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Insights into the functional roles of α_1-adrenoceptor subtypes in mouse carotid arteries using knockout mice

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1 α_1-Adrenoceptor (AR) subtypes in mouse carotid arteries were characterised using a combination of agonist/antagonist pharmacology and knockout (KO) mice.

2 Phenylephrine (PE) was most potent in the α_{1B}-KO ($pEC_{50} = 6.9 \pm 0.2$) followed by control ($pEC_{50} = 6.3 \pm 0.06$) and α_{1D}-KO ($pEC_{50} = 5.5 \pm 0.07$). Both *N*-[5-(4,5-dihydro-1*H*-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide (A-61603) and 5-hydroxytryptamine (5-HT) were more potent in the α_{1D}-KO ($pEC_{50} = 7.4 \pm 0.27$ and 7.4 ± 0.05, respectively) than the control ($pEC_{50} = 6.9 \pm 0.09$ and 6.9 ± 0.08, respectively) and equipotent with the control in the α_{1B}-KO ($pEC_{50} = 6.7 \pm 0.07$ and 6.8 ± 0.04). Maximum responses to PE and A-61603 were reduced in the α_{1D}-KO compared to control; there was no difference in maximum responses to 5-HT.

3 In control arteries, prazosin and 5-methylurapidil acted competitively with pA_2 of 9.6 and 7.5, respectively. BMY7378 produced antagonism only at the highest concentration used (100 nM; pK_B 8.3).

4 Prazosin, 5-methylurapidil and BMY7378 acted competitively in α_{1B}-KO carotid arteries with pA_2 of 10.3, 7.6 and 9.6, respectively.

5 In the α_{1D}-KO, against PE, 5-methylurapidil produced a pA_2 of 8.1. pK_B values were calculated for prazosin (10.6) and BMY7378 (7.0). Against A-61603, 5-methylurapidil had a pA_2 of 8.5, prazosin 8.6, while BMY7378 had no effect.

6 In conclusion, the α_{1B}-KO mediates contraction solely through α_{1D}-ARs and the α_{1D}-KO through α_{1A}-ARs. Extrapolating back to the control from the knockout data suggests that all three subtypes could be involved in the responses, but we propose that the α_{1D}-AR causes the contractile response and that the role of the α_{1B}-AR is mainly regulatory.
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Abbreviations: AR, adrenoceptor; CRC, concentration–response curve; 5-HT, 5-hydroxytryptamine; KO, knockout; NA, noradrenaline; PE, phenylephrine; A-61603, *N*-[5-(4,5-dihydro-1*H*-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide

Introduction

Three native α_1-adrenoceptor (AR) subtypes, defined by ligand binding and functional pharmacology, α_{1A}, α_{1B} and α_{1D}, correspond to three cloned subtypes, α_{1a}, α_{1b} and α_{1d} (Bylund *et al*., 1994). It is not known whether the three subtypes have different biological roles. Several tissues, including arteries, express more than one subtype. The mRNA and protein for all three α_1-AR subtypes are expressed in the major blood vessels of the rat (Piascik *et al*., 1995; Scofield *et al*., 1995; Piascik *et al*., 1997; Hrometz *et al*., 1999). However, separating the responses mediated by these subtypes has proved difficult, due to the limitations of selectivity of antagonists between the three receptors and the proposition that they might all be involved in the same type of response, namely contraction of vascular smooth muscle.

A handful of α_{1A}-AR-selective antagonists are available, such as 5-methylurapidil, WB4101 and RS100329 (Gross *et al*., 1988; Schwinn *et al*., 1995; Williams *et al*., 1999), while BMY7378 is the only widely accepted α_{1D}-AR-selective antagonist (Saussy *et al*., 1994; Goetz *et al*., 1995; Kenny *et al*., 1995). A major pharmacological complication when attempting to subtype α_1-ARs is the lack of a selective competitive antagonist for the α_{1B}-AR. This appears to be a situation in which receptor knockouts might simplify the pharmacological analysis.

Most studies of vascular α_1-ARs, either as an undivided class or as subtypes, have been carried out in rats, rabbits and dogs and until recently little data has been available for the mouse. However, α_1-AR knockout (KO) mice are now available for

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the α_{1B}- and α_{1D}-ARs (Cavalli *et al*., 1997; Tanoue *et al*., 2002). These provide novel environments to study and subtype the remaining two possible α_1-ARs.

We have chosen to study the carotid artery since this vessel has greater potential as an experimental model, being accessible to surgical manipulation *in vivo* and amenable to perfusion studies *in vitro*. There is also controversy over whether contraction is mediated by the α_{1B}- or α_{1D}-ARs according to species (dog: α_{1B} (Muramatsu *et al*., 1991; Kohno *et al*., 1994); rabbit: α_{1B} (Muramatsu *et al*., 1995); rat: α_{1D} (Villalobos-Molina & Ibarra, 1996; de Oliveira *et al*., 1998)). Theoretically, this presents a relatively straightforward scenario for observing the consequences of knocking out each of these subtypes. Previous work has shown that (1) knockout of the α_{1B}-AR produces little change in the size or sensitivity of responses to phenylephrine (PE) in the aorta and carotid arteries; the antagonist pharmacology is more consistent with α_{1D}-AR pharmacology, suggesting a major role for the α_{1D}-AR and a minor one for the α_{1B}-AR (Daly *et al*., 2002), and (2) knockout of the α_{1D}-AR produced a significant reduction in sensitivity and maximum response to PE in the aorta, consistent with the loss of the major contractile α_1-AR (Tanoue *et al*., 2002).

