The $\alpha_{1D}$-adrenergic receptor directly regulates arterial blood pressure via vasoconstriction

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To investigate the physiological role of the $\alpha_{1D}$-adrenergic receptor ($\alpha_{1D}$-AR) subtype, we created mice lacking the $\alpha_{1D}$-AR ($\alpha_{1D}^{-/-}$) by gene targeting and characterized their cardiovascular function. In $\alpha_{1D}^{-/-}$ mice, the RT-PCR did not detect any transcript of the $\alpha_{1D}$-AR in any tissue examined, and there was no apparent upregulation of other $\alpha_1$-AR subtypes. Radioligand binding studies showed that $\alpha_1$-AR binding capacity in the aorta was lost, while that in the heart was unaltered in $\alpha_{1D}^{-/-}$ mice. Non-anesthetized $\alpha_{1D}^{-/-}$ mice maintained significantly lower basal systolic and mean arterial blood pressure conditions, relative to wild-type mice, and they showed no significant change in heart rate or in cardiac function, as assessed by echocardiogram. Besides hypotension, the pressor responses to phenylephrine and norepinephrine were decreased by 30–40% in $\alpha_{1D}^{-/-}$ mice. Furthermore, the contractile response of the aorta and the pressor response of isolated perfused mesenteric arterial beds to $α_1$-AR stimulation were markedly reduced in $\alpha_{1D}^{-/-}$ mice. We conclude that the $\alpha_{1D}$-AR participates directly in sympathetic regulation of systemic blood pressure by vasoconstriction.


Introduction

The sympathetic nervous system plays an important role in regulating the tone of the peripheral circulation and hence in the control of blood pressure. Catecholamines cause vascular smooth muscle contraction by activating $\alpha_1$-adrenergic receptors ($\alpha_1$-ARs) (1). Recent extensive efforts have been made to classify the three known $\alpha_1$-AR subtypes ($\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$) by molecular cloning (2–7) and pharmacological analyses (8–11); however, the contribution of each $\alpha_1$-AR subtype to catecholamine-induced physiological responses still has not been well characterized (12, 13). Studies aimed at assessing functional role(s) mediated by distinct $\alpha_1$-AR subtypes have been hampered, in part because the available subtype-selective drugs are only moderately selective and may interact with other adrenergic and nonadrenergic receptors and because native tissues can express all three subtypes. Thus, the functional implications of $\alpha_1$-AR heterogeneity and their physiological relevance remain largely unknown.

Gene disruption (knockout) experiments have proved to be useful in defining the function of a target molecule in vivo. Gene targeting of each receptor subtype ought to be useful in determining their functional role(s). The power to reveal novel functions and mechanisms of action can be greatly enhanced when pharmacological tools are used in conjunction with these genetic techniques (14, 15). Among the three $\alpha_1$-AR subtypes, this technique has been used to disrupt expression of the $\alpha_{1B}$-AR subtype (16). $\alpha_{1B}$-AR knockout mice were shown to be normotensive, but displayed a moderate decrease in pressor responses to $\alpha_1$-AR stimulation (16), providing evidence that the $\alpha_{1B}$-AR participates in the regulation of vasoconstriction and hence blood pressure. However, pharmacological studies with the “$\alpha_{1D}$-AR-selective” antagonist BMY7378 suggest that the $\alpha_{1D}$-AR plays a predominant role in the vascular contractions induced by $\alpha_1$-AR agonists in the rat (17). Also, by examining transgenic mice overexpressing the $\alpha_{1B}$-AR Zuscik et al. (18) very recently have reported that the $\alpha_{1B}$-AR is not directly involved in blood pressure–related vasoconstriction. Hence, the functional role of the $\alpha_{1D}$-AR in the control of vascular tone and blood pressure needs to be clarified.

In this study, we describe the gene targeting of the mouse $\alpha_{1D}$-AR and the initial functional characterization of knockout mice lacking this receptor subtype. The clinical efficacy of $\alpha_1$-AR antagonists as antihypertensive drugs reflects the important physiological role of $\alpha_1$-ARs in vascular function and in the maintenance of arterial blood pressure. We therefore focused on functional characterization of the $\alpha_{1D}$-AR knockout model in terms of cardiovascular functions. Our study shows that the $\alpha_{1D}$-AR is a mediator of the vasoconstrictive and pressor responses to catecholamines.
Methods

