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Two α_1-adrenergic receptor subtypes regulating the vasopressor response have differential roles in blood pressure regulation

Chihiro Hosoda*, Taka-aki Koshimizu*, Akito Tanoue, Yoshihisa Nasa, Ryo Oikawa, Takashi Tomabechi, Shinya Fukuda, Hitomi Shinoura, Sayuri Oshikawa, Satoshi Takeo, Tadaichi Kitamura, Susanna Cotecchia, and Gozoh Tsujimoto

Department of Molecular, Cell Pharmacology, National Research Institute for Child Health and Development, 3-35-31, Taishido, Setagaya-ku, Tokyo, 154-8567, Japan (C. H., A. T., T-a. K., H. S., and S. O.)

Department of Pharmacology, Tokyo University of Pharmacy and Life Science, 1432-1, Horinouchi, Hachioji, Tokyo, 192-0392, Japan (Y. N., R. O., T. T., S. F, and S. T.)

Department of Urology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan (C. H., and T. K.)

Institut de Pharmacologie et Toxicologie, Universit de Lausanne, 1005 Lausanne, Switzerland (S. C.)

Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan (G. T.)

*; These authors equally contributed to this work.

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Corresponding author: Gozoh Tsujimoto, M.D., Ph. D.

Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

Tel: ++81-3-3419-2476, Fax: ++81-3-3419-1252, E-mail: gtsujimoto@nch.go.jp

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Abstract

To study the functional role of individual α_1-AR subtypes in blood pressure (BP) regulation, we used mice lacking the α_{1B}-AR and/or α_{1D}-AR with the same genetic background, and further studied their hemodynamic and vasocontractile responses. Both the α_{1D}-AR knockout and α_{1B}-/α_{1D}-AR double knockout, but not the α_{1B}-AR knockout mice, had significantly ($p < 0.05$) lower levels of basal systolic and mean arterial BP than wild type mice in non-anesthetized condition and they showed no significant change in heart rate or in cardiac function, as assessed by echocardiogram. All mutants showed a significantly ($p < 0.05$) reduced catecholamine-induced pressor and vasoconstriction responses. Notably, the infusion of norepinephrine did not elicit any pressor response at all in α_{1B}-/α_{1D}-AR double knockout mice. In an attempt to further examine α_1-AR subtype, which is involved in the genesis or maintenance of hypertension, BP after salt loading was monitored by tail-cuff readings and confirmed at the end point by direct intra-arterial recording. After salt-loading, α_{1B}-AR knockout mice developed a comparable level of hypertension to wild type mice, while mice lacking α_{1D}-AR had significantly ($p < 0.05$) attenuated BP and lower levels of circulating catecholamines. Our data indicated that α_{1B}- and α_{1D}-AR subtypes participate co-operatively in BP regulation; however, the deletion of the functional α_{1D}-AR, not α_{1B}-AR, leads to an anti-hypertensive effect. The study shows differential contributions of α_{1B}- and α_{1D}-ARs in BP regulation.

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Introduction

Catecholamines released from sympathetic nerve terminals cause vascular smooth muscle contraction primarily by activating α_1-adrenergic receptors (α_1-ARs) in arteries (Hoffman, 2001). Thus, blockade of α_1-AR leads to a fall in peripheral vascular resistance. Because of their consistent effect in lowering systemic blood pressure (BP), α_1-AR blockers have been widely used as an anti-hypertensive drug. However, a large clinical trial unexpectedly disclosed that doxazosin, a nonselective α_1-AR antagonist, was associated with an increased incidence of heart failure (ALLHAT Collaborative Research Group, 2000). This raised a serious concern about the long-term use of α_1-AR antagonists in the treatment of hypertension (HT) (ALLHAT Collaborative Research Group, 2000). On the other hand, clinical efficacy of a subtype-selective inhibition of α_1-AR has not been fully determined.

The three known α_1-AR subtypes (α_{1A}-, α_{1B}- and α_{1D}-AR) participate in the constellation of α_1-adrenergic activities in the cardiovascular system (Hieble et al., 1995). All of these receptors are coupled to Ca^{2+} signaling, leading to smooth muscle contraction (Esbenshade et al., 1995; Hieble et al., 1995). Elucidation of the physiological and pathophysiological roles of the specific α_1-AR subtype has been hampered, because the α_1-AR subtypes are co-expressed in the same arterial smooth muscles with different ratios (Michelotti et al., 2000; Piascik and Perez, 2001), and that sufficiently subtype-selective agonists and antagonists have not been available (Esbenshade et al., 1995; Guimaraes and Moura, 2001; Piascik and Perez, 2001). In addition, recent biochemical and pharmacological studies confirmed the potential role of dimerization of distinct α_1-AR subtypes in controlling their expression and pharmacological properties (Stanasila et al., 2003; Uberti et al., 2003). Therefore, complex interactions of subtypes could be expected, when subtypes are co-expressed in a same smooth muscle cell.

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In this study, we used three mice groups specifically lacking the α_{1B}-AR and/or α_{1D}-AR subtypes ($\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$, and $\alpha_{1BD}^{-/-}$) to examine the individual and cooperative roles of these receptor subtypes in BP regulation, both under basal conditions and in the development of salt-induced experimental HT. In addition, responsiveness to intravenous infusion of catecholamines was compared by direct measurement of arterial pressure. Our data showed that α_{1B}- and α_{1D}-ARs play distinctive contributions to the resting and agonist-stimulated BP regulations, particularly to the progression of hypertensive state.

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Materials and Methods

Generation of mice lacking both the α_{1B}-AR and α_{1D}-AR subtypes. $\alpha_{1B}^{-/-}$and $\alpha_{1D}^{-/-}$ mice were previously generated and characterized (Cavalli et al., 1997; Tanoue et al., 2002b). Disruption of the α_{1B}- or α_{1D}-AR gene was achieved using a positive-negative selection strategy to effect homologous recombination in embryonic stem cells, using the targeting construct. The strain background of both $\alpha_{1B}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice was a mixture of 129Sv and C57Bl6/J. Double knockout $\alpha_{1BD}^{-/-}$ mice were generated by mixture of homozygous $\alpha_{1B}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice. The resulting F1 generation of compound heterozygotes was subsequently intercrossed to generate F2 mice with all possible combinations of α_{1B}- and α_{1D}-AR gene disruptions. Mice were genotyped for both α_{1B}- and α_{1D}-AR disruptions by Southern blotting or PCR of mouse tail biopsies (Cavalli et al., 1997; Tanoue et al., 2002b). According to Mendelian's law, 1/16 of progeny were predicted to be homozygous-deficient for α_{1B}- and α_{1D}-AR, and 1/16 of progeny were predicted to be wild type (WT) for both α_{1B}- and α_{1D}-AR. The F2 double knockout $\alpha_{1BD}^{-/-}$ mice were bred to produce double knockout mice used in our experiments. The wild type F2 mice were bred to produce WT controls. Thus, the overall strain contributions in the WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice were equivalent. Animals were housed in micro-isolator cages in a pathogen-free barrier facility. All experimentation was performed under approved Institutional Guidelines. All mice used in this study were 7-9 weeks old male ones.