The objective of the present study was to apply a consistent antagonist analysis using the two knockouts to allow us to explore the functional relationship between α_1-AR subtypes; for example, what are the consequences of deleting each receptor? Does this show how they interact? Do other subtypes upregulate to compensate? We used the 'definitive' antagonists (prazosin, 5-methylurapidil and BMY7378) and the α_1-AR-selective agonist PE (eliminates possible complications from α_2- and β-ARs). We also used the α_{1A}-AR-selective agonist *N*-[5-(4,5-dihydro-1*H*-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide more commonly known as A-61603 (Knepper *et al*., 1995) to reinforce the antagonist analysis.

Latterly, we applied the knowledge obtained from mouse carotid arteries to data collected from our laboratory a number of years ago on the rat carotid artery, which at the time were difficult to interpret.

Methods

Animals used and set-up procedure

All transgenic mice (C57 Black genetic background; for a full description of genetic background see Cavalli *et al*. (1997) and Tanoue *et al*. (2002)) were bred at the University of Glasgow. Mice were killed by lethal overdose of carbon dioxide. The common carotid arteries were removed, placed in cold oxygenated Krebs and dissected free of connective tissue with the aid of a dissecting microscope.

Experiments were carried out in a four-chamber wire myograph (J.P. Trading, Aarhus, Denmark). Arteries were cut into approximately 2 mm lengths and mounted on two 40 μm wires. One wire was attached to a fixed head, while the other was attached to a head connected to a force transducer. The force transducer was in turn connected to a Linseis pen recorder to allow recordings of the force achieved.

Vessels were allowed to equilibrate in Krebs (37°C and gassed with 95% O_2, 5% CO_2) for 15 min after which time the vessels were set under their optimal resting length tensions: previously calculated to be 250 mg for the control, α_{1B}- and α_{1D}-KO mouse carotid arteries (Deighan, 2001). The vessels were left to equilibrate at this tension for 30–45 min with washes every 15 min. Prior to the start of each experiment, vessels were challenged with a sensitising concentration of 0.3 μM PE (control and α_{1B}-KO mouse), 10 μM PE (α_{1D}-KO), 10 μM A-61603 (α_{1D}-KO) or 1 μM 5-hydroxytryptamine (5-HT) (all mice strains). The contraction was allowed to plateau and then washed with Krebs. This was repeated three times to minimise changes in sensitivity to further challenges with agonists. Cumulative concentration–response curves (CRC) were carried out to either PE (1 nM–1 mM), A-61603 (1 nM–300 μM) or 5-HT (1 nM–30 μM). Subsequent CRCs to PE or A-61603 were carried out in the presence of antagonists (prazosin, 5-methylurapidil and BMY7378), which were equilibrated with the tissue for 30 min prior to beginning the CRC. Time controls were carried out in parallel with antagonist curves.

A similar analysis was carried out on carotid artery rings from male Wistar rats (320–400 g) suspended between two wire hooks and recorded isometrically. The protocol was identical to that in mice except that the vessels were equilibrated under 2.5 g of tension and noradrenaline (NA; 1 nM–10 μM) was used as the agonist.

Data analysis

Responses to agonists are expressed as tension in grams or as a percentage of the maximum response of the first CRC. The pEC_{50} was calculated as the negative logarithm of the concentration of agonist that produces half the maximal response. pEC_{50} values for PE, A-61603 and 5-HT in control, α_{1B}- and α_{1D}-KO mice were analysed using a one-way analysis of variance (ANOVA) followed by a Bonferroni post test.

The pEC_{50} values, Hill slopes and maximum responses calculated from the antagonist data in mouse carotid arteries were analysed using a two-way ANOVA followed by a Bonferroni post test. For both one- and two-way ANOVA, a *P*-value of less than 0.05 was considered significant. The agonist concentration ratios (CRs) were determined from the ratio of the EC_{50} of the agonist in the presence and absence of the antagonist and used for Schild analysis where the log[antagonist] is plotted against log(CR−1) (Arunlakshana & Schild, 1959). Linear regression produces an *x*-intercept that is equal to the pA_2 of the antagonist. If the slope of the Schild plot is equal to 1, then $pA_2 = pK_B$ and is indicative of competitive binding. Where a pA_2 value could not be calculated (e.g. where there is only a small shift with antagonist), a pK_B value was calculated instead using the equation

$$pK_B = \log(CR - 1) - \log[B]$$

where pK_B is the negative logarithm of the dissociation constant K_B and [B] is the concentration of antagonist.

Solutions and drugs

The Krebs–Henseleit solution was of the following composition (mM): NaCl (119), KCl (4.7), $MgCl_2$ (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25), $NaHPO_4$ (1.2), glucose (11.5) and Na_2EDTA (0.023).

