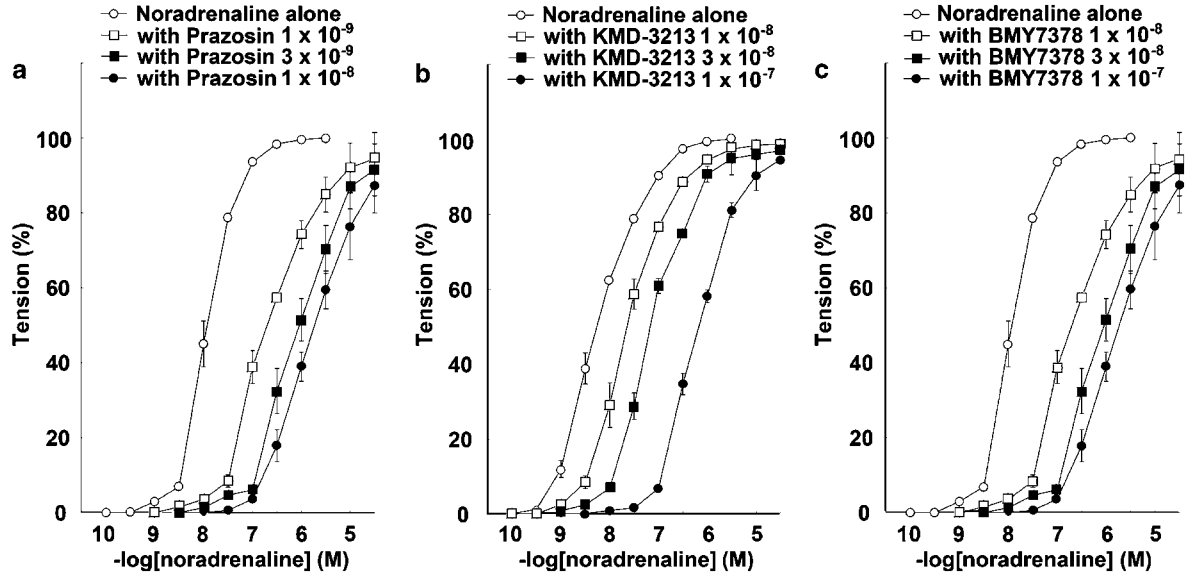

Figure 2 Effects of prazosin, KMD-3213, and BMY7378 on NAd-induced contraction in thoracic aortas from WT mice. Ordinate: contraction, expressed as percentages of the maximum response. Abscissa: negative log concentration (M) of NAd. Each value represents the mean ± s.e. of 5–10 experiments. Aortic segments were exposed to vehicle (control) or different concentrations of (a) prazosin, (b) KMD-3213, or (c) BMY7378 before the addition of cumulative concentrations of NAd.

Table 1 pA_2 values for antagonists against NAd in the thoracic aorta of WT and α_{1B}-KO mice

	WT mice		*α_{1B}-KO mice*	
Antagonist	*pA_2 value*	*Slope*	*pA_2 value*	*Slope*
Prazosin	9.99 ± 0.15	1.18 ± 0.08	10.41 ± 0.04	0.92 ± 0.05
KMD-3213	8.25 ± 0.04	1.06 ± 0.04	8.49 ± 0.11	1.09 ± 0.06
BMY7378	8.46 ± 0.22	0.99 ± 0.01	9.13 ± 0.10	1.06 ± 0.05

Each value represents the mean ± s.e. of 5–10 experiments.

the Schild regression lines were not significantly different from unity (Table 1).

pA_2 values for antagonists in the mesenteric arteries of α_{1D}-KO mice and α_{1BD}-KO mice

The responses to NAd in the mesenteric arteries of α_{1D}-KO and α_{1BD}-KO mice were antagonized by the presence of prazosin in a concentration-dependent manner. Prazosin shifted the CRCs for NAd rightwards. The pA_2 value for prazosin in the mesenteric arteries of α_{1D}-KO mice was 9.30 ± 0.26 ($n = 4$) and in α_{1BD}-KO mice it was 9.43 ± 0.31 ($n = 5$), and these were not significantly different from each other. However, prazosin produced shallow Schild slopes, which were significantly different from unity (data not shown).