RT-PCR analysis. Total RNA from different mouse tissues was prepared using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan). Total RNA of 5 μg was treated with RNase-free DNase (TaKaRa Bio Co., Tokyo, Japan) and reverse-transcribed using random hexamers, (Tanoue et al., 1990). One-tenth of each cDNA sample was amplified by PCR with a receptor-specific primer set and a

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primer set specific for 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 Bio 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 AGGCTGCTCAAGTTTTCTCG and CAGATTGGTCCTTTGGCACT for α_{1A}-AR (275 bp), GGGAGAGTTGAAAGATGCCA and TTGGTACTGCTGAGGGTGTC for α_{1B}-AR (752 bp), and CGCTGTGGTGGGAACCGGCAG and ACAGCTGCACTCAGTAGCAGGTCA for α_{1D}-AR (282 bp). The upstream primer for the α_{1A}-AR or the α_{1B}-AR gene was located within the first exon, and the downstream primer for the α_{1A}-AR or the α_{1B}-AR gene was located within the second exon. The primers for the α_{1D}-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 α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR sequences (Alonso-Llamazares et al., 1995). The GAPDH primers (5'→3') were GGTCATCATCTCCGCCCCTTC upstream and CCACCACCCTGTTGCTGTAG 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 ^{32}P-labeled probes (Alonso-Llamazares et al., 1995).

Radioligand ligand binding study. Radioligand binding studies were performed on membrane preparations of mouse native tissues (Shibata et al., 1995). Briefly, whole brain, heart, liver,

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kidney and aorta were dissected from mice, placed in a lysis buffer A (250 mM sucrose, 5 mM Tris-HCl and 1 mM $MgCl_2$, pH 7.4), and homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) at 4°C, at speed 7 for 10 sec. The homogenate was then centrifuged at 1,000 g at 4°C for 10 min to remove the nuclei. The supernatant fraction was centrifuged at 35,000 g for 20 min at 4°C. The resulting pellet was re-suspended in binding buffer B (50 mM Tris-HCl, 10 mM $MgCl_2$ and 10 mM EGTA, pH 7.4) and was frozen at -80°C until assay. Protein concentration was measured using the bicinchoninic acid protein assay kit (PIERCE, Rockford, IL, USA). Radioligand binding studies were performed using ^{125}I-(2-β-(4-hydroxyphenyl)-ethylaminomethyl)-tetralone ([^{125}I]-HEAT; 2,200 Ci/mmol; NEN, Boston, MA, USA) or [7-methoxy-^{3}H]-prazosin ([^{3}H]-prazosin; 83.8 Ci/mmol; NEN). Briefly, 20 - 100 μg of membrane protein from brain, aorta and heart were incubated with [^{125}I]-HEAT and membranes from liver and kidney was incubated with [^{3}H]-prazosin in a final volume of 250 μl binding buffer B in the presence or absence of competing drugs for 60 min at 25°C. The incubation was terminated by addition of the ice-cold buffer B and immediate filtration through Whatmann GF/C glass fiber filters with a Brandel Cell Harvester (Model-30; Brandel, Gaithersburg, MD, USA). Each filter was collected and the radioactivity was measured. Binding assays were always performed in duplicate. For competition curve analysis, each assay contained about 100 pM [^{125}I]-HEAT or [^{3}H]-prazosin. Nonspecific binding was defined as binding displaced by phentolamine (10 μM).

Measurement of BP and HR. Systolic BP (SBP) and heart rate (HR) were measured in conscious mice with a computerized tail-cuff system (BA-98A system; Softron Co., Tokyo, Japan) that determines SBP using a photoelectric sensor (Tanoue et al., 2002b). Before the study

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was initiated, at least 3 days of training sessions were provided for the mice to become accustomed to the tail-cuff procedure. Sessions of recorded measurements were then made form 1 to 5 PM daily on 3 consecutive days. Each session included more than ten tail-cuff measurements. Mean arterial pressure (MAP) and HR were also measured in non-anesthetized mice by an intra-arterial catheter (Tanoue et al., 2002b). After cervical incision on mice anesthetized with sodium pentobarbital (40 mg/kg, i.p.), a stretched intramedic PE10 polyethylene catheter (Clay Adams, Parsippany, NJ, 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 of 24 hour recovery, mice were placed in Plexiglas tubes to partially restrict their movements, and the saline-filled catheter was removed from the pouch and connected to a pressure transducer (model DX-360; Nihonkohden, Tokyo, Japan) and MAP was recorded on a thermal pen recorder (model RTA-1200; Nihonkohden). Measurement of HR was triggered from changes in MAP (model AT-601G; Nihonkohden). In order to examine pressor responses in non-anesthetized mice, drugs in ~30 μl of injection volume (1 μl/g of mouse body weight) were administered through catheter inserted into the right femoral vein as a bolus at 15-20 min intervals after ensuring MAP and HR had returned to baseline levels.

The effect of α_1-antagonists on the norepinephrine-induced pressor response was examined in each mouse group. Following propranolol (1mg/kg) treatment, either bunazosin hydrochloride (10 μg/kg, i.v.; Eisai Co. Ltd., Tokyo, Japan) or BMY7378 (100 μg/kg, i.v.; Research Biochemicals Inc, Natick, MA, USA) was administered 10 min prior to the continuous infusion of norepinephrine (1 μg/kg/min i.v. for 5 min) using a micro-syringe pump (CFV-2100; Nihonkohden).