The following compounds were used: A-61603 hydrobromide (Tocris, U.K.), BMY 7378 dihydrochloride (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione; Research Biochemicals International, U.K.), 5-HT (Sigma, U.K.), 5-methylurapidil (Research Biochemicals International, U.K.), noradrenaline hydrochloride (Sigma, U.K.), phenylephrine hydrochloride (Sigma, U.K.) and prazosin hydrochloride (Sigma, U.K.).

All drugs were dissolved in deionised water and then diluted (1 : 10) to give the concentrations used for the CRCs.

Results

The genetic controls for the α_{1B}- and α_{1D}-KO were found to be pharmacologically similar in their sensitivities and maximum responses to PE and 5-HT (Figure 1). Therefore, only one set of control experiments was required.

Control, α_{1B}-KO and α_{1D}-KO mouse carotid arteries

All agonist data (pEC_{50} values, maximum responses, Hill slopes and statistical comparisons) are presented in Table 1.

PE produced concentration-dependent contractions in carotid arteries from all three strains of mice. Sensitivity to PE was found to vary between mouse strains (Figure 2a). The α_{1B}-KO was the most sensitive to PE, followed by the control carotid artery and finally the least sensitive was the α_{1D}-KO. All pEC_{50} values were significantly different between the three mouse strains. Control and α_{1B}-KO carotid arteries produced similar maximum responses, whereas the maximum response from the α_{1D}-KO carotid artery was significantly smaller. CRCs to PE demonstrated shallow Hill slopes significantly different from unity in both control and α_{1B}-KO carotid arteries. This was not the case in α_{1D}-KO carotid arteries; the PE CRC had a Hill slope that was not significantly different from unity.

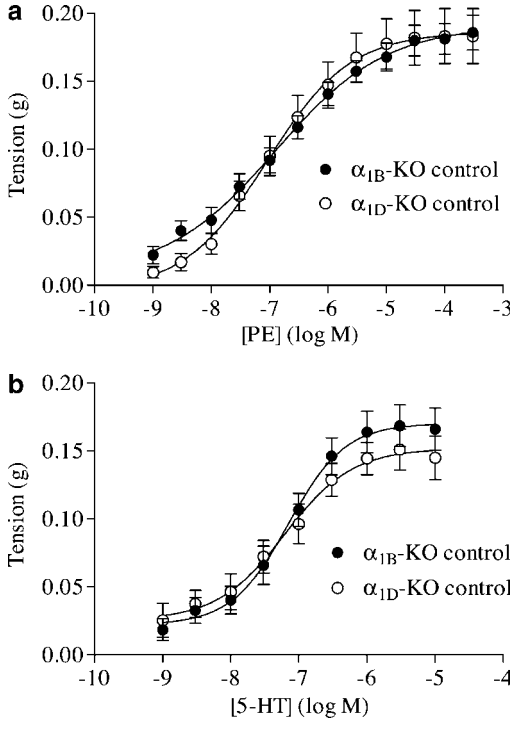

Figure 1 Mean concentration response data to (a) PE and (b) 5-HT in α_{1B}-KO and α_{1D}-KO control mouse carotid arteries expressed as tension in grams. Both agonists produced similar responses in the two strains of control mice. Mean curves were generated using nonlinear regression upon which the mean ± s.e.m. data have been superimposed ($n > 9$).

Table 1 Comparison of pEC_{50} values, maximum responses and Hill slopes for agonists producing contractions in (a) control, (b) α_{1B}-KO and (c) α_{1D}-KO mouse carotid arteries

Agonist	*pEC_{50}*	*Max. response* (g)	*Hill slope (95% CI)*
(a) Control			
PE	6.3 ± 0.06	0.37 ± 0.01	0.5 (0.4–0.6)#
(R)-A-61603	6.9 ± 0.09	0.27 ± 0.01	0.65 (0.5–0.8)#
5-HT	6.9 ± 0.08	0.27 ± 0.04	1.15 (0.6–1.7)
(b) α_{1B}-KO			
PE	6.9 ± 0.22*	0.33 ± 0.01	0.4 (0.2–0.6)#
(R)-A-61603	6.7 ± 0.07	0.28 ± 0.01	0.65 (0.5–0.8)#
5-HT	6.8 ± 0.04	0.27 ± 0.05	1.3 (0.9–1.7)
(c) α_{1D}-KO			
PE	5.5 ± 0.07*	0.21 ± 0.02*	0.85 (0.6–1.1)
(R)-A-61603	7.4 ± 0.27*	0.11 ± 0.01*	0.4 (0.1–0.7)#
5-HT	7.4 ± 0.05*	0.26 ± 0.06	1.0 (0.7–1.3)

pEC_{50} values and maximum responses are expressed as mean ± s.e.m. and the Hill slopes are given along with their 95% confidence intervals (95% CI). *$P < 0.05$ compared to control; #Hill slope significantly different from unity.

A-61603 produced concentration-dependent contractions in all three mouse strains (Figure 2b). The α_{1D}-KO was more sensitive to A-61603 than the control or the α_{1B}-KO carotid arteries. However, the efficacy of A-61603 in the α_{1D}-KO was reduced compared to the other two mouse strains. The maximum responses produced by the control and α_{1B}-KO carotid arteries were similar, while the α_{1D}-KO response was smaller. All three strains of mice produced shallow Hill slopes significantly different from unity.