Gene targeting. The murine α1D-AR gene consists of two exons and one intron, spanning more than 10 kb (19). Restriction fragments of 3 kb (HindIII/SacII) and 5 kb (SacI/SalI) were subcloned from the mouse α1D-AR genomic clone (Figure 1) into pBlueScript. These two fragments were inserted into a plasmid with a 1.6-kb cassette containing the neomycin resistance gene (Neo), under the control of the phosphoglycerate kinase promoter, as described (20). As a result, the 0.3-kb SacI-SalI region, including the first AUG codon (−131 to +181, relative to AUG initiation codon), in the first exon of the α1D-AR gene was replaced with the Neo cassette. The diphtheria toxin A fragment gene was used as a negative selection marker (21). The 1.8-kb diphtheria toxin cassette (DT) was inserted into the plasmid to obtain the targeting vector NeoDT (Figure 1). After its linearization with NotI, the targeting vector contained two regions of homology with the α1D-AR gene: 3 kb of the 5′ untranslated sequences flanking the first exon and a 5-kb fragment containing the first exon and intron. The linearized targeting vector was electroporated into 129Sv embryonic stem (ES) cells, which were then subjected to selection with G418. Southern blot analysis was performed on 288 neomycin-resistant ES cell clones. Genomic DNA was digested with EcoRI, electrophoresed on a 0.8% agarose gel, transferred to a membrane, and hybridized with the 5′ probe, derived from the α1D-AR locus (Figure 1). Digestion of genomic DNA with EcoRI generated 12-kb and 4-kb restriction fragments for the wild-type and disrupted alleles, respectively. Seven clones positive for the 5′ probe and a 5-kb fragment containing the first exon and intron were expanded and subjected to further Southern blot analysis with 3′ and Neo probes, revealing that three of these clones were positive for the correct targeting event. The three positive ES cell clones were independently microinjected into C57Black/6J mouse blastocysts, which were then transferred into pseudopregnant NMRI females. This generated 12 chimeric mice based on coat color. Male chimeras were then mated to C57Black/6J mice, and evidence of germ-line transmission was monitored by agouti coat color contributed from the 129Sv-derived ES cell genome.

Males and females with different genotypes were intercrossed to obtain α1D+/+, α1D+/−, and α1D−/− progeny. Mice were screened by genotyping using Southern blot analysis and PCR for α1D-AR gene. All mice analyzed were from F1 to F5, which carried the genetic background of 129Sv and C57Black/6J strains, and α0p−/− littermates were used for analysis as the wild-type mice. Since cardiovascular physiology could differ depending on the difference of mouse strains (22), mice with the same genetic background were always compared as the wild-type. Animals were housed in microisolator cages in a pathogen-free barrier facility. All experimentation was performed under approved institutional guidelines.

RT-PCR analysis. Total RNA from different mouse tissues was prepared using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan). Total RNA (5 μg) was treated with RNase-free DNase (TaKaRa Shuzo Co., Tokyo, Japan) and reverse-transcribed using random hexamers, as described (23). One-tenth of each cDNA sample was amplified by PCR with a receptor-specific primer set and a primer set specific for GAPDH (24). 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 MgCl2, and 2.5 U of Taq DNA polymerase (TaKaRa Shuzo Co.). Thermal cycling was performed for 1 minute at 94°C, 1 minute at 56°C, and 2 minutes at 72°C for 27 cycles. The upstream and downstream primers (5′→3′) were AGGGTCGT-CGAATTTCCTCG and CAGATTTGCTCTTGGACACT for α1A (275 bp), GGGAGGTTGAAAGATCCCA and TTGGTACTGCTAGGTTGTC for α1B (752 bp), and CGCTGTGGTTGACGCGACT and ACAAGTGCAGTCAGTCAGCA-GGTC for α1D (282 bp). The upstream primer for the α1A-AR or the α1B-AR gene was located within the first exon, and the downstream primer for the α1A-AR or the α1B-AR gene was located within the second exon. The primers for the α1D-AR gene were located within the first exon, and the forward primer was within the region replaced with the Neo in the mutant allele. The primers were derived from the murine α1A (25), α1B (25), and α1D (19, 25) sequences. The GAPDH primers (5′→3′) were GGTTCATCATTCCGGGCCCTC upstream and CCACACCCCTGTTTGCTTAG downstream (662 bp). Control PCR reactions also were performed on non–reverse-transcribed RNA to exclude any contamination by genomic DNA. The amplified DNAs were analyzed on a 1.5% agarose gel with 100 bp DNA marker (New England Biolabs Inc., Beverly, Massachusetts, USA). The specificity of the amplified DNA fragments was determined by Southern blot analysis using receptor-specific 32P-labeled probes (cDNAs of the murine α1A-AR, α1B-AR, and α1D-AR; ref. 25).

TaqMan assay. For rigorous quantification of RT-PCR products, the TaqMan 5′ nucleotide fluorogenic quantitative PCR assay was conducted according to manufacturer’s instructions, using total RNA from the brain of α1D+/+, α1D+/−, and α1D−/− mice. The cDNAs were synthesized from total RNA (5 μg), as described above. TaqMan assays (Applied Biosystems Japan Ltd., Tokyo, Japan) were then carried out using the following oligonucleotides (5′→3′): α1D forward primer CGCTGTGGTGGAAAAGCCGCAG, α1D reverse primer AGTGGGTGACGCTGTCGAAGT, α1D probe 6FAM-CGGGCACAACCTTCTCGTATCCCTTCTC-TAMRA, α1A forward primer GGGTGGACGCTCTTATGCT, α1A reverse primer TCACCAACTGATCCGCTGCTGG, α1A probe 6FAM-CATGCGGCGCTCCTGGCCTCCTC-TAMRA, α1B forward primer CCTGTCATGCCTGCTGGCAG, α1B reverse primer GACTCCCCGTCCAAGTCCT, α1B probe 6FAM-TCTACTGCGGCAAAAGAGGACACC-TAMRA. All primers used for TaqMan assays were derived from the nucleotide sequences within the first exon of each gene.