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Measurement of blood chemistries. After 1 hour of stable anesthesia (80mg/kg pentobarbital, intraperitoneally), blood was drawn slowly from the right carotid arterial line to measure total plasma catecholamine levels (epinephrine, norepinephrine and dopamine), angiotensin I and II levels, creatinine levels, blood cell counts, hematocrit and serum electrolytes. Plasma catecholamine levels were determined by high pressure liquid chromatography using commercially available reagents (Tosho Co., Tokyo, Japan). Plasma angiotensin I and II were measured with a radio-immunoassay kit (PerkinElmer ^{TM} Life Science; Wellesley, MA, USA) and plasma creatinine by a colorimetric kit (Sigma Diagnostics, St. Louis, MO, USA).

Measurement of aortic contraction. The thoracic aorta was prepared for aortic contractile responses to drugs as described (Tanoue et al., 2002b). Briefly, the excised thoracic aorta was cleaned and cut into 1 mm 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% CO_2 and 95%O_2 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 min under a resting tension of 0.5 g, and the buffer was replaced every 15 min. 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 (40 mM)-induced aortic contraction of mice weighing 20-23g. 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 mesenteric arterial beds were

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prepared to measure the perfusion pressure (Nasa et al., 1998). The superior mesenteric artery of diethylether-anesthetized mice was dissected and a stainless-steel cannula (27G syringe) was inserted. The preparations were perfused with Krebs-Henseleit solution equilibrated with a mixture of 95% O_2 and 5% CO_2 (PO_2 > 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 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; Nihonkohden) connected to a carrier amplifier (AP-621G; Nihonkohden) and recorded on a thermal pen recorder (WT-645G; Nihonkohden). The preparations were equilibrated for 30 min before administration of phenylephrine.

Histological analysis of heart and thoracic aorta. Heart to body weight ratios were calculated as mg/g. For histological analysis, heart and thoracic aorta were fixed with perfusion of 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.

Echocardiography. Quantitative echocardiographic measurements were performed on lightly anesthetized, spontaneously breathing mice (Tanoue et al., 2002b). Mice were anesthetized (40 mg/kg pentobarbital, i. p.), and the chest area was shaved, and ultrasonic gel was applied. The measurements with the SONOS-5500 system (Philipus Medical Systems, Andover, MA, 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 quantitative analysis, measurements were performed in three

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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 HR. LVIDd, and LVIDs were normalized to body weight and percent fractional shortening (%FS) was calculated as 100 x [(LVIDd - LVIDs)/LVIDd]. Cardiac output (CO) was calculated from doppler echocardiography using the following equation, $[\pi \times (Ao)^2 \times VTI \times HR]/4$, 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 electrocardiogram.

Nephrectomy and salt-induced HT. Mice, weighing 18 to 23 g, were subjected to two steps of nephrectomy protocol (Johns et al., 1996). Briefly, both poles of the left kidney were excised under anesthesia with intraperitoneal sodium pentobarbital (50mg/kg), leaving a small amount of residual renal tissue around the hilum and preserving the ureter and hilar vessels. The excised renal tissues were weighed, and the ratios of those organs to the body weights were calculated. After a seven-day recovery period, the right kidney was removed, leaving 25% of the total renal mass. Twenty-four hours after the second operation, the animals were maintained with 1% saline as drinking water for 35 days. SBP and HR were monitored by tail-cuff system as described above. At the end point, SBP and HR were recorded for three consecutive days and averaged. HT was defined as follows: tail-cuff SBP that reached 150mmHg, or an increase of > 40mmHg above the baseline.

Data Analysis. All values are expressed as means ± SEM. Differences among each group of mice were assessed by ANOVA with subsequent Bonferroni post hoc test for multiple

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comparisons. Data from the radioligand binding study were analyzed using the iterative non-linear regression program, LIGAND (Munson and Rodbard, 1980). The presence of one, two or three different binding sites was assessed using the *F*-test in the program. Cumulative survival curves were constructed by the Kaplan-Meier method (Kaplan and Meier, 1958), and differences between the curves were tested for significance using the log-rank statistic. Statistical significance was established at a value of $p < 0.05$. Apparent pD_2 value, agonist dose or concentration that gives half-maximal response, was calculated from dose-response or concentration-response curves constructed from experiments. Difference among the concentration-response curves was evaluated by two-way ANOVA, if applicable.

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Results

The $\alpha_{1B}^{-/-}$ and $\alpha_{1D}^{-/-}$ single knockout mice, which have the same genetic background, were crossed to produce the $\alpha_{1BD}^{-/-}$ double knockout mice. The $\alpha_{1BD}^{-/-}$ mice were viable at the expected Mendelian ratios from heterozygote intercrosses. They developed normally and showed no gross abnormalities. Analysis of venous blood samples from these mutant mice showed no significant difference in the following parameters: plasma creatinine, plasma catecholamine, epinephrine, norepinephrine, angiotensin I and II levels (baseline values in Table 1), blood cell counts, hematocrit and serum electrolytes (data not shown).

Expression of α_1-AR in $\alpha_{1BD}^{-/-}$ mice — We confirmed the lack of α_{1B}-AR and α_{1D}-AR expression in the mutant mice by RT-PCR and by radioligand binding studies. RT-PCR analysis showed that the $\alpha_{1BD}^{-/-}$ mice expressed neither α_{1B}-AR nor α_{1D}-AR mRNA in any tissue examined (brain, heart, aorta, kidney and liver; data not shown), and had no apparent compensatory up-regulation of α_{1A}-AR mRNA. Corresponding well with the RT-PCR results, radioligand binding studies showed a decreased α_1-AR binding capacity in the brain, heart and kidney of knockout mice (Table 2A). α_{1B}-AR and α_{1D}-AR are predominant α_1-AR in the liver and aorta, respectively (Cavalli et al., 1997; Tanoue et al., 2002b) and α_1-AR ligand-binding capacities in the liver and aorta of $\alpha_{1BD}^{-/-}$ mice were markedly diminished (Table 2A), indicating that no significant compensatory increase of α_1-AR binding site in these mice. In accordance with data of saturation binding experiments, competition binding experiments using the α_{1A}-AR-selective antagonist KMD-3213 (Shibata et al., 1995) showed that only high-affinity binding site for KMD-3213 was detected in the brain of $\alpha_{1BD}^{-/-}$ mice (K_H = 0.26 nM), whereas two sites were detected in WT, $\alpha_{1B}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice (Table 2B).