Expression of each α_1-AR subtype in the vessels of WT mice

Real-time PCR analysis revealed that the mRNA expression level of α_{1D}-AR was the highest of the α_1-AR subtypes in both the thoracic aorta and mesenteric artery ($n = 5$, each group) (Figure 7a and b). The expression level of α_{1D}-AR mRNA in the mesenteric artery was approximately half that in the thoracic aorta. In the thoracic aorta, the level of expression of α_{1B}-AR mRNA was approximately one-quarter that of α_{1D}-AR, and the expression level of α_{1A}-AR was approximately one-tenth that of α_{1D}-AR (Figure 7a). In the mesenteric artery, the level of expression of α_{1A}-AR mRNA was approximately half that of α_{1D}-AR, and expression of α_{1B}-AR was approximately one-sixth that of α_{1D}-AR (Figure 7b). The expression of the mRNA of each α_1-AR subtype in order of rank was α_{1D}-AR > α_{1B}-AR > α_{1A}-AR in the mouse thoracic aorta and α_{1D}-AR > α_{1A}-AR > α_{1B}-AR in the mouse mesenteric artery. In addition, the total level of expression of the mRNAs of all the α_1-AR subtypes was significantly higher in the thoracic aorta than in the mesenteric artery ($P < 0.05$).

Expression of each α_1-AR subtype in the thoracic aortas of knockout mice

Real-time PCR analysis showed that the level of expression of α_{1D}-AR mRNA in the thoracic aortas of α_{1B}-KO mice tended to be higher ($P = 0.066$) than in WT mice ($n = 5$, each) (Figure 8). However, no other trends in the levels of expression of mRNA of α_1-AR subtypes in the thoracic aorta were observed ($n = 5$, each) (Figure 8).

Expression of each α_1-AR subtype in the mesenteric arteries of knockout mice

Real-time PCR analysis revealed that the expression level of α_{1A}-AR mRNA in the mesenteric arteries of both α_{1D}-KO and α_{1BD}-KO mice tended to be higher than in those of WT mice ($P = 0.087$ in α_{1D}-KO vs WT and $P = 0.074$ in α_{1BD}-KO vs WT, $n = 5$ each) (Figure 9). No higher tendency in the levels of expression of mRNA of different α_1-AR subtypes was observed in the mesenteric artery ($n = 5$, each) (Figure 9).