Ligand binding. Radioligand binding studies were performed on membrane preparations from the monkey kidney COS cell line (COS) transiently expressing each
mouse α1-AR subtype and on mouse native tissues, as described previously (26). Briefly, whole brain, heart, liver, kidney, and aorta were dissected from mice (8–18 weeks old), placed in a lysis buffer (250 mM sucrose, 5 mM Tris-HCl, and 1 mM MgCl₂, pH 7.4), and homogenized with a Polytron homogenizer (Kinematica AG, Littau-Luzern, Switzerland) at 4°C, at speed 7 for 10 seconds. The homogenate was centrifuged at 35,000 g for 20 minutes at 4°C. The resulting pellet was resuspended in binding buffer B (50 mM Tris-HCl, 10 mM MgCl₂, and 10 mM EGTA, pH 7.4), and was frozen at −80°C until assayed. A membrane preparation of COS cells transiently expressing mouse α1A-, α1B-, or α1D-AR was also used for binding studies. The collected cells were placed in ice-cold buffer A and disrupted in a sonicator (SONIFER 250, Branson Ultrasounds Corp., Danbury, Connecticut, USA) at setting 5 for 8 seconds. They were then centrifuged at 3,000 g at 4°C for 10 minutes to remove the nuclei. The supernatant fraction was centrifuged at 35,000 g for 20 minutes at 4°C. Protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, Illinois, USA). Radioligand binding was measured using [125I]-HEAT (125I-(2-b-(4-hydroxyphenyl)-ethylamino)methyl)-tetralone; specific activity, 2,200 Ci/mmol; NEN Life Science Products Inc., Boston, Massachusetts, USA), as described (26). Briefly, measurement of specific [125I]-HEAT binding was performed by incubating 0.1 ml membrane preparation (~1–5 µg protein for COS cell membranes and ~30–200 µg for native tissues), with [125I]-HEAT for 45 minutes at 25°C in the presence or absence of competing drugs. For competition curve analysis, each assay contained about 100 pM [125I]-HEAT. Nonspecific binding was defined as binding displaced by phentolamine (10 µM).

Heart/body weight ratio. Age-matched (3–5 months) α1D+/– or α1D–/– male mice were anesthetized with lethal doses of pentobarbital (200 mg/kg intraperitoneally). The mice were weighed, then their hearts were excised, blotted three times on filter paper, and weighed. Heart/body-weight ratios were calculated and expressed as milligrams per gram.

Histological analysis. Heart and thoracic aorta from α1D+/– or α1D–/– male mice (12–18 weeks old) were perfusion fixed in PBS plus 10% formalin. Several sections of hearts and aorta were obtained for gross morphological analysis, then paraffin embedded for thin sectioning followed by hematoxylin and eosin staining.

Measurement of blood pressure. Systolic blood pressure (SBP) and heart rate (HR) were measured in conscious 12- to 18-week-old male mice (mean body weights were 28.8 g for α1D+/– and 28.9 g for α1D–/– mice, respectively) (28). After a cervical incision was made on mice anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally), a stretched Intramedic PE10 polyethylene catheter (Clay Adams, Parsippany, New Jersey, USA) was inserted into the right carotid artery. The catheter was tunneled through the neck and then placed in a subcutaneous pouch in the back. After a minimum 24-hour recovery, mice were placed in Plexiglas tubes to partially restrict their movements, the saline-filled catheter was removed from the pouch and connected to a pressure transducer (DX-360; Nihon Kohden Corp., Tokyo, Japan) and MAP was recorded on a thermal pen recorder (RTA-1200; Nihon Kohden Corp.). Measurement of HR was triggered from changes in MAP (AT-601G; Nihon Kohden Corp.). To examine pressor responses in unanesthetized mice, drugs in approximately 30 µl of injection volume (1 µl/g of mouse body weight) were administered through the catheter inserted into the right femoral vein as a bolus at 15- to 20-minute intervals after ensuring MAP and HR had returned to baseline levels.

In some experiments, the effect of α1-agonists on the norepinephrine-induced pressor response was examined in male mice (10–12 weeks old) anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally). Following propranolol (1 mg/kg) treatment, either bunazosin hydrochloride (10 µg/kg, intravenously; Eisai Co., Tokyo, Japan) or BMY7378 (100 µg/kg, intravenously; Research Biochemicals International, Natick, Massachusetts, USA) was administered 10 minutes prior to the continuous infusion of norepinephrine (1 µg/kg/min intravenously for 10 minutes) using a microsyringe pump (CFV-2100; Nihon Kohden Corp.).

Echocardiography. Quantitative echocardiographic measurements were performed on lightly anesthetized, spontaneously breathing mice according to a previously published transthoracic method (29). The male mice (12–18 weeks old) were anesthetized (40 mg/kg pentobarbital, intraperitoneally), the chest area was shaved, and ultrasonic gel was applied. The measurements with the SONOS-5500 system (Philips Medical Systems, Andover, Massachusetts, USA) employed a dynamically focused symmetrical annular array transducer (12.5 MHz) for two-dimensional, M-mode, and Doppler imaging. The parasternal long and short axes and four chamber views were visualized. For
For a quantitative analysis, measurements were performed in three to five consecutive cardiac cycles. Cardiac parameters determined include interventricular septal thickness (IVS), posterior wall thickness (PW), left ventricular internal dimension in diastole (LVIDd) and in systole (LVIDs), and heart rate (HR). IVS, PW, LVIDd, and LVIDs were normalized to body weight, and percentage of fractional shortening (%FS) was calculated as 100 × [(LVIDd – LVIDs)/LVIDd]. Cardiac output (CO) was calculated from Doppler echocardiography using the following equation, π × (Ao)² × VTI × HR / 4, where Ao was the diameter of the aortic artery, VTI was the Doppler velocity time integral in left ventricular outflow, and HR was determined from the simultaneous monitoring of electrocardiograms.