5-HT produced concentration-dependent contractions in all three mouse strains (Figure 2c). Desensitisation occurred at the higher concentrations of 5-HT; therefore, CRCs were stopped as soon as the maximum response began to decline. All three mouse strains produced similar responses to 5-HT, with no differences observed in maximum responses or Hill slopes. However, α_{1D}-KO carotid arteries were found to be more sensitive to 5-HT than either control or α_{1B}-KO arteries.

In control, α_{1B}-KO and α_{1D}-KO carotid arteries, the *subtype-selective* antagonists produced a rightward displacement of the PE curve without a depression in the maximum response (Figure 3). Prazosin was found to cause a decrease in the maximum response at 1 and 10 nM in α_{1D}-KO arteries and at 100 nM in control and α_{1B}-KO arteries (Figure 3). The pA_2 values for prazosin and 5-methylurapidil in control tissue were calculated to be 9.6 and 7.5, respectively, with slopes that were not significantly different from unity, indicating competitive antagonism (Table 2). Only the highest concentration of BMY7378 (100 nM) produced a significant shift in the CRC to PE. Therefore, a pA_2 value could not be calculated. Instead, a pK_B value was calculated at 100 nM BMY7378 and was found to be 8.3. In the α_{1B}-KO carotid artery, all antagonists acted

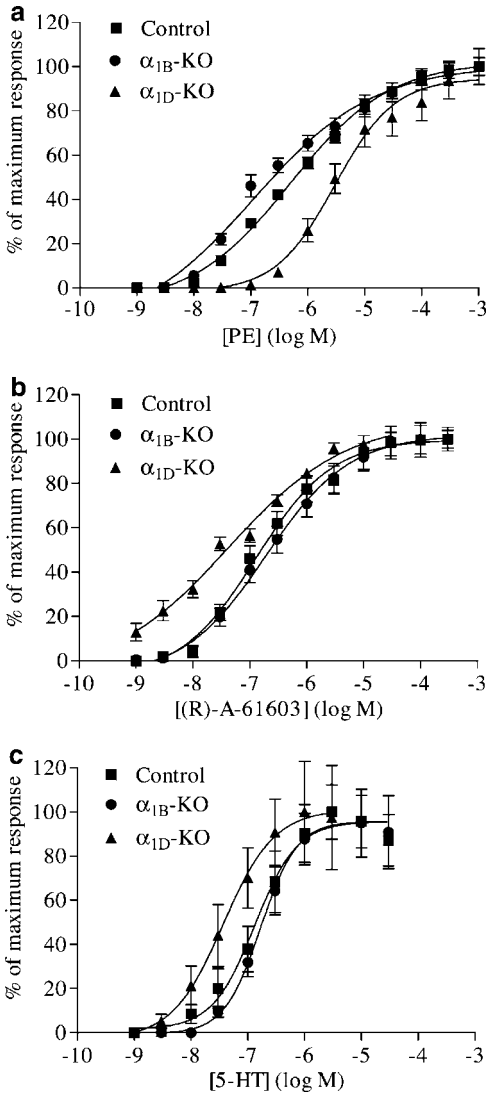

Figure 2 Mean concentration response data to (a) PE, (b) A-61603 and (c) 5-HT in carotid arteries from control, α_{1B}-KO and α_{1D}-KO mice expressed as a percentage of their own maximum response. Mean curves were generated using nonlinear regression upon which the mean $\pm$ s.e.m. data have been superimposed ($n > 9$).

competitively. The pA_2 values for prazosin, 5-methylurapidil and BMY7378 were found to be 10.3, 7.6 and 9.6, respectively (Table 2). In the α_{1D}-KO, it was possible to calculate a pA_2 value only for 5-methylurapidil, which was 8.1. Prazosin antagonised the contractions to PE with such potency that we had to dilute the concentrations used in control and α_{1B}-KO by a factor of 10 to allow us to obtain four consecutive CRCs. Despite this, it was still possible to calculate EC_{50} values only for the smallest concentration used (0.1 nM). This was the concentration used to calculate a pK_B for prazosin, which was 10.6. In contrast BMY7378 could only weakly antagonise the contraction to PE in α_{1D}-KO carotid arteries. A pK_B value could be calculated only at 100 nM, which was 7.0 (Table 2).

In addition to the antagonist data obtained to PE, we repeated these experiments with A-61603 as the agonist in the α_{1D}-KO (Figure 4). As with PE, prazosin produced a rightward displacement of the curve. However, unlike PE, there was no depression in the maximum response. A pA_2 was calculated and found to be 8.6 with a slope not significantly different from unity, indicating competitive antagonism. 5-Methylurapidil potently inhibited contractions to A-61603 in the α_{1D}-KO with a higher pA_2 than had been previously calculated for any of the strains of mice used, including the pA_2 obtained against PE contractions in the α_{1D}-KO. As with prazosin, 5-methylurapidil acted competitively. Relative to the time control, no significant shift occurred with BMY7378. Therefore, no pA_2 or pK_B could be calculated. pA_2 values and slope parameters are presented in Table 3.