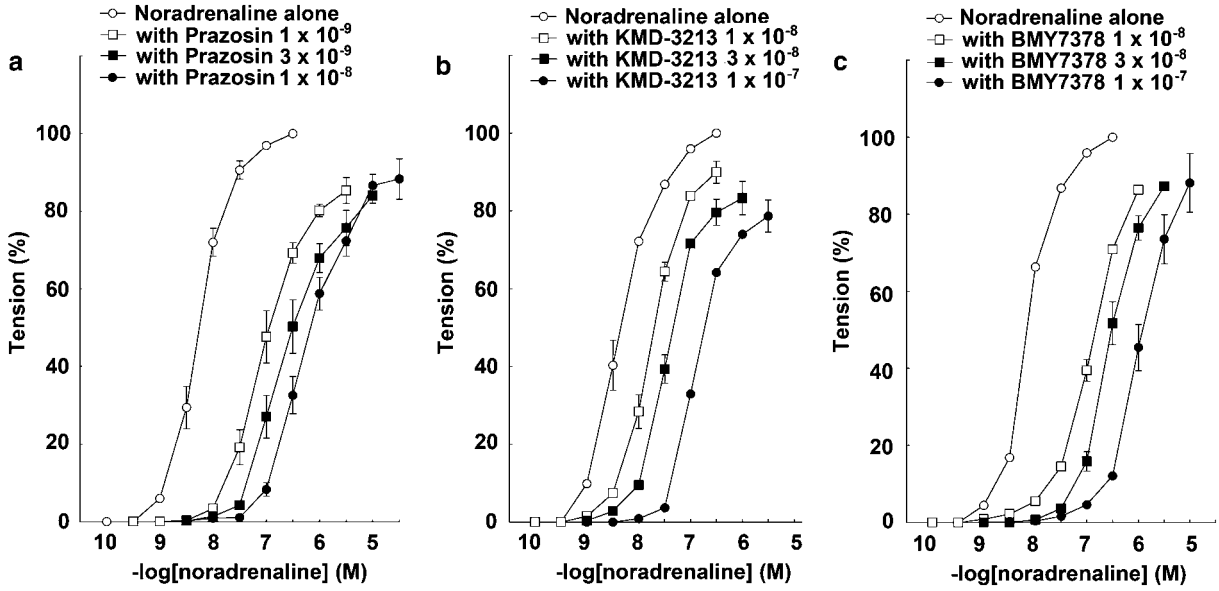

Figure 3 Effects of prazosin, KMD-3213, and BMY7378 on NAd-induced contraction in thoracic aortas from α_{1B}-KO mice. Ordinate: contraction, expressed as percentages of the maximum response. Abscissa: negative log concentration (M) of NAd. Each value represents the mean ± s.e. of 7–10 experiments. Aortic segments were exposed to vehicle (control) or different concentrations of (a) prazosin, (b) KMD-3213, or (c) BMY7378 before the addition of cumulative concentrations of NAd.

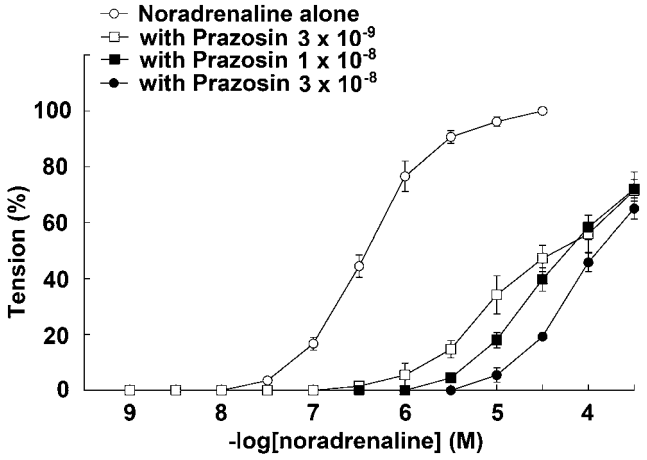

Figure 4 Effects of prazosin on NAd-induced contraction in thoracic aortas from α_{1D}-KO mice. Ordinate: contraction, expressed as percentages of the maximum response. Abscissa: negative log concentration (M) of NAd. Each value represents the mean ± s.e. of 10 experiments. Aortic segments were exposed to vehicle (control) or to different concentrations of prazosin before the addition of cumulative concentrations of NAd.

Discussion

We examined the pharmacological characteristics of the thoracic aorta and mesenteric artery in WT and mutant mice with the same genetic background. The CRC for NAd in the thoracic aorta of α_{1BD}-KO mice was almost completely abolished (Figure 1a), revealing that α_{1A}-AR plays almost no role in α_1-AR-mediated contraction of the mouse thoracic aorta. α_{1D}-AR was effectively the only α_1-AR expressed in the thoracic aortas of α_{1B}-KO mice and, similarly, α_{1B}-AR was in effect the only α_1-AR expressed in the thoracic aortas of α_{1D}-KO mice. Taking into account the pD_2 values of the thoracic aortas of α_{1B}-KO and α_{1D}-KO mice (7.77 and 6.44, respectively), contraction mediated through α_{1D}-AR appeared to be approximately 20 times greater than that evoked by α_{1B}-AR. Therefore, the vasoconstrictor role of each α_1-AR subtype in the mouse thoracic aorta in rank order of predominance was α_{1D}-AR > α_{1B}-AR $\gg$ α_{1A}-AR. In the mouse mesenteric artery, α_{1B}-AR appears to be of little importance in α_1-AR-mediated contraction, since no statistically significant difference was observed between CRCs for NAd between WT and α_{1B}-KO mice or between α_{1D}- and α_{1BD}-KO mice. In addition, the CRC for NAd in the mesenteric artery showed that the maximum response in α_{1D}-KO mice was remarkably reduced compared to that in WT mice (173 ± 19 mg in WT and 35 ± 8 mg in α_{1D}-KO). These results indicate that the majority of α_1-ARs were absent from the mesenteric arteries of α_{1D}-KO mice, suggesting that α_{1D}-AR could play a major vasoconstrictor role and that the vasoconstrictor activity of the other α_1-ARs (α_{1A}-AR and α_{1B}-AR) in the mouse mesenteric artery might be minor. Furthermore, CRCs for NAd in the mesenteric arteries of α_{1BD}-KO mice, previously considered to be mediated through only α_{1A}-AR, were not abolished as they were in the thoracic arteries, even though there was lower sensitivity to NAd and a lower maximum response. These findings suggest that α_{1A}-AR might play a minor vasoconstrictor role in the mouse mesenteric artery. Therefore, the rank order of predominance of the vasoconstrictor activity of each α_1-AR subtype in the mesenteric artery was α_{1D}-AR > α_{1A}-AR $\gg$ α_{1B}-AR. Sensitivity to NAd in α_1-AR-mediated contraction is much greater in the thoracic aorta than in the mesenteric artery (approximately 1/50th sensitivity of that in the thoracic aorta) in mice (Figure 1a and b).