Measurement of aortic contraction. The thoracic aorta was excised from mice (12–18 weeks old), cleaned, and cut into 1-mm-long segments. These segments were suspended in isolated tissue baths filled with 10 ml Krebs-Henseleit bicarbonate buffer containing timolol (3 µM), continuously bubbled with a gas mixture of 5% CO₂/95%O₂ at 37 °C. One end of the aortic segment was connected to a tissue holder and the other to an isometric force transducer. Aortic segments were equilibrated for 60 minutes under a resting tension of 0.5 g, and the buffer was replaced every 15 minutes. In a preliminary experiment, the length of the smooth muscle was increased stepwise during the equilibration period to adjust passive wall tension to 0.5 g; this resting tension was found to be optimal for KCl-induced (40 mM) aortic contraction of mice weighing 22–28 g. Care was taken to avoid endothelial damage; functional integrity of the endothelium was assessed using acetylcholine (10 µM). Only intact segments were used for further analysis.

Pressor response in perfused mesenteric arterial beds. The perfused mesenteric arterial bed was prepared according to the methods described previously (30). The superior mesenteric artery of diethyl ether-anesthetized mice (12–18 weeks old) was dissected, and a stainless-steel cannula (27 G syringe) was inserted. The preparations were perfused with Krebs-Henseleit solution equilibrated with a mixture of 95% O₂ and 5% CO₂ (PO₂ > 600 mmHg). The entire ileum was dissected longitudinally at the opposite site of mesenteric vasculature. The preparation was placed in a chamber with a warm water jacket to maintain the temperature at 37 °C. The perfusion flow rate was maintained at 1.0 ml/min using a peristaltic pump. Perfusion pressure was measured through a branch of the perfusion cannula by means of a pressure transducer (TP-400T; Nihon Kohden Corp.) connected to a carrier amplifier (AP-621G; Nihon Kohden Corp.) and recorded on a thermal pen recorder (WT-645G; Nihon Kohden Corp.). The preparations were equilibrated for 30 minutes before administration of phenylephrine.

Measurement of serum catecholamines. After 1 hour of stable anesthesia (80 mg/kg pentobarbital, intraperitoneally), an abdominal incision was made, and blood samples were obtained from mice (12–18 weeks old) by venipuncture of the vena cava. Total plasma catecholamine levels (epinephrine, norepinephrine, and dopamine) were determined in 200 µl of plasma samples by HPLC using commercially available reagents (Toho Co., Tokyo, Japan).

Statistics. All values are expressed as means plus or minus SEM. Statistical analysis was performed using two-way ANOVA. A P value less than 0.05 by a Student t test was considered statistically significant.
tion data from the radioligand binding study were analyzed using the iterative nonlinear regression program, LIGAND (31). The presence of one, two, or three different binding sites was assessed using the F test in the program. The model adopted was that which provided the significant best fit (P < 0.05).

Results
Targeted disruption of the mouse α1D-AR gene. The strategy for inactivating one copy of the α1D-AR gene in ES cells is described in Figure 1a. Homologous recombinants were identified by Southern blot analysis of genomic DNA. Three of the positive ES clones confirmed by Southern blot analysis with the 5′, 3′, and Neo probe were independently microinjected into C57Black/6J blastocyst-stage embryos. Five of 12 chimeric mice were mated to C57Black/6J mice, and germline transmission of the mutant allele was confirmed by genomic Southern analysis of tail DNA from F1 progeny. Mating between heterozygous male and female mice generated F2 progeny with all three genotypes: homozygous mutant, heterozygous mutant, and wild-type mice (Figure 1b). The wild-type allele generates a 7-kb EcoRV fragment, and the mutant allele generates a 4-kb EcoRV fragment. Analysis of the α1D-AR genotype frequencies after intercrosses of heterozygous mutant mice did not reveal any deviation from Mendelian expectations (α1D+/+ 30%, α1D+/− 44%, α1D−/− 26%, n = 212). Monitoring of mice body weight at 4 weeks old did not reveal any significant difference in growth among mice of different α1D-AR genotypes.