Time controls for control, α_{1B}-KO and α_{1D}-KO carotid arteries showed no significant change in sensitivity or maximum response to PE or A-61603 (data not shown).

Rat carotid arteries

NA produced concentration-dependent contractions in rat carotid arteries with a pEC_{50} of 7.9 ± 0.06 and a maximum response of 1.1 ± 0.06 g ($n = 6$) (graphs not shown).

All antagonists used shifted the CRC to NA to the right in a concentration-dependent manner. There was no decrease in the maximum response for any of the antagonists. Schild regression produced pA_2 values of 10.0 for prazosin with a Schild slope indicative of competitive antagonism. 5-Methylurapidil and BMY7378 had pA_2 values of 9.1 and 9.2, respectively, accompanied by shallow Schild slopes, significantly different from unity (Table 4). Time controls showed no significant change in sensitivity or maximum response to NA (data not shown).

Discussion

This study demonstrates that when the α_{1B}-KO and α_{1D}-KO strains of mice are used in conjunction with antagonists, a different pharmacological situation emerges relative to control mice and to each other. However, the pharmacological differences between strains cannot simply be explained in terms of the effects of removing one of the subtypes. Interpreting the pharmacology of the control remains complex and suggests interactions between the subtypes beyond their effects on smooth muscle contraction.

In the α_{1B}-KO, the α_{1D}-ARs were apparently isolated, producing robust vasoconstrictor responses that were amenable to classical pharmacological analysis. In contrast, the α_{1D}-KO responses were less sensitive to PE (though not the α_{1A}-AR-selective A61603), had a smaller maximum response and responded to selective antagonists with the characteristics of an α_{1A}-AR. This raises a few related questions: (1) why is there no evidence for an α_{1A}-AR-mediated response in the control or α_{1B}-KO? (2) is the α_{1A}-AR-mediated response present in these arteries but has not been identified with the antagonists used or (3) has it been upregulated?

Receptor subtypes as revealed by agonists

The pattern of the relative potency of agonists for the three mouse strains was inverted for the two agonists tested. For PE, the order of potency was α_{1B}-KO > control > α_{1D}-KO and, for A-61603, it was α_{1D}-KO > control > α_{1B}-KO (Figure 2 and Table 1). Knepper *et al.* (1995) have shown that A-61603 is much more potent than PE at α_{1A}-AR and less potent than PE