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Hemodynamic parameters — We measured systemic BP in the series of mutant mice to delineate consequences of deleting two α_1-AR subtypes. When resting SBP of conscious mice was monitored by tail-cuff, the mean SBP values were significantly low in two mouse groups deleted with α_{1D}-AR gene, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$, compared to those of WT and $\alpha_{1B}^{-/-}$ mice (99 ± 2 mmHg for WT, n=10; 99 ± 3 mmHg for $\alpha_{1B}^{-/-}$, n=9; 93 ± 2 mmHg for $\alpha_{1D}^{-/-}$, n=9; 92 ± 2 mmHg for $\alpha_{1BD}^{-/-}$, n=9, $p < 0.05$). These differences were also confirmed by direct pressure measurement by intra-arterial catheter (Table 3A). Averaged HR during the BP monitoring were a similar level in all mice groups in tail-cuff recording (542 ± 16 bpm for WT, n=10; 520 ± 20 bpm for $\alpha_{1B}^{-/-}$ mice, n=9; 533 ± 17 bpm for $\alpha_{1D}^{-/-}$ mice, n=9; 516 ± 28 bpm for $\alpha_{1BD}^{-/-}$ mice, n=9) and in direct intra-arterial recording (Table 3A).

We next examined the pressor responses to several vasoactive agents in non-anesthetized mice. As α_1-AR agonists, we administered phenylephrine (0.1-300 μg/kg), norepinephrine (0.1-10.0 μg/kg) or the α_{1A} selective agonist, A61603 (0.01-3.0 μg/kg). The analysis showed a significant difference in the pressor response curves to phenylephrine between WT and $\alpha_{1BD}^{-/-}$ mice (Figure 1A). The pD_2 values for phenylephrine in WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice were 9 ± 2, 10 ± 2, 12 ±1 and 19 ± 3 μg/kg (n=15-22), respectively. The responses to norepinephrine of each mutant mouse were also significantly less than that of WT (Figure 1B). The maximal plateau level of pressor responses to norepinephrine could not be monitored, because high doses of norepinephrine frequently caused circulatory collapse due to its cardiac toxicity. Unlike phenylephrine or norepinephrine, WT and $\alpha_{1BD}^{-/-}$ mice showed similar pressor response to A61603 infusion (Figure 1C). None of the mutant mice exhibited a significant alteration in pressor responses to non-adrenergic vasoactive stimuli, such as angiotensin II or vasopressin.

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Increases in BP by intravenous administration of angiotensin II (100 ng/kg) were 31.4 ± 2.8, 33.4 ± 2.4, 30.8 ± 3.6, and 33.4 ± 2.4 mmHg in WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ (n=6-11), respectively. Vasopressin (100 ng/kg)-induced BP increases were 12 ± 0.6, 19.5 ± 3.2, 23.8 ± 8.7, and 22.4 mmHg in WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ (n=4-9), respectively.

We further assessed the contribution of each subtype to the α_1-AR-mediated pressor response. Continuous infusion of norepinephrine (1 μg/kg/min, for 5 min) promptly induced a significant increase in BP (23 ± 2 mmHg, n=16), which lasted during the administration of norepinephrine in WT mice (Figure 1D). This BP increase was suppressed partly by 100 μg/kg BMY7378, an α_{1D}-selective antagonist (Goetz et al., 1995), and almost lost by 10 μg/kg of bunazosin, a nonselective α_1-AR antagonist (Takeo et al., 1988). In $\alpha_{1B}^{-/-}$ mice, the norepinephrine-induced increase in MAP was similar to that of WT (21 ± 2 mmHg, n=16), and was inhibited by pretreatment with either BMY7378 or bunazosin (Figure 1E). In $\alpha_{1D}^{-/-}$ mice, the norepinephrine-induced MAP increase was significantly less than that of WT mice (19 ± 1 mmHg, n=12, $p < 0.05$). BMY7378 pretreatment to $\alpha_{1D}^{-/-}$ had no inhibitory effect, whereas bunazosin almost completely inhibited the pressor response (Figure 1F). In $\alpha_{1BD}^{-/-}$ mice, the infusion of norepinephrine at the same rate of 1 μg/kg/min did not elicit any increase in BP (n=14, Figure 1G).

Vascular responsiveness of mutant mice – We measured contractile forces of isolated aortic segment induced by α_1-AR agonists. Norepinephrine and phenylephrine induced concentration-dependent contractile responses in thoracic aortic segments from WT, $\alpha_{1B}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice; however, the potency of norepinephrine was slightly reduced in $\alpha_{1B}^{-/-}$ mice, and the reduction was more pronounced in $\alpha_{1D}^{-/-}$ mice (Figure 2A and 2B). The pD_2 values for

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norepinephrine-induced contraction in WT, $\alpha_{1B}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice were 3.8 ± 0.5 nM, 5.3 ± 0.5 nM, and 190 ± 40 nM (n=10-16), respectively, and corresponding values for phenylephrine were 20 ± 2 nM, 70 ± 10 nM, and 840 ± 40 nM (n=10-16) for WT, $\alpha_{1B}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice, respectively. In contrast, the contractile response was apparently lost in $\alpha_{1BD}^{-/-}$ mice (Figure 2A and 2B). All mice had same levels of response to serotonin stimuli (Figure 2C, n=10-16).

We next examined perfusion pressure of mesenteric arterial beds isolated from WT and mutant mice. The increase in the pressure to phenylephrine stimulation was significantly attenuated in $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice as compared with WT and $\alpha_{1B}^{-/-}$ mice (Figure 2D).

Cardiac functions – The cardiac output of WT and mutant mouse groups were similar level (Table 4). Vascular resistances for the systemic vascular beds were calculated from cardiac output and MAP, and the calculated values were significantly decreased in $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice. Vascular resistances were (in mmHg/litter/min) 8845 ± 3191, 7754 ± 2934, 7240 ± 1842, and 6782 ± 1983, for WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$, and $\alpha_{1BD}^{-/-}$, respectively. Myocardial contractility was monitored with either fractional shortening (FS) or ejection fraction (EF), and was significantly lower in $\alpha_{1B}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice than in WT and $\alpha_{1D}^{-/-}$ mice (Table 4). The left ventricular wall thickness, measured at the interventricular septum and posterior wall by echocardiogram, was comparable in all groups of mice (data not shown). The heart-weight/body-weight ratio did not significantly differ among the groups of mice (Table 1). Also, there were no obvious differences among the groups of mice with respect to gross morphology or microscopic myocyte appearance of the hearts and aorta (data not shown).