We then examined the pharmacological characteristics of these vessels using the nonselective α_1-antagonist prazosin. Prazosin effectively antagonized NAd-induced contraction of thoracic aortas from WT, α_{1B}- and α_{1D}-KO mice, and of mesenteric arteries from WT, α_{1B}-, α_{1D}-, and α_{1BD}-KO mice,

which showed that the response to NAd was mediated through α_1-ARs (Tables 1 and 2, Figures 2–6). The pA_2 values for prazosin against NAd from the Schild plot were all very similar to each other (9.99 in WT thoracic aorta, 10.41 in α_{1B}-KO thoracic aorta, 9.30 in α_{1D}-KO thoracic aorta, 9.92 in WT mesenteric artery, 9.83 in α_{1B}-KO mesenteric artery, 9.30 in α_{1D}-KO mesenteric artery, and 9.43 in α_{1BD}-KO mesenteric artery), and were in good agreement with those of mice in our previous study (pA_2 value for prazosin was 9.71 in mouse thoracic aorta and 9.93 in mouse mesenteric artery; Yamamoto & Koike, 2001a, b). The slopes of the Schild regression lines for the thoracic aorta and mesenteric artery in WT and α_{1B}-KO mice indicated the competitive nature of the antagonism. On the other hand, prazosin produced shallow Schild slopes, which were significantly different from unity, in the thoracic aortas of α_{1D}-KO mice and the mesenteric arteries of α_{1D}- and

Table 2 pA_2 values for antagonists against NAd in the mesenteric artery of WT and α_{1B}-KO mice

	WT mice		*α_{1B}-KO mice*	
Antagonist	*pA_2 value*	*Slope*	*pA_2 value*	*Slope*
Prazosin	9.92±0.11	0.96±0.07	9.83±0.06	1.24±0.14
KMD-3213	8.34±0.40	1.36±0.19	8.11±0.05	1.06±0.06
BMY7378	7.47±0.18	1.08±0.12	7.82±0.10	1.02±0.11

Each value represents the mean±s.e. of 6–10 experiments.