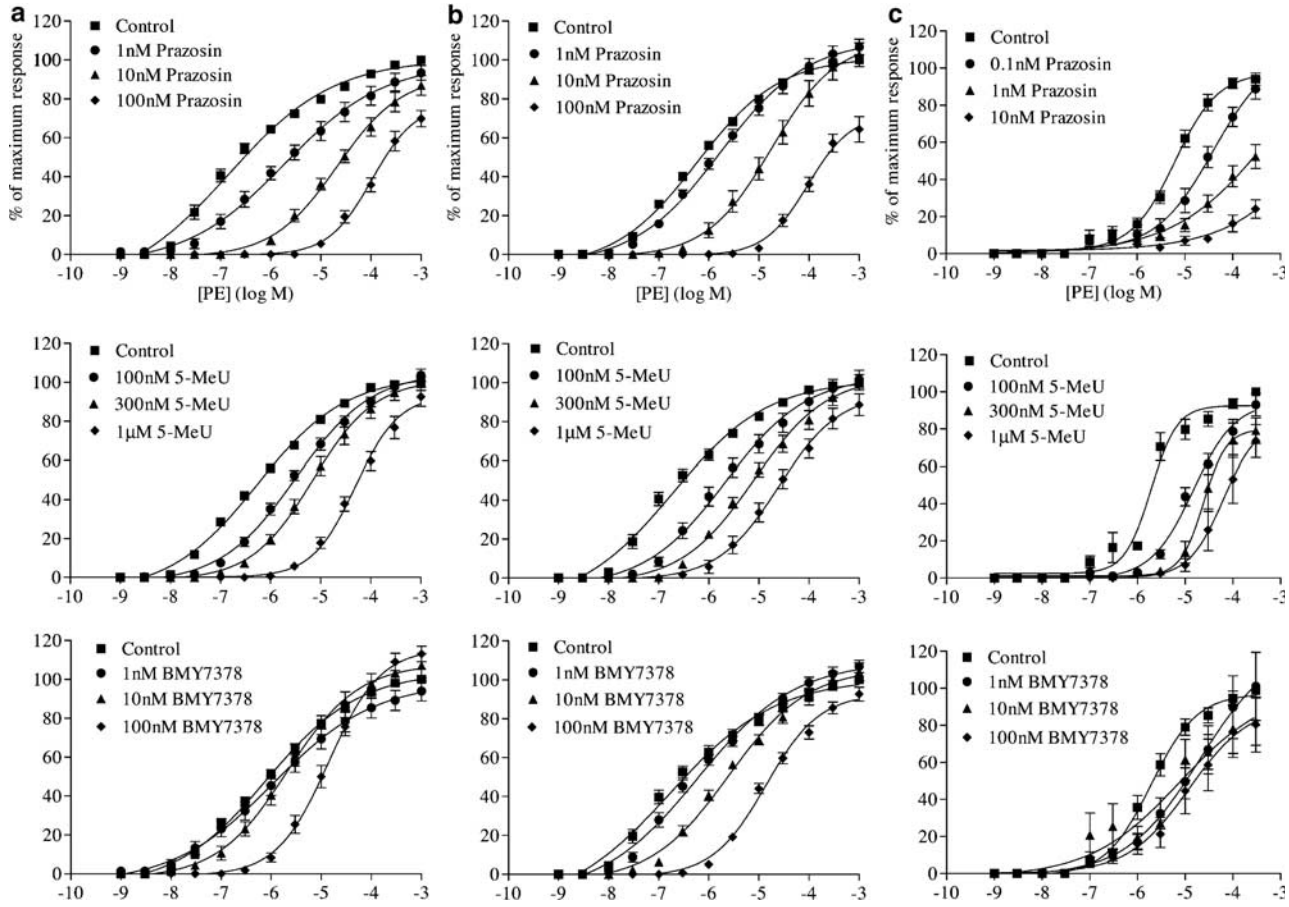

Figure 3 Mean concentration response data to PE in the presence of increasing concentrations of antagonists in (a) control, (b) α_{1B}-KO and (c) α_{1D}-KO mouse carotid arteries. Mean curves were generated using nonlinear regression upon which the mean ± s.e.m. data have been superimposed ($n > 6$).

Table 2 pA_2 or pK_B values and slope parameters of antagonists in control, α_{1B}-KO or α_{1D}-KO mouse carotid arteries

	Control		*α_{1B}-KO*		*α_{1D}-KO*	
Antagonist	pA_2/pK_B	*Slope*	pA_2/pK_B	*Slope*	pA_2/pK_B	*Slope*
Prazosin	9.6	0.93 (0.77–1.08)	10.3	0.92 (0.68–1.2)	10.6	NA
5-MeU	7.5	1.1 (0.73–1.5)	7.6	1.1 (0.77–1.5)	8.1	0.82 (0.4–1.3)
BMY7378	8.3	NA	9.6	0.9	7.0	NA

CRCs were constructed to PE. Values in parentheses are the 95% confidence limits for the slope value. 5-MeU, 5-methylurapidil; NA, not applicable.

at α_{1D}- and α_{1B}-ARs. In these terms, the responses in the α_{1D}-KO are strongly correlated with α_{1A}-AR, while the control and the α_{1B}-KO correlate better with either α_{1B}- or α_{1D}-ARs.

The α_{1D}-KO does not produce such large contractions as the control or α_{1B}-KO in response to the α_1-AR agonists PE or A-61603. The maximum response achieved is approximately half of the response produced by the control and the α_{1B}-KO, yet the responses to 5-HT produced by the three strains of mice are not significantly different. This suggests that the α_1-AR(s) mediating contraction in control and α_{1B}-KO carotid arteries are either more efficiently coupled to contraction than the α_1-AR mediating contraction in the α_{1D}-KO or that there are fewer receptors present in the α_{1D}-KO to mediate a response.

The maximum to 5-HT was not significantly different between the three mouse strains, indicating that the decreased response to PE and A-61603 in the α_{1D}-KO is not due to a general decline in agonist-mediated responses (indeed sensitivity to 5-HT was enhanced) but seems likely to be a consequence of deleting the α_{1D}-AR. This deserves closer analysis to determine whether it represents engagement of a subpopulation of smooth muscle cells or a submaximal excitation of each cell; however, the data presented here seem to show that the remaining α_{1A}- and/or α_{1B}-ARs are not as efficient as the α_{1D}-AR when it comes to mediating contraction in the carotid artery, perhaps consistent with their different physiological roles as discussed below.

The increase in sensitivity to 5-HT in the α_{1D}-KO suggests heterologous upregulation in response to the loss of sensitivity to catecholamines *via* α_1-ARs. A similar observation has been made in the aorta of the α_{1D}-KO mouse by Tanoue *et al.* (2002). Both 5-HT_{1A} receptors and α_1-ARs are coupled to $G_{q/11}$ (Alexander *et al.*, 2004). Therefore, it seems likely that they will share common pathways that are subject to feedback modulation and may be capable of compensating for one another in a KO mouse.