Nephrectomy and Salt-induced hypertension model — During subtotal nephrectomy and 1%

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saline loading, six of the 16 WT, six of the 15 $\alpha_{1B}^{-/-}$, seven of the 16 $\alpha_{1D}^{-/-}$, and six of the 15 $\alpha_{1BD}^{-/-}$ died with general edema within 6-8 days without an appreciable change in BP, and 9 to 10 surviving mice in each group were used for analysis. Hence, the Kaplan-Meier analysis at the 35th day showed no significant effect of the α_{1B} gene and α_{1D} gene ablation on cumulative survival (data not shown). Besides, no significant difference was observed with respect to the following parameters: plasma creatinine levels, ratios of residual kidney weight to body weight, blood cell counts, hematocrit and serum electrolytes (data not shown). Plasma creatinine levels and heart weight to body weight ratios significantly ($p < 0.05$) increased at the endpoint, and angiotensin I and II levels significantly ($p < 0.05$) decreased in all four groups, as compared with those at baseline (Table 1). However, these endpoint data, except those of plasma catecholamines, did not show any significant differences among the groups of mice (Table 1). Although plasma catecholamine levels at baseline were not significantly different, at the endpoint plasma norepinephrine, dopamine and total catecholamine levels of the WT and $\alpha_{1B}^{-/-}$ mice were significantly higher than those of $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice (Table 1).

Figure 3 shows the time course of SBP and HR changes during the 1% saline drinking period, as measured by tail cuff monitoring. The baseline SBP values of WT and $\alpha_{1B}^{-/-}$ mice were significantly higher than those of $\alpha_{1D}^{-/-}$ or $\alpha_{1BD}^{-/-}$ mice, whereas there was no significant difference in HR among the groups of mice. After three weeks from the beginning of salt loading, the WT and $\alpha_{1B}^{-/-}$ mice showed significantly higher SBP than the $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice (Figure 3A). The endpoint SBP values were (in mmHg) 144 ± 3, 141 ± 4, 124 ± 4 and 120 ± 4 for WT (n=10), $\alpha_{1B}^{-/-}$ (n=9), $\alpha_{1D}^{-/-}$ (n=9), and $\alpha_{1BD}^{-/-}$ (n=9), respectively (Figure 3A). Eight of ten surviving WT and seven of nine surviving $\alpha_{1B}^{-/-}$ mice satisfied the HT criteria, as defined in the Methods section; however, only two of the nine surviving $\alpha_{1D}^{-/-}$ mice and two of the nine surviving $\alpha_{1BD}^{-/-}$ mice satisfied the

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criteria. The endpoint HR by tail-cuff recording were 596 ± 13 bpm for WT mice (n=10), 567 ± 30 bpm for $\alpha_{1B}^{-/-}$ mice (n=9), 584 ± 25 bpm for $\alpha_{1D}^{-/-}$ mice (n=9), 583 ± 28 bpm for $\alpha_{1BD}^{-/-}$ mice (n=9), respectively (Figure 3B). Unlike the SBP response, the HR change did not differ significantly among the groups at any time point during salt loading (Figure 3B). At the endpoint, MAP and HR were confirmed directly under non-anesthetized conditions (Table 3B). Consistent with the tail-cuff SBP measurements, the endpoint direct intra-arterial MAP of $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice was significantly ($p < 0.05$) lower than that of WT and $\alpha_{1B}^{-/-}$ mice (Table 3B). No significant difference was observed in direct intra-arterial HR among the groups of mice at the endpoint (Table 3B).

Vascular contraction of the HT model mice – To assess whether the salt-induced HT procedure caused altered catecholamine sensitivity in the vasculature, we examined the pressure responses to phenylephrine in the perfused arterial beds. As shown in Figure 3C, the maximal pressure responses to phenylephrine at the endpoint were significantly ($p < 0.05$) enhanced compared to the baseline ones (Figure 2D) in all groups of mice; however, the phenylephrine-induced changes in perfused pressure (> 10 nmol) were significantly ($p < 0.05$) lower in $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice, compared to WT and $\alpha_{1B}^{-/-}$ mice. The response was not significantly different either between $\alpha_{1B}^{-/-}$ and WT mice, or between $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice (Figure 3C).

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Discussion

We investigated the consequences of simultaneous deletion of α_{1B}- and α_{1D}-AR function in BP regulation. RT-PCR and radioligand binding studies confirmed the deletion of both α_{1B}-AR and α_{1D}-AR gene in $\alpha_{1BD}^{-/-}$ double knockout mice, and also indicated that the $\alpha_{1BD}^{-/-}$ mice had no apparent compensatory up-regulation of α_{1A}-AR. Non-anesthetized $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice had significantly lower basal SBP and MAP relative to WT and $\alpha_{1B}^{-/-}$ mice, while all mice showed no significant change in HR. The pressor response of perfused mesenteric arterial beds to α_1-AR stimulation, however, was not affected in $\alpha_{1B}^{-/-}$, and significantly reduced in both $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice. Furthermore, in the mice lacking α_{1D}-AR, but not $\alpha_{1B}^{-/-}$ mice, development of HT was significantly attenuated compared with WT mice, further extended our previous observation that α_{1D}-AR plays an important role in salt-sensitive BP increase, irrespective of co-expression of α_{1B}-AR (Tanoue et al., 2002b). The present study shows that both α_{1B}-AR and α_{1D}-AR subtypes are involved in α_1-AR-mediated pressor and vasoconstrictive responses, but to different extents.

RT-PCR and radioligand binding studies showed that the $\alpha_{1BD}^{-/-}$ mice expressed neither α_{1B}-AR nor α_{1D}-AR mRNA in any tissue examined, and had no apparent compensatory up-regulation of α_{1A}-AR. Saturation binding studies showed that the reduction of Bmax in $\alpha_{1BD}^{-/-}$ mice well corresponds to the summation of reduced Bmax values of $\alpha_{1B}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice. Thus, the reductions in Bmax of α_{1B}-AR (in $\alpha_{1B}^{-/-}$ mice) and α_{1D}-AR (in $\alpha_{1D}^{-/-}$ mice) were 41 and 11 % in brain, 55 and 10 % in heart, and 2 and 18 % in kidney, respectively (from Table 2), and those in $\alpha_{1BD}^{-/-}$ mice were 51 % in brain, 64% in heart, and 15 % in kidney, respectively. Also, competition binding experiment in the brain with KMD3213 confirmed the reduction of low affinity sites in $\alpha_{1B}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice, and no low affinity site for KMD 3213 was detected in

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$\alpha_{1BD}^{-/-}$ double knockout mice.