Figure 2
RT-PCR analysis of the RNA from tissues of α1D+/+, α1D+/−, and α1D−/−. (a) Ethidium bromide staining of RT-PCR fragments (left). The α1A-, α1B-, and α1D-AR mRNA transcripts were detected and are shown in the upper, middle, and lower panels as 275-, 752-, and 282-bp fragments, respectively, indicated by the arrows. RT-PCR analysis was controlled by detection of the 662-bp fragment of GAPDH message, indicated by the arrowhead. Southern blots of the RT-PCR fragments are shown on the right. The specificity of the amplified fragments was assessed using 32P-labeled probes specific for each receptor subtype. M, 100-bp DNA marker; B, Brain; H, Heart; Lu, Lung; K, Kidney; Li, Liver; A, Aorta; S, Spleen. (b) TaqMan assay. Total RNA was isolated from whole brain and reverse-transcribed. Relative RNA levels of each α1-AR subtype, standardized against GAPDH levels, were obtained by semiquantitative PCR using the Taq-Man system. Values represent the mean ± SEM of five independent experiments.
interaction of BMY7378 with α1D-AR subtypes in membrane preparations from mouse tissues

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>α1D+/+ (fmol/mg protein)</th>
<th>α1D+/- (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain</td>
<td>101.2 ± 6.6</td>
<td>92.2 ± 1.7</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>247.0 ± 23.2</td>
<td>153.0 ± 30.6</td>
</tr>
<tr>
<td>Aorta</td>
<td>47.1 ± 13.0</td>
<td>ND</td>
</tr>
<tr>
<td>Heart</td>
<td>47.9 ± 7.5</td>
<td>43.8 ± 7.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>30.2 ± 1.3</td>
<td>31.5 ± 1.9</td>
</tr>
</tbody>
</table>

Each value is the mean ± SEM of six different experiments.

Table 2

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Tissue</th>
<th>Kα (nM)</th>
<th>Kβ (nM)</th>
<th>Rα (%)</th>
<th>Rβ (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1D+/-</td>
<td>Whole brain</td>
<td>149 ± 20</td>
<td>342 ± 50.5</td>
<td>0</td>
<td>89 ± 13</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Cerebral cortex</td>
<td>2.8 ± 0.1</td>
<td>814 ± 79.9</td>
<td>24 ± 2</td>
<td>76 ± 9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Hippocampus</td>
<td>0.4 ± 0.2</td>
<td>304 ± 37.9</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>α1D-/-</td>
<td>Cerebral cortex</td>
<td></td>
<td>423 ± 122</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hippocampus</td>
<td></td>
<td></td>
<td>0</td>
<td>100</td>
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</table>

Inhibition of specific [125I]-HEAT binding by BMY7378 was determined in membrane preparations from each tissue, as described. The best two-site fit was determined by nonlinear regression analysis of the averaged curve, and high-affinity (Rα) and low-affinity (Rβ) sites for BMY7378 were determined as described. Kα, Kβ value at high-affinity site. Kα, Kβ value at low-affinity site. The P value for the best two-site fit compared with the best one-site fit is given. Each value is the mean ± SEM of six different experiments.
mouse α₁D-AR expressed in COS-7 cells (Kᵣ values for mouse α₁A- and α₁B-ARs were 490 ± 30 nM and 410 ± 5 nM, n = 6 each, respectively). Competition binding studies with other α₁ antagonists (prazosin or the α₁A-AR–selective antagonist KMD-3213) showed no difference in their affinities between α₁D⁺/⁺ and α₁D⁻/⁻ mice, indicating that the remaining α₁A- and α₁B-ARs were not much changed with respect to their pharmacological properties (data not shown).

**Heart weight and histological analysis.** Heart-weight/body-weight ratio did not significantly differ between α₁D⁺/⁺ and α₁D⁻/⁻ mice (4.96 ± 0.31 mg/g, n = 10, and 5.22 ± 0.24 mg/g, n = 14, in α₁D⁺/⁺ and α₁D⁻/⁻, respectively). There were no obvious differences between α₁D⁺/⁺ and α₁D⁻/⁻ mice with respect to gross morphology or microscopic myocyte appearance of hearts and aorta (data not shown).

**Measurement of blood pressure.** The HR and blood pressure were analyzed in male mice 12–18 weeks of age. The resting SBP, measured by tail-cuff reading or MAP, measured by direct intra-arterial recording under unanesthetized conditions, were significantly (P < 0.05) lower in α₁D⁻/⁻ mice compared with α₁D⁺/⁺ mice (SBP: 108.7 ± 1.9 mmHg, n = 31, and 99.1 ± 1.7 mmHg, n = 23, in α₁D⁺/⁺ and α₁D⁻/⁻, respectively; MAP: 116.5 ± 2.2 mmHg, n = 14, and 106.9 ± 3.7 mmHg, n = 18, in α₁D⁺/⁺ and α₁D⁻/⁻, respectively); however, there was no significant difference in HR in beats per minute (bpm) between the two groups monitored by either tail-cuff reading or the intra-arterial measurements (554 ± 13 bpm, n = 31, and 529 ± 13 bpm, n = 23, by tail cuff reading in α₁D⁺/⁺ and α₁D⁻/⁻, respectively; 616 ± 12 bpm, n = 14, and 638 ± 17 bpm, n = 18, by intra-arterial measurements in α₁D⁺/⁺ and α₁D⁻/⁻, respectively).

We next examined the pressor responses to several vasoactive agents in nonanesthetized mice. Increasing doses of phenylephrine or norepinephrine progressively increased the blood pressure in both α₁D⁺/⁺ and α₁D⁻/⁻ mice. As shown in Figure 3, a and b, these pressor responses were considerably reduced in the α₁D⁻/⁻ as compared with the α₁D⁺/⁺ mouse; however, the pressor responses caused by higher doses of phenylephrine (>100 μg/kg) were not significantly different.