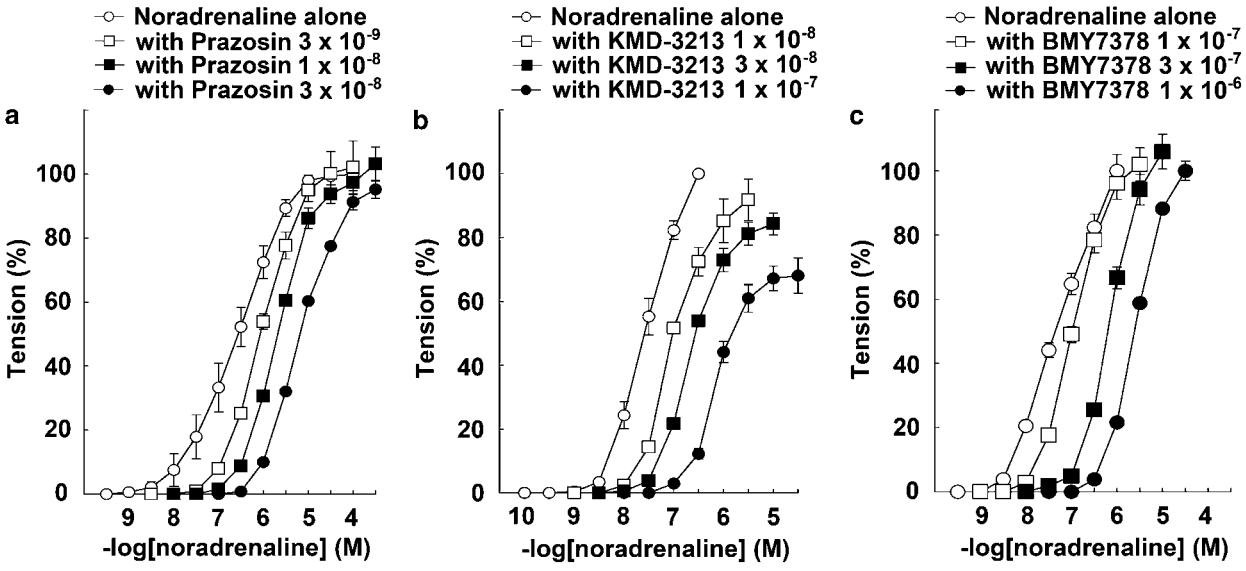

Figure 5 Effects of prazosin, KMD-3213, and BMY7378 on NAd-induced contraction in mesenteric arteries from WT mice. Ordinate: contraction, expressed as percentages of the maximum response. Abscissa: negative log concentration (M) of NAd. Each value represents the mean±s.e. of 7–10 experiments. Mesenteric arterial segments were exposed to vehicle (control) or to different concentrations of (a) prazosin, (b) KMD-3213, or (c) BMY7378 before the addition of cumulative concentrations of NAd.

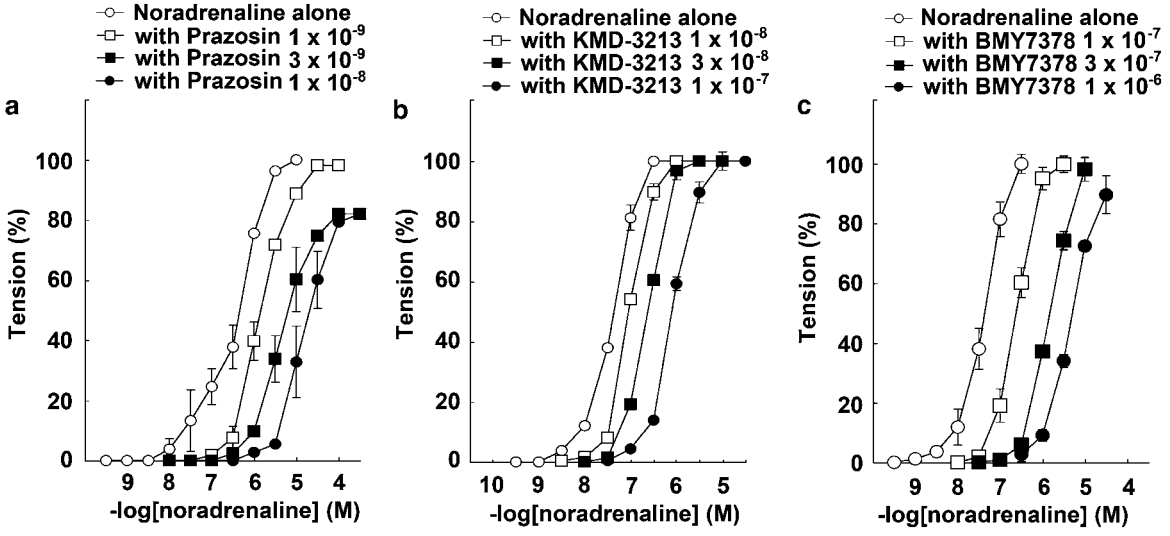

Figure 6 Effect of prazosin, KMD-3213, and BMY7378 on NAd-induced contraction in mesenteric arteries from α_{1B}-KO mice. Ordinate: contraction, expressed as percentages of the maximum response. Abscissa: negative log concentration (M) of NAd. Each value represents the mean±s.e. of 6–10 experiments. Mesenteric arterial segments were exposed to vehicle (control) or to different concentrations of (a) prazosin, (b) KMD-3213, or (c) BMY7378 before the addition of cumulative concentrations of NAd.