One of the possible cardiovascular parameters that contribute to the lower resting BP of $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ is a reduced systemic vascular resistance, as demonstrated in this study. Although the $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ partially retained the ability to increase blood pressure in response to intravenously administered α_1-agonists, circulating catecholamines of $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ are similar level with WT and are not high enough to compensate decrease in resting BP. Both angiotensin II and vasopressin evoked similar blood pressure changes in WT and double knockout mice, suggesting that responsiveness to non-adrenergic stimuli was largely preserved in $\alpha_{1BD}^{-/-}$ mice. On the other hand, α_{1A}-AR knockout mouse ($\alpha_{1A}^{-/-}$), but not $\alpha_{1AB}^{-/-}$ double knockout mouse, has been reported to have lower resting BP (O'Connell et al., 2003; Rokosh and Simpson, 2002). Because cardiac functions of $\alpha_{1A}^{-/-}$ are in normal range, α_{1A}-AR is required to maintain resting BP (Rokosh and Simpson, 2002). The BP of $\alpha_{1AB}^{-/-}$ mice was not different from that of WT, although cardiac output of the male knockout mouse was significantly decreased (O'Connell et al., 2003). In male $\alpha_{1AB}^{-/-}$ mice, the remaining vascular α_1-AR, especially α_{1D}-AR subtype, appears to compensate a negative effect of decreased cardiac output to maintain normal resting blood pressure, however, this assumption needs to be verified experimentally.

Interestingly, the maximum MAP of $\alpha_{1A}^{-/-}$ upon phenylephrine stimulation was about 10–15 % lower than that of WT control, suggesting roles of α_{1B}- and α_{1D}-AR in the pressor response to the α_1-agonist (Rokosh and Simpson, 2002). In contrast, maximum MAP response of $\alpha_{1BD}^{-/-}$ to phenylephrine stimulation was about 10 % lower compared with WT. Expression analysis and radioligand binding studies performed on the $\alpha_{1A}^{-/-}$ (Rokosh and Simpson, 2002) and $\alpha_{1BD}^{-/-}$ knockout mice suggested that deletion of α_1-AR resulted in a consistent reduction of total binding sites and up-regulation of remaining α_1-AR gene was scarcely apparent. These results

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indicate that each α_1-AR subtype could participate in the vasopressor response to circulating α_1-agonist and that redundancy of α_1-AR exists compared to the required number of α_1-AR to obtaining maximum blood pressure response. Such a finding might have a clinical importance when studies on these knockout mice are translated to a clinical field of antihypertensive therapy.

The question as to which α_1-AR subtype is involved in vasoconstricive responses in a particular vascular bed is not easy to clarify, as vascular smooth muscles express more than one α_1-AR subtype (Guimaraes and Moura, 2001; Zhong and Minneman, 1999). In addition, the distribution of the α_1-AR subtype in blood vessels markedly varies depending on species and vessels (Daniel et al., 1999; Piascik and Perez, 2001). We studied contractile responses in two types of blood vessels: thoracic aorta and mesenteric artery. Our data on aortic contractile response and on perfusion pressure of mesenteric vascular beds are in good agreement with previous reports (Cavalli et al., 1997; Daly et al., 2002; Hedemann and Michel, 2002). Contraction of mouse aorta was shown to be mainly mediated by α_{1D}-AR (Cavalli et al., 1997; Daly et al., 2002) and of mesenteric artery via α_{1A}-AR (Hedemann and Michel, 2002). Hence, our data confirmed the previous observation by Daly et al. that α_{1B}-AR plays a relatively small role in α_1-AR-mediated contraction of mouse aorta (Daly et al., 2002). Also, relatively small contribution of α_{1D}-AR in the contraction of mesenteric arterial beds was observed in a previous study (Hedemann and Michel, 2002). This, however, does not necessarily mean that the role of α_{1D}-AR in blood pressure regulation is small. It was recently reported in the contraction of small femoral resistant arteries that α_{1A}-AR, but not α_{1D}-AR, mainly mediates the contractile responses to exogenous norepinephrine, while α_{1D}-AR appear to be activated by neurally released norepinephrine (Zacharia et al., 2004a; Zacharia et al., 2004b). As both studies by Hedemann and Michel and ours examined the contractile responses to the exogenously applied catecholamines,

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the relative contribution of α_{1D}-AR appears to be small. Hence, further studies will be required to clarify the relative contribution of each subtype in the sympathetic regulation of neuronally stimulated and blood-borne catecholamine-stimulated pressor responses, *in vivo* in particular.

The α_1-AR-stimulated pressor responses seen in $\alpha_{1BD}^{-/-}$ mice in the present study may further support the idea that the remaining α_1-AR, which is mainly regarded to be probably α_{1A}-AR subtype, is a vasopressor expressed in resistance arteries (Rokosh and Simpson, 2002). A number of previous pharmacological and mRNA expression studies have indicated the contribution of α_{1A}-AR to vascular contraction (Leech and Faber, 1996; Piascik and Perez, 2001; Rokosh and Simpson, 2002). Furthermore, Rokosh and Simpson (Rokosh and Simpson, 2002) showed histochemically that Lac-Z, whose gene substituted for the α_{1A}-AR gene in their α_{1A}-KO mouse, was expressed in peripheral arteries, such as mesenteric artery, but not expressed in the major conducting arteries, such as the thoracic aorta. Also, the pressor response to a potent α_{1A}-AR-selective agonist, A61063, in $\alpha_{1BD}^{-/-}$ mice was intact and comparable to WT, indicating that this ligand is selective for α_{1A}-AR-mediated function.