![Figure 3](http://www.jci.org)  
**Figure 3**  
Blood pressure responses in α₁D⁺/⁺ and α₁D⁻/⁻ mice. Phenylinephrine (a), norepinephrine (b), angiotensin II or vasopressin (c) was injected intravenously as a bolus to male nonanesthetized α₁D⁺/⁺ (open circles, n = 8) or α₁D⁻/⁻ mice (filled circles, n = 12) (12–18 weeks old). The effects on blood pressure are shown and expressed as the change in MAP (in mmHg). Responses to phenylephrine or norepinephrine in α₁D⁻/⁻ mice were significantly decreased at doses as indicated compared with the wild-type response. The maximal increase in blood pressure is shown. Points represent the mean ± SEM. *P < 0.05 as compared with α₁D⁺/⁺ mice.

**Figure 4**  
Effects of BMY7378 or bunazosin on blood pressure responses in α₁D⁺/⁺ and α₁D⁻/⁻ mice under anesthesia. Inhibitory effects of BMY7378 or bunazosin on the pressor response to norepinephrine in α₁D⁺/⁺ (upper) and α₁D⁻/⁻ (lower) mice. β-Blocker, propranolol (1 mg/kg) was preadministered, and BMY7378 (100 μg/kg) or bunazosin (10 μg/kg) was injected into male α₁D⁺/⁺ or α₁D⁻/⁻ mice (10–12 weeks old) 10 minutes prior to continuous infusion of norepinephrine (1 μg/kg/min, for 10 minutes). Points represent the mean ± SEM of eight mice. Open squares, norepinephrine infusion; open circles, norepinephrine infusion + BMY7378 pretreatment; filled circles, norepinephrine infusion + bunazosin pretreatment. *P < 0.05 as compared with norepinephrine infusion.
Figure 5  
Vascular contraction in α_{1D}^{+/+} and α_{1D}^{-/-} mice. The contractile response to norepinephrine (NE), phenylephrine (PE), and serotonin (5-HT). Concentration-response curves for norepinephrine-induced (a), phenylephrine-induced (b), and serotonin-induced contractions (c) in aortic segments from α_{1D}^{+/+} (filled circles) or α_{1D}^{-/-} (open circles) mice. The results are the mean ± SEM of 15–27 preparations for NE, PE, or 5-HT. Effects of BMY7378 on norepinephrine-induced contractions in aortic segments of α_{1D}^{+/+} (d) or α_{1D}^{-/-} (e) mice. Aortic segments were exposed to vehicle (filled circles, control) or different concentrations of BMY7378 (open circles, 1 nM; filled squares, 10 nM; open squares, 100 nM), prior to the addition of cumulative concentrations of norepinephrine (NE). Inset (d): A Schild plot derived from the data from α_{1D}^{-/-} mice was fitted by a straight line (R² = 0.92) with a slope of 1.06 ± 0.11. Data represent the mean ± SEM of six different aortic segments for each group. (f) Concentration-response curves for phenylephrine-induced pressor response in perfused mesenteric arterial beds of α_{1D}^{+/+} (filled circles) or α_{1D}^{-/-} (open circles, n = 7). Two-way ANOVA showed that concentration-response curve for phenylephrine-induced pressor response of α_{1D}^{-/-} mice was significantly (P < 0.05) different from that of the α_{1D}^{+/+} mice. *P < 0.05 as compared with α_{1D}^{+/+}.

The maximal plateau level of pressor responses by norepinephrine was not successfully monitored, because administration of higher doses of norepinephrine frequently caused circulatory collapse, probably due to its cardiac toxicity. Despite the diminished response to phenylephrine and norepinephrine in the α_{1D}^{-/-} mice, the increase in blood pressure induced by angiotensin II (0.1 µg/kg) or vasopressin (0.01 µg/kg) did not significantly differ between the α_{1D}^{+/+} and α_{1D}^{-/-} mice (Figure 3c). Pretreatment with the nonselective α-antagonist bunazosin (10 µg/kg), on the other hand, more strongly inhibited the norepinephrine-induced pressor response in both α_{1D}^{+/+} and α_{1D}^{-/-} mice (Figure 4).

Cardiac function. The cardiac output (CO) was similar in the α_{1D}^{+/+} and control mice (18.6 ± 1.6 ml/min, n = 15, and 16.3 ± 1.2 ml/min, n = 18) in the α_{1D}^{-/-} and α_{1D}^{+/+}, respectively. Myocardial contractility monitored with %FS also showed no significant difference between the α_{1D}^{+/+} and α_{1D}^{-/-} mice (2.1% ± 2.1% in α_{1D}^{+/+}, n = 15, and 49.5% ± 49.7% in α_{1D}^{-/-}, n = 18, in α_{1D}^{+/+} and α_{1D}^{-/-}, respectively). The left ventricular wall thickness measured at the IVS and PW was similar in the two groups (data not shown). During echocardiography, HRs were similar in the two groups (501 ± 27 bpm, n = 15, and 522 ± 23 bpm, n = 18, in α_{1D}^{+/+} and α_{1D}^{-/-}, respectively).