α_{1BD}-KO mice. These phenomena originally suggested a change in the nature of prazosin from a competitive to a noncompetitive-like antagonist, according to the Schild and Gaddum equations (Arunlakshana & Schild, 1959; Tallarida *et al*., 1979). However, we have already confirmed that prazosin acts as a competitive antagonist of NAd in the thoracic aorta and mesenteric artery in the mouse (Yamamoto & Koike, 2001a, b) Also, no structural change in the residual α_1-ARs in α_1-AR subtype knockout mice has yet been reported (Cavalli *et al*., 1997; Rokosh & Simpson, 2001; Daly *et al*., 2002; Tanoue *et al*., 2002). In addition, these phenomena were observed only in the vessels of mice lacking the α_{1D}-AR gene, a major vasoconstrictor of these vessels. These conflicting phenomena suggest a remarkable reduction in the quantity of α_1-ARs in these vessels in mice lacking α_{1D}-AR gene compared with WT mice, rather than a change in the nature of prazosin from that of a competitive to a noncompetitive-like antagonist. However, a reduction in receptor number does not, theoretically, change the nature of the antagonism. It is not clear why the reduction in α_{1D}-ARs leads to apparent noncompetitive antagonism, because no similar study using α_{1D}-KO mice has yet been reported.

The pharmacological characterization of these vessels using the α_{1A}-selective antagonist KMD-3213 found that the mean pA_2 values of KMD-3213 against NAd-induced contraction in the thoracic aorta (pA_2 value for WT mice was 8.25 and for α_{1B}-KO mice was 8.49, Table 1) and in the mesenteric artery (pA_2 value for WT mice was 8.34 and for α_{1B}-KO mice was 8.11, Table 2) were in good agreement with the negative logarithm of dissociation constant (pK_i value) of human cloned α_{1d}-AR (pK_i values were 10.5, 7.5, and 8.5 for human α_{1a}-, α_{1b}-, and α_{1d}-AR, respectively; Shibata *et al*., 1995) and with the pA_2 value of functional α_{1D}-AR (α_{1A}-AR, 10.0 in rat caudal artery; α_{1B}-AR, 7.7 in dog carotid artery; α_{1D}-AR, 8.3 and 8.13 in rat thoracic aorta) (Ford *et al*., 1996; Yamagishi *et al*., 1996; Murata *et al*., 1999, respectively). The slopes of the Schild regression lines in these vessels indicated that the antagonism is competitive. These previous findings strongly show that contraction of these vessels in WT and α_{1B}-KO mice was mediated mainly through α_{1D}-AR.

The mean pA_2 value of the α_{1D}-selective antagonist BMY7378 (8.46, Table 1) against NAd-induced contraction in the thoracic aortas of WT mice (Goetz *et al*., 1995; Saussy *et al*., 1996) was similar to that reported in our previous study (pA_2 value 8.43; Yamamoto & Koike, 2001b), and was in good agreement with the generally accepted value for the rat thoracic aorta (pA_2 value, 8.5; Ford *et al*., 1996). In addition, the mean pA_2 value (9.13, Table 1) for BMY7378 against NAd-induced contraction in the thoracic aortas of α_{1B}-KO mice tended to be higher ($P=0.062$) than that of WT mice and was similar to that reported previously (9.3) in the thoracic aortas of these mice (Daly *et al*., 2002). The slopes of the Schild regression lines for the thoracic aortas of WT and α_{1B}-KO mice indicated that the antagonism is competitive. On the other hand, in the mouse mesenteric arteries, the mean pA_2 values of BMY7378 against NAd-induced contraction in WT (7.47) and α_{1B}-KO (7.82) mice indicated low affinity for BMY7378 (Table 2). The slopes of the Schild regression lines again indicate a competitive antagonism. The effects of BMY7378 on NAd-induced contraction in the mesenteric arteries of WT

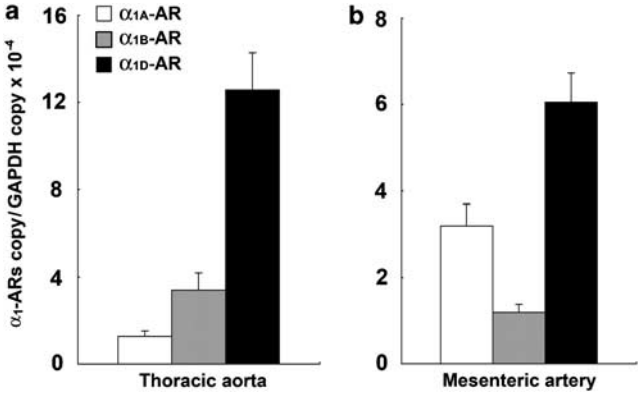

Figure 7 α_1-AR subtype mRNA expression in the thoracic aortas (a) and mesenteric arteries (b) of WT mice. Ordinate: relative level of expression of mRNA of each α_1-AR subtype, standardized against the GAPDH level. Abscissa: vessels from which the total RNA was isolated. Values represent means ± s.e. of five independent experiments.