Our next focus in this study was on a direct comparison of α_1-AR subtypes *in vivo*, in terms of their causative roles of salt-sensitive hypertension. Distinct BP patterns of $\alpha_{1B}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice were clear evidence indicating the α_{1D}-AR plays a crucial role in raising BP in this model. Observations in this and previous studies suggest a critical role of α_{1D}-AR on increased sensitivity to vasoconstriction, especially in hypertensive state (Chalothorn et al., 2003; Clements et al., 1997; Daly et al., 2002; Tanoue et al., 2002a). Altered sympathetic activity is another prominent feature of HT. We found that the circulating catecholamine levels of $\alpha_{1BD}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice under salt loading were less than those of WT. Because plasma catecholamine levels correlate well with spillover from sympathetic nerves in organs (Esler and Kaye, 2000; Grassi,

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1998), lower plasma catecholamine levels of $\alpha_{1BD}^{-/-}$ and $\alpha_{1D}^{-/-}$ mice indicate suppression of sympathetic outflow. In fact, the α_1-blocker prazosin has been shown to act on α_1-AR in the central nervous system (CNS) and suppress sympathetic outflow (Hoffman, 2001). In mouse CNS, about 10 % of α_1-AR is α_{1D}-AR subtype, as seen in this study. Although contribution of central α_{1D}-AR to the regulation of sympathetic outflow need to be further examined, our current data clearly indicates that α_{1D}-AR gene knockout leads to decrease in plasma catecholamine levels and in the antihypertensive effects on salt-sensitive hypertension. Using the same hypertension model, the mice lacking one copy of α_{2B}-AR gene had attenuated BP increase compared to the WT group (Makaritsis et al., 1999). It is, therefore, of interest to explore a possibility of functional relationship between α_{2B}-AR and α_{1D}-AR subtypes, because both are found in the CNS and activated by the same agonist (Gavras and Gavras, 2001; Tanoue et al., 2002b).

In conclusion, three α_1-AR subtypes differently participate in systemic blood pressure regulation. Ablation of α_{1D}-AR, but not of α_{1B}-AR, reduced resting blood pressure by reducing peripheral resistance. Pressor response to α_1-agonist is suppressed according to the number of α_1-AR gene deleted, however, an increase in BP of double knockout mice suggests that functional redundancy could exist in α_1-AR-mediated pressor response. Furthermore, α_{1D}-AR is an important receptor subtype in the development of secondary HT accompanying acute renal dysfunctions.

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Footnotes

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Reprint request to; Gozoh Tsujimoto, M.D., Ph. D.

Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

Tel: ++81-3-3419-2476, Fax: ++81-3-3419-1252, E-mail: gtsujimoto@nch.go.jp

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Legends for Figures

Figure 1. BP responses of WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice to adrenergic agonists.

Following propranolol (1mg/kg) treatment, increasing doses of vasoactive agents, phenylephrine (A), norepinephrine (B) or A61603 (C), were administered as a bolus at 15-20 min intervals. BP and HR had returned to baseline levels during the interval. The changes in MAP from basal levels are shown in mmHg. Data points are the means ± SEM from analyses of 15-22 mice.

Inhibitory effects of BMY7378 or bunazosin on the pressor response to norepinephrine were assessed in WT (D), $\alpha_{1B}^{-/-}$ (E), $\alpha_{1D}^{-/-}$ (F) and $\alpha_{1BD}^{-/-}$ mice (G). After pretreatment of propranolol (1mg/kg), either BMY7378 (100 μg/kg) or bunazosin (10 μg/kg) was injected into the mice 10 min prior to continuous infusion of norepinephrine (1 μg/kg/min for 5 min). Data points are the means ± SEM. from analyses of 10-16 mice.

Figure 2. Vascular contraction in WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice.

Concentration-response curves for norepinephrine-induced (A), phenylephrine-induced (B) and serotonin-induced contractions (C) were constructed using thoracic aortic segments from WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice. The contraction (%) is expressed as a percentage of the maximum contraction induced by 40 mM KCl. The results are the means ± SEM of 10-16 preparations of norepinephrine, phenylephrine and serotonin. (D) Concentration response for the phenylephrine-induced pressor response in perfused mesenteric arterial beds of WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice. Two-way ANOVA showed that the concentration-response for the phenylephrine-induced pressor response of $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice was significantly ($p < 0.05$) different from that of WT and $\alpha_{1B}^{-/-}$ mice. Values represent the means ± SEM of 6-9 independent experiments.

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*p <0.05, $\alpha_{1D}^{-/-}$ or $\alpha_{1BD}^{-/-}$ vs. WT or $\alpha_{1B}^{-/-}$.

Figure 3. Salt-induced hypertension model of knockout and WT mice.

After nephrectomy, SBP (A) and HR (B) values of $\alpha_{1B}^{-/-}$ (n=9), $\alpha_{1D}^{-/-}$ (n=9), $\alpha_{1BD}^{-/-}$ (n=9) and WT (n=10) were monitored by tail cuff recording. The monitoring was performed from 1:00 PM to 5:00 PM every two or three days. The SBP change in WT or $\alpha_{1B}^{-/-}$ mice showed significant differences as compared with those of $\alpha_{1D}^{-/-}$ or $\alpha_{1BD}^{-/-}$ mice (p <0.05, $\alpha_{1D}^{-/-}$ or $\alpha_{1BD}^{-/-}$ vs. WT or $\alpha_{1B}^{-/-}$ by two-way ANOVA). *p <0.05, $\alpha_{1D}^{-/-}$ or $\alpha_{1BD}^{-/-}$ vs. WT or $\alpha_{1B}^{-/-}$. (C) Concentration-response relationship for the phenylephrine-induced pressor response in perfused mesenteric arterial beds of WT, $\alpha_{1B}^{-/-}$, $\alpha_{1D}^{-/-}$ and $\alpha_{1BD}^{-/-}$ mice at the endpoint. Two-way ANOVA showed that the concentration-response curves of $\alpha_{1D}^{-/-}$ or $\alpha_{1BD}^{-/-}$ mice were significantly (p < 0.05) different from those of WT and $\alpha_{1B}^{-/-}$ mice. Values represent the means ± SEM of 6-9 independent experiments. *p <0.05, $\alpha_{1D}^{-/-}$ or $\alpha_{1BD}^{-/-}$ vs. WT or $\alpha_{1B}^{-/-}$.