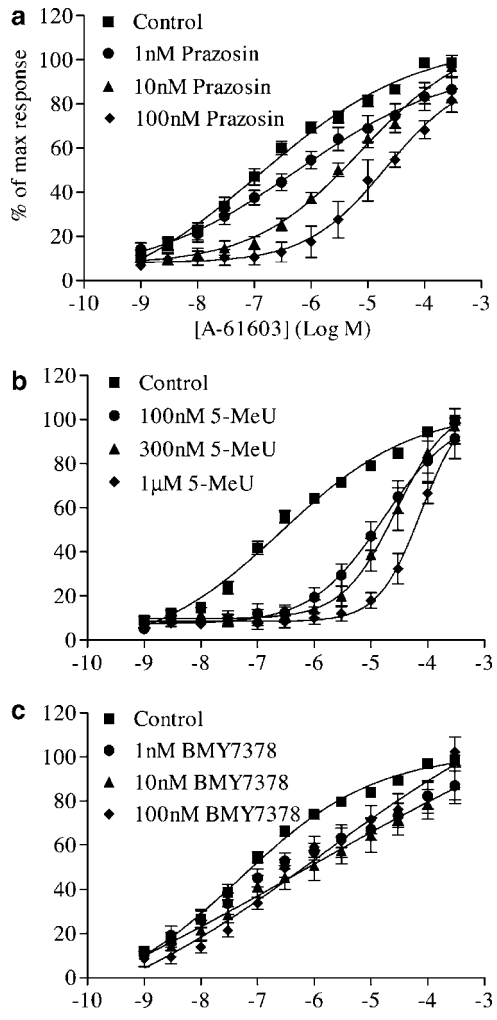

Figure 4 Mean concentration response data to A-61603 in the presence of increasing concentrations of antagonists in α_{1D}-KO mouse carotid arteries. Mean curves were generated using nonlinear regression upon which the mean ± s.e.m. data have been superimposed ($n > 6$).

Table 3 pA_2 values and slope parameters of antagonists in α_{1D}-KO mouse carotid arteries

Antagonist	*p*A$_2$	*Slope*
Prazosin	8.6	1.2 (0.7–1.8)
5-MeU	8.5	0.9 (0.2–1.8)
BMY7378	ND	ND

CRCs were constructed to A-61603. Values in parentheses are the 95% confidence limits for the slope value. ND, not determined.

Receptor subtypes as revealed by agonist–antagonist interactions

In the α_{1B}-KO, the estimated affinity for BMY7378 increased compared with controls. This would be expected if the primary response in the control is α_{1D}-AR mediated and a secondary (α_{1A}- or α_{1B}-AR) component is present. There is no positive evidence for the presence of α_{1A}-ARs in the control; 5-methylurapidil has lower affinity than in vessels believed to utilise α_{1A}-AR (Jarajapu *et al*., 2001a, b; Daly *et al*., 2002) and the control shows a relatively low sensitivity to A-61603 (Knepper *et al*., 1995). The analysis of control and α_{1B}-KO data together suggest that control carotid arteries mediate contraction through α_{1D}-ARs (primary response) and α_{1B}-ARs (secondary response) while α_{1B}-KO carotid arteries mediate contraction solely through α_{1D}-ARs.

Table 4 pA_2 values and slope parameters of antagonists in rat carotid artery

Antagonist	*p*A$_2$	*Slope*
Prazosin	10.7	1.1 (0.4–1.4)
5-MeU	9.0	0.6 (0.3–0.8)
BMY7378	9.8	0.5 (0.3–0.7)

Values in parentheses are the 95% confidence limits for the slope value.

In the α_{1D}-KO, where only α_{1A}- or α_{1B}-AR can be present, the affinity of 5-methylurapidil increases compared with the control, pointing to the presence of α_{1A}-ARs. To test this hypothesis, 5-methylurapidil was tested against the α_{1A}-AR agonist A-61603 in the α_{1D}-KO. 5-Methylurapidil showed still higher affinity, suggesting that α_{1A}-ARs were indeed contributing to contraction.

There is no positive evidence from control or α_{1B}-KO data to suggest an α_{1A}-AR component to their contractions. Therefore, if the α_{1A}-AR is involved in the functional response of the α_{1D}-KO, it may be as a result of upregulation of the α_{1A}-AR from a subfunctional level as a consequence of losing the preferred, dominant receptor, the α_{1D}-AR.

Comparison with published studies

Evidence has been presented for and against the presence of functional α_{1A}-ARs in large arteries. Several analyses have favoured α_{1D}- and/or α_{1B}-ARs over α_{1A}-ARs (Muramatsu *et al*., 1991; 1995; Aboud *et al*., 1993; Kohno *et al*., 1994; Testa *et al*., 1995a, b; Villalobos-Molina & Ibarra, 1996; de Oliveira *et al*., 1998; Martinez *et al*., 1999). Furthermore, Rokosh & Simpson (2002) created an α_{1A}-KO mouse and showed histochemically that Lac-Z, whose gene substituted for the α_{1A}-ARs gene, was not detected in the major conducting arteries, including the carotid artery. However, there is some evidence in favour of an α_{1A}-AR response in rat conducting arteries. Gisbert *et al*. (2003) showed in rat aorta that there was an α_{1A}-AR-mediated component to the production of inositol phosphates by NA. In addition, our own data from the rat carotid artery show that it is difficult to define the subtype involved in the contractile response to NA. The subtype-selective antagonists BMY7378 and 5-methylurapidil both produced high pA_2 values, although low slopes indicate a complex response consistent with multiple subtypes (Table 4). In the light of the mouse data, we now propose that the rat carotid artery expresses a mixture of α_{1A}- and α_{1D}-ARs. The high sensitivity of antagonists to both α_{1A}- and α_{1D}-ARs implies not only the presence of both of these subtypes but that they may act synergistically, allowing all antagonists to be effective.