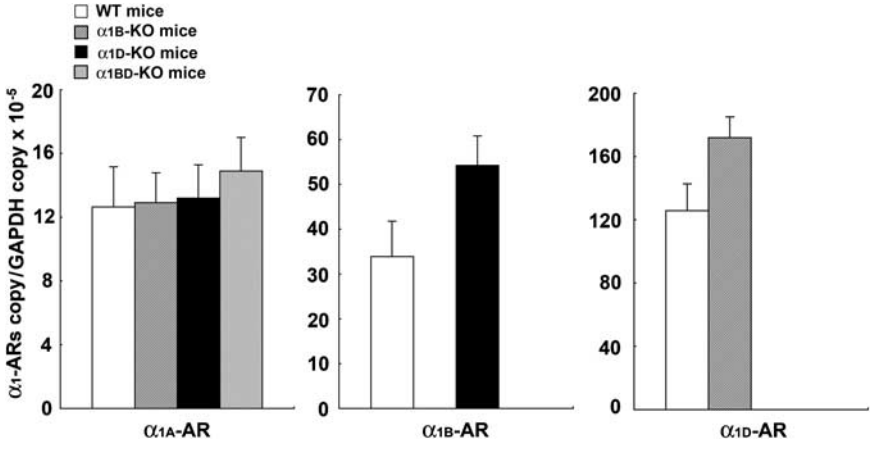

Figure 8 α_1-AR subtype mRNA expression in thoracic aortas of each group of mice. Ordinate: relative level of expression of mRNA of each α_1-AR subtype, standardized against the GAPDH level. Abscissa: each α_1-AR subtype, as expressed in the thoracic aortas of each group of mice. Values represent means ± s.e. of five independent experiments.

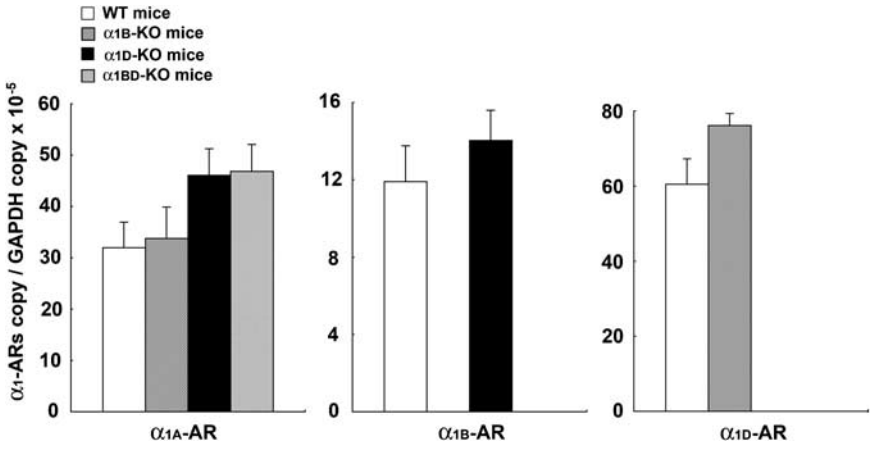

Figure 9 α_1-AR subtype mRNA expression in mesenteric arteries of each group of mice. Ordinate: relative level of expression of mRNA of each α_1-AR subtype, standardized against the GAPDH level. Abscissa: each α_1-AR subtype, as expressed in the mesenteric arteries of each group of mice. Values represent means ± s.e. of five independent experiments.

and α_{1B}-KO mice were in good agreement with those in our previous report (pA_2 value for mice mesenteric artery, 7.69; Yamamoto & Koike, 2001a).