Vascular contraction. To assess whether α_{1D}-AR was directly involved in vascular smooth muscle contraction, we measured the effect of norepinephrine and phenylephrine on the contraction of isolated aortic segments from male α_{1D}^{+/+} and α_{1D}^{-/-} mice. As shown in Figure 5a, norepinephrine induced concentration-dependent contractile responses in aortic segments from α_{1D}^{+/+} and α_{1D}^{-/-} mice. However, the potency of the response was markedly reduced in aortic segments from α_{1D}^{-/-} as compared with α_{1D}^{+/+} mice (Figure 5a). A similar decrease in potency was also observed with phenylenephrine-induced contractions (Figure 5b). The E_C50 values of norepinephrine and phenylephrine were increased approximately 50- and 40-fold in α_{1D}^{-/-} mice compared with α_{1D}^{+/+} mice (50% effective dose [E_C50] val-
ues: 3.8 ± 0.5 nM, n = 26, in α₁D+/− mice, and 190 ± 40 nM, n = 15, in α₁D+/− mice for norepinephrine; 20.0 ± 2.0 nM, n = 27, in α₁D+/− mice, and 840 ± 40 nM, n = 15, in α₁D+/− mice for phenylephrine, respectively). The contractile response induced by serotonin was not decreased. Rather, the concentration-response curve of serotonin-induced contraction was slightly shifted to the left in α₁D+/− mice compared with α₁D+/− mice (EC₅₀ values: 28.0 ± 2.1 nM in α₁D+/− mice and 12.1 ± 2.4 nM in α₁D+/− mice, n = 15 each) (Figure 5c).

The contractile response induced by norepinephrine was competitively antagonized by BMY7378 in α₁D+/− mice (Figure 5d), but only to a small extent in α₁D+/− mice (Figure 5e). Competitive antagonism was shown by Schild analysis in which the negative logarithms of the dissociation constant (pA₂) value was 8.61 ± 0.2 and the slope was 1.06 ± 0.11 (n = 6) for BMY7378 in α₁D+/− mice. This pA₂ value was in good agreement with Kᵩ values (~1 nM) obtained in binding studies with the cloned mouse α₁D-AR and aorta.

The contractile response to α₁-AR in aorta was observed to be reduced in α₁D+/− mice, clearly showing that α₁D-AR mediate aortic contraction; however, aorta is a conduit artery that may not directly control blood pressure. Hence, we further examined the α₁-AR–mediated vascular response in the resistance arteries of mesenteric arterial beds. As shown in Figure 5f, the pressor response of isolated perfused mesenteric arterial beds to phenylephrine was significantly attenuated in α₁D+/− compared with α₁D+/− mice.

**Discussion**

Using gene targeting to create a mouse model lacking the α₁D-AR, we investigated the functional role of the α₁D-AR subtype in the cardiovascular system. By RT-PCR and radioligand binding studies, we confirmed a loss of α₁D-AR expression in α₁D+/− mice and observed little apparent compensatory upregulation of the other subtypes. The α₁D−/− mice showed a modest hypotension under unanesthetized conditions without a notable increase in heart rate. Also, there was no significant alteration in ventricular function or in the circulating catecholamine levels between the α₁D+/− and α₁D+/− mice. Consistent with the loss of α₁D-AR expression, α₁D+/− mice showed reduced pressor responses to α₁-AR stimulation, and the contractile responses of the aorta and mesenteric arterial beds to α₁-agonists were markedly suppressed. The present study provides clear evidence that the α₁D-AR mediates a pressor response to catecholamines by directly regulating vasoconstriction.

Our study showed that the α₁D-AR regulates not only the vasopressor response to α₁-AR stimulation, but also the resting blood pressure. Conscious α₁D+/− mice showed a slight but significant decrease in the resting blood pressure measured by the tail-cuff method as well as by direct intra-arterial measurement. Because cardiac outputs assessed by echocardiogram were similar between the α₁D+/− and α₁D+/− mice, the modest hypotension observed in α₁D+/− mice is considered to be mainly due to the reduction in total peripheral resistance. However, an increase in heart rate, an expected compensatory response to a low blood pressure, was not observed in α₁D+/− mice. The mechanism for the lack of reflex tachycardia in α₁D+/− mice cannot be fully explained from the present study, but interestingly, chronic administration of α₁-AR blocking drugs has been reported to lower blood pressure without causing reflex tachycardia in patients with essential hypertension (32).

Although in vitro as well as in vivo pharmacological studies (33–36) have implicated a predominant role for α₁D-AR in the vascular contractions caused by α₁-AR agonists, our present study clearly shows, we believe for the first time, that α₁D-ARs directly mediate α₁-AR-stimulated vascular smooth muscle contraction. As shown in RT-PCR and radioligand binding studies, murine aorta predominantly expresses the α₁D-AR. Corresponding to the loss of α₁D-AR expression, we observed a marked reduction of α₁D-stimulated aortic contractile response in α₁D+/− mice, showing that α₁D-ARs are predominantly responsible for α₁D-stimulated aortic contraction. This observation obtained in a conduit artery of aorta, however, may not be directly extrapolated to the resistance vessels in general, because many studies have shown that the dominant contractile α₁-AR varies with vascular bed type (37–40). Hence, we further examined the α₁-AR–mediated vascular response in the resistance artery that more directly controls blood pressure and observed a significant reduced α₁-AR–stimulated pressor response in isolated perfused mesenteric arterial preparation of α₁D+/− mice. The results, therefore, show that the α₁D-AR contributes to the regulation of not only the conduit-type vasculatures (such as the aorta), but also the muscular-type resistance vessels, which are more responsible for pressor reactivity. Taken together, our functional examinations in α₁D+/− mice show that the α₁D-AR plays a significant role in direct regulation of peripheral vascular tone. However, pressor response experiments in α₁D+/− mice did not completely exclude the possibility that subtypes other than α₁D-AR are involved in α₁-AR-stimulated pressor response. In fact, the dose-pressor response for phenylephrine (Figure 3a) showed that the pressor responses in α₁D+/− mice were significantly reduced only at the midrange doses, and the pressor responses at maximum doses were comparable to control. These data may indicate not only that α₁D-AR is involved in vasopressor response to phenylephrine, but also that other α₁-AR subtypes are involved in vasopressor response.