Overall, the present data suggest that large conducting arteries have the potential to express and employ all three α_1-AR subtypes. There is positive evidence for the α_{1D}-AR in the conducting arteries of mice and rats. There is more controversial evidence that it can be accompanied by an α_{1B}-AR in normal

mice and by either or both of the α_{1B}-AR and the α_{1A}-AR in rats. In the α_{1B}-KO mouse, the α_{1D}-AR component becomes dominant as expected from simple removal of the α_{1B}-AR. However, in the α_{1D}-KO, the remaining response shows characteristics of the α_{1A}-AR. This suggests that the vessel can withstand the loss of its minor receptor without compensation, but that when its major receptor is lost it compensates by upregulating the α_{1A}-AR. This is not the first time we have observed this phenomenon. The α_{1A}-AR is upregulated in the liver of the α_{1B}-KO mouse (Deighan *et al*., 2004), which in control mice expresses only the α_{1B}-AR (Garcia-Sainz *et al*., 1994; Cavalli *et al*., 1997; Deighan *et al*., 2004). Compensatory upregulation of another α_1-AR subtype may be a general response to loss of the major subtype in any particular tissue, be it α_{1B}- or α_{1D}-ARs.

Physiological relevance

In general, there seems to be a reciprocal presence of the α_{1A}-AR and the α_{1D}-AR in blood vessels. Large noninnervated conductance arteries are associated with expressing the α_{1D}-AR (Kenny *et al*., 1995; de Oliveira *et al*., 1998; Daly *et al*., 2002; Tanoue *et al*., 2002), while small innervated resistance vessels are associated with the α_{1A}-AR (Stassen *et al*., 1998; Jarajapu *et al*., 2001b; Daly *et al*., 2002). The innervated vessels, on the whole, are less sensitive to agonists. This suggests a physiological basis for the relative balance of subtypes based on the balance of humoral and neurogenic control; that is, developmentally, when vessels become innervated they lose α_{1D}-ARs and gain α_{1A}-ARs, making them less sensitive to circulating catecholamines but gaining a graded sensitivity to locally released NA, at a higher concentration range. Conducting arteries do not become innervated, so remain sensitive to catecholamines through their α_{1D}-AR. This would explain why α_1-AR agonists, such as NA or PE, are more potent at α_{1D}-ARs than at the other subtypes (Minneman *et al*., 1994; Knepper *et al*., 1995; Yang *et al*., 1997). The present work shows that if they are deprived of this natural selection process by deletion of the preferred receptor's gene, they upregulate the alternative catecholamine contractile-signalling receptor that is best able to contract vascular smooth muscle, the α_{1A}-AR. However, the lower efficiency of the α_{1A}-AR can only partly compensate in functional terms, as witnessed by the weak submaximal contractions elicited to both PE and A-61603 in the α_{1D}-KO carotid artery.

Our data seem to suggest the presence of α_{1B}-ARs in the control; yet, when it is conclusively absent in the α_{1B}-KO, this has little effect on the artery's contractile ability and in the α_{1D}-KO there is no evidence for its presence. If it is indeed present in the control, then what functional role does the α_{1B}-AR have in these blood vessels? Regulatory interactions between the subtypes involving heterodimerisation of α_{1B}-ARs and the other two subtypes have been proposed from studies of fluorescent-labelled recombinant receptors. The α_{1B}-AR, which of the three subtypes is the most susceptible to agonist-mediated endocytosis (Chalthorn *et al*., 2002), can form heterodimers with the other subtypes that can affect their cellular location and expression levels (Uberti *et al*., 2003; Stanasila *et al*., 2003). The formation of the α_{1A}/α_{1B} heterodimer allowed the α_{1A}- and α_{1B}-ARs to cointernalise and consequently increased the extent of agonist-mediated internalisation of the α_{1A}-AR (Stanasila *et al*., 2003). In the case of the α_{1B}/α_{1D} heterodimer, the presence of the α_{1B}-AR was found to relocate the α_{1D}-AR from intracellular sites to the plasma membrane (Uberti *et al*., 2003; Hague *et al*., 2004). The present data are the first to show that the functions of α_1-ARs are influenced by the presence of the other subtypes.

The heterodimersation studies may show potential interactions that play a part in long-term receptor regulation involving other factors. The absence of the α_{1B}-AR seems to cause some sensitisation of the mainly α_{1D}-AR-mediated contraction of mouse carotid, which might indicate the loss of a regulation of receptor expression or of some other essentially negative effects of the α_{1B}-AR. The apparent absence of an α_{1B}-AR-mediated contraction in the α_{1D}-KO, where the α_{1A}-AR has taken over function, may indicate an adaptation to counteract the negative influence of the α_{1B}-AR since α_1-AR function is already compromised. Overall, it seems probable that the α_{1B}-AR will emerge as a regulatory receptor capable of fine-tuning the properties of the α_{1A}- and α_{1D}-ARs.

To summarise, normal mouse carotid arteries have antagonist absolute affinity values that are not consistent with a single α_1-AR subtype but correspond to those expected from a mixed population of at least two and possibly all three subtypes. The α_{1B}-KO mouse presents a straightforward picture of contractile α_{1D}-ARs, while the α_{1D}-KO mouse utilises α_{1A}-ARs. The emergence of α_{1A}-ARs when the major subtype, α_{1D}-AR, is knocked out suggests compensatory upregulation of the α_{1A}-AR. The α_{1B}-AR may have a regulatory role to play in control carotid artery by influencing the expression and cellular location of the other subtypes.

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