We derived pharmacological characterizations for these vessels using these antagonists and found that contraction of the vessels in WT and α_{1B}-KO mice might be mediated primarily through α_{1D}-AR. An exception is in the case of the low affinity for BMY7378 in the mesenteric arteries of WT and α_{1B}-KO mice. Although the pK_i value for BMY7378 against α_{1d}-AR of mice was not previously known, other studies have proved that pK_i values for BMY7378 against α_{1d}-AR vary from 8.2 (rats) to 9.4 (humans) (Goetz *et al*., 1995). Moreover, the mouse mesenteric artery is less sensitive to NAd than the thoracic aorta, suggesting that it has lower concentrations of α_1-AR subtypes (especially the major vasoconstrictor) and that α_{1A}-AR is a more important vasoconstrictor in the mesenteric artery than in the thoracic aorta. These findings suggest that there is a lower concentration of α_{1D}-AR, and a higher concentration of α_{1A}-AR, in the mesenteric artery than in the thoracic aorta of the mouse. This may cause a lowering of the affinity for BMY7378 in the mouse mesenteric artery. Although the affinity for the α_{1D}-selective antagonist BMY7378 tended to be higher in the thoracic aortas of α_{1B}-KO mice, the patterns of contraction in the thoracic aortas and mesenteric arteries of α_{1B}-KO mice were similar to those in WT mice. This similarity suggests that α_{1B}-AR may play only a minor vasoconstrictor role, or no vasoconstrictor role at all, in contraction of the mouse thoracic aorta and mesenteric artery. The results of our pharmacological analysis using α_1-AR antagonists were therefore similar to those found in our comparison of the CRCs for NAd in these vessels.

We investigated the correlation between the vasoconstrictor role of each α_1-AR subtype and its mRNA expression in these vessels. There was reasonably close correlation in the rank order of predominance of each α_1-AR subtype in terms of vasoconstrictor role and mRNA expression level, as follows: α_{1D}-AR > α_{1B}-AR > α_{1A}-AR in the mouse thoracic aorta and α_{1D}-AR > α_{1A}-AR > α_{1B}-AR in the mouse mesenteric artery (Figure 7). Furthermore, the mouse thoracic aorta scored more highly than the mesenteric artery in terms of both sensitivity to NAd and total copy number of α_1-AR mRNAs.

Recent studies in animal vessels have investigated the correlation between mRNA or protein expression levels of α_1-AR subtypes and the functional roles of these receptors in vasoconstriction (Guarino *et al*., 1996; Miller *et al*., 1996). In most cases, although all α_1-AR subtypes are expressed at the protein level (Hrometz *et al*., 1999) or mRNA level (Xu *et al*., 1997), the correlation between protein or mRNA expression of one α_1-AR subtype and the functional roles of these receptors in vasoconstriction has been elusive (Piascik *et al*., 1997; Hussain & Marshall, 2000). Use of the quantitative technique of real-time PCR enabled us to quantify the mRNA expression of each α_1-AR subtype. Although the mRNAs of all three α_1-AR subtypes were expressed in the mouse thoracic aorta and mesenteric artery, it appears that not all α_1-AR subtypes mediate NAd-induced contraction in these vessels. In terms of compensatory expression of mRNA of α_1-AR subtypes, the level of expression of α_{1D}-AR mRNA in the thoracic aortas of α_{1B}-KO mice tended to be higher than that in WT mice ($P = 0.066$) (Figure 8). This tendency was in good agreement with the tendency toward a higher pA_2 value for BMY7378 in the thoracic aortas of α_{1B}-KO mice ($P = 0.062$) than those of WT mice (Table 1). However, in each mutant strain, no dramatic compensatory change in expression of the other α_1-AR subtypes was observed. This result was in good agreement with that of our previous report (Tanoue *et al*., 2002) and suggests that the difference in sensitivity to NAd in these vessels reflects the deletion of α_{1B}- and/or α_{1D}-AR.

In conclusion, we were able to evaluate the rank orders of the vasoconstrictor role and mRNA expression level of each α_1-AR subtype in these mouse vessels. Our resulting pharmacological analysis and mRNA expression profile correlated well and demonstrated that α_{1D}-AR is primarily predominant and that other α_1-AR subtypes are secondary and differ between vessels in terms of both NAd-stimulated vasoconstriction and mRNA expression level.

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