Our α₁D+/− mice showed little adrenergic compensatory effect on α₁D-AR at the cardiovascular level. The TaqMan assay and binding study in α₁D+/− mice showed that other α₁-AR subtypes are apparently not upregulated to compensate for the loss of α₁D-AR. Furthermore, comparison of the inhibitory effects of the non-selective α₁-antagonist bunosin and the α₁D-AR-
selective antagonist BMY7378 on norepinephrine-induced pressor responses in \( \alpha_{1D}^{+/+} \) and \( \alpha_{1D}^{-/-} \) mice showed little adrenergic compensatory effect; rather, it suggested the contribution of other subtype(s) to the \( \alpha_1 \)-AR–mediated pressor response. The results clearly show that despite the presence of multiple \( \alpha_1 \)-AR subtypes in the same tissue, at best there is only partial functional redundancy in cardiovascular tissue (i.e., multiple \( \alpha_1 \)-AR subtypes can mediate the vasopressor response, but cannot compensate for each other). A similar observation regarding functional redundancy has been made in a study of \( \alpha_{1B} \)-AR knockout mice (16). Together with previous observations in \( \alpha_{1B} \)-AR knockout mice (16), our study supports the idea that \( \alpha_{1B} \) as well as \( \alpha_{1D} \)-ARs contribute to the \( \alpha_1 \)-AR–mediated pressor response. Moreover, together with information on tissue distribution and from observations in \( \alpha_{1A} \)-AR knockout mice (16), our present study would provide further important definition of the role(s) of each \( \alpha_1 \)-AR subtype in the cardiovascular system. Thus, \( \alpha_{1A} \)-ARs may have a specific effect on the vascular system, while having little effect on the heart. The \( \alpha_{1B} \)-AR subtype may be linked to both cardiac and vascular effects (16, 41, 42). The \( \alpha_{1A} \)-AR, however, may regulate cardiac function, since \( \alpha_{1A} \)-AR knockout mice mainly display impaired cardiac function (43). These findings are of particular importance for better understanding of the cardiovascular effects of drugs acting at the \( \alpha_1 \)-AR and for more precisely defining goals linked to the development of \( \alpha_1 \)-AR subtype–selective ligands.

Because \( \alpha_1 \)-AR subtype expression is known to be markedly varied depending on vessel type and species, one must be careful in directly extrapolating findings obtained from knockout mice to human vascular \( \alpha_1 \)-AR physiology. At present, the distribution of \( \alpha_1 \)-AR subtypes in blood vessels is relatively well characterized at mRNA or protein levels, but the available information regarding \( \alpha_1 \)-AR subtypes mediating vasoconstriction is still very scarce in humans (44). Furthermore, in mice, little is known about either the distribution or function of \( \alpha_1 \)-AR subtypes in blood vessels. Studies have been hampered both by the lack of drugs sufficiently selective for the three subtypes and by cross-reactivity of \( \alpha_1 \)-AR ligands with other receptors. In fact, the \( \alpha_{1D} \)-AR subtype–selective antagonist BMY7378 used in the present study is known to have a broader pharmacological profile (also acting as a 5-HT1A receptor partial agonist). Using \( \alpha_{1D}^{-/-} \) mice, however, we showed that BMY7378 is selective for \( \alpha_{1D} \)-AR–mediated function. As exemplified in the present study, the knockout mice of each \( \alpha_1 \)-AR subtype would be of use in developing and evaluating subtype-selective pharmacological agents.

In conclusion, our knockout mouse study has demonstrated the physiological role of \( \alpha_{1D} \)-AR in the cardiovascular system; thus, the \( \alpha_{1D} \)-AR mediates the pressor response to catecholamines by directly regulating vasoconstriction. Enhanced activity of the \( \alpha_{1D} \)-AR has been suggested to be involved in the pathogenesis and/or maintenance of hypertension (45–47) and age-related changes in vascular responsiveness (48) and also other physiological effects, such as vascular smooth muscle cell growth and hypertrophy (49, 50). The \( \alpha_{1D} \)-AR knockout mice would be of value in studying mechanisms involved in the control of vascular physiology and its dysregulation. \( \alpha_1 \)-AR subtype knockout mice (single, double, and triple knockouts) should constitute useful models to clarify the functional specificity of each \( \alpha_1 \)-AR subtype and provide a valuable experimental platform for assessing and developing new therapeutic agents.

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