Table 1. Physical and laboratory parameters before and after nephrectomy and salt loading

	WT		$\alpha_{1B}^{-/-}$		$\alpha_{1D}^{-/-}$		$\alpha_{1BD}^{-/-}$	
Parameters	Baseline	Endpoint	Baseline	Endpoint	Baseline	Endpoint	Baseline	Endpoint
BW (g)	22 ± 0.6	22 ± 1	21 ± 0.8	21 ± 1	21 ± 0.7	21 ± 1	20 ± 1.1	21 ± 1
Creatinine (mmol/L)	12 ± 1	20 ± 3 *	10 ± 1	20 ± 1 *	12 ± 1	19 ± 2 *	10 ± 1	20 ± 4 *
HW/BW	3.3 ± 0.1	6.4 ± 0.3 *	3.3 ± 0.1	5.9 ± 0.3 *	3.3 ± 0.1	6.1 ± 0.2 *	3.4 ± 0.1	6.4 ± 0.4 *
ExKW/BW	3.3 ± 0.1	NA	3.3 ± 0.1	NA	3.2 ± 0.1	NA	3.2 ± 0.1	NA
Total CA (nmol/L)	35 ± 4	190 ± 13 *, **	28 ± 3	188 ± 20 *, **	32 ± 5	110 ± 12 *	31 ± 7	98 ± 3 *
EP (nmol/L)	20 ± 4	45 ± 6 *	16 ± 3	44 ± 13 *	19 ± 4	38 ± 7 *	18 ± 5	34 ± 6 *
NEP (nmol/L)	13 ± 2	141 ± 8 *, **	10 ± 1	141 ± 9 *, **	11 ± 2	70 ± 9 *	10 ± 2	61 ± 6 *
DA (nmol/L)	2.3 ± 0.2	3.5 ± 0.5 *, **	2.1 ± 0.2	3.4 ± 0.4 *, **	2.3 ± 0.3	2.2 ± 0.2	2.3 ± 0.1	2.3 ± 0.2
AT I (pg/ml)	867 ± 58	376 ± 68 *	703 ± 81	364 ± 48 *	725 ± 57	411 ± 112 *	822 ± 86	371 ± 51 *
AT II (pg/ml)	1243 ± 110	589 ± 136 *	1230 ± 95	567 ± 34 *	1323 ± 98	603 ± 135 *	1246 ± 100	536 ± 26 *

BW, body weight; HW, heart weight; ExKW, excised kidney weight; CA, catecholamine; EP, epinephrine; NEP, norepinephrine; Dopamine, DA; and AT, angiotensine. Values shown are the mean ± SEM from 8-15 mice.* $p < 0.05$: Before and after salt-loading. # $p<0.05$

Table 2. Radioligand binding studies

(A) Saturation binding studies

Tissue	Bmax (fmol/mg protein)			
	WT	$\alpha_{1B}^{-/-}$	$\alpha_{1D}^{-/-}$	$\alpha_{1BD}^{-/-}$
Brain	103.2 ± 2.4	61 ± 2.5*	90.8 ± 2.0*	50.3 ± 3.1*
Aorta	48.1 ± 4.3	45.5 ± 3.8	N.D.	N.D.
Liver	42.3 ± 3.8	1.7 ± 0.3*	40.8 ± 4.1	1.1 ± 0.1*
Heart	45.6 ± 2	20.5 ± 2.2*	40.9 ± 0.9*	16.3 ± 1.9*
Kidney	34 ± 1.1	32.9 ± 2.2	28.3 ± 0.3*	29 ± 0.5*

Specific binding was measured with [^{125}I]-HEAT for the brain, aorta and heart, and with [^{3}H]-prazosin for the liver and kidney as described in Methods. Each value is the means ± SEM of 4-6 different experiments.

Table 2.

(B) Competition binding study using [^{125}I]HEAT binding by KMD-3213

		Two-site analysis			
		WT	$\alpha_{1B}^{-/-}$	$\alpha_{1D}^{-/-}$	$\alpha_{1BD}^{-/-}$
K_H	(nM)	0.29 ± 0.09	0.23 ± 0.02	0.23 ± 0.05	0.26 ± 0.03
K_L	(nM)	40 ± 7	27 ± 5	35 ± 1	n.d.
R_H	(%)	28 ± 5	42 ± 2	32 ± 5	100
R_L	(%)	72 ± 5	58 ± 2	68 ± 5	0

Inhibition of specific [125I]HEAT binding by KMD-3213 was determined in membrane preparations from the mouse brain. The best two-site fit was determined by nonlinear regression analysis of the averaged curve, and high- and low-affinity sites for KMD-3213

Table 3. MAP measurements with intra-carotid catheter

	WT	$\alpha_{1B}^{-/-}$	$\alpha_{1D}^{-/-}$	$\alpha_{1BD}^{-/-}$
Basaline				
MAP (mmHg)	118 ± 3	111 ± 5	109 ± 3*	103 ± 6*, #
HR (bpm)	622 ± 26	630 ± 25	645 ± 17	640 ± 32
Endopoint				
MAP (mmHg)	145 ± 6	144 ± 5	126 ± 5*, #	119 ± 7*, #
HR (bpm)	610 ± 18	605 ± 15	633 ± 41	630 ± 28

MAP and HR were measured in non-anesthetized mice as described in Methods. Values are the mean ± SEM of 9-18 mice. *$p < 0.05$, compared to WT mice;#$p<0.05$, compared to $\alpha_{1B}^{-/-}$ mice.

Table 4. Cardiac function assessed by echocardiogram

	WT	$\alpha_{1B}^{-/-}$	$\alpha_{1D}^{-/-}$	$\alpha_{1BD}^{-/-}$
	(n=14)	(n=9)	(n=18)	(n=12)
LVIDd (mm/gBw)	0.093 ± 0.01	0.11 ± 0.005	0.104 ± 0.007	0.123 ± 0.006*
LVIDs (mm/gBw)	0.048 ± 0.006	0.069 ± 0.005*	0.055 ± 0.005	0.073 ± 0.005*
FS (%)	48 ± 4	37 ± 3*	47 ± 2	40 ± 2*
EF (%)	86 ± 2	75 ± 3*	85 ± 2	79 ± 2*
HR (bpm)	497 ± 30	541 ± 22	525 ± 26	484 ± 20
CO (ml/min)	14.9 ± 2	16.1 ± 2	15.9 ± 1	16.4 ± 2

LVIDd, left ventricular internal dimension in diastole; LVIDs, left ventricular internal dimension in systole; %FS, percent fractional shortening; EF, ejection fraction; HR, heart rate; CO, cardiac output. Values are the mean ± SEM. *$p < 0.05$ as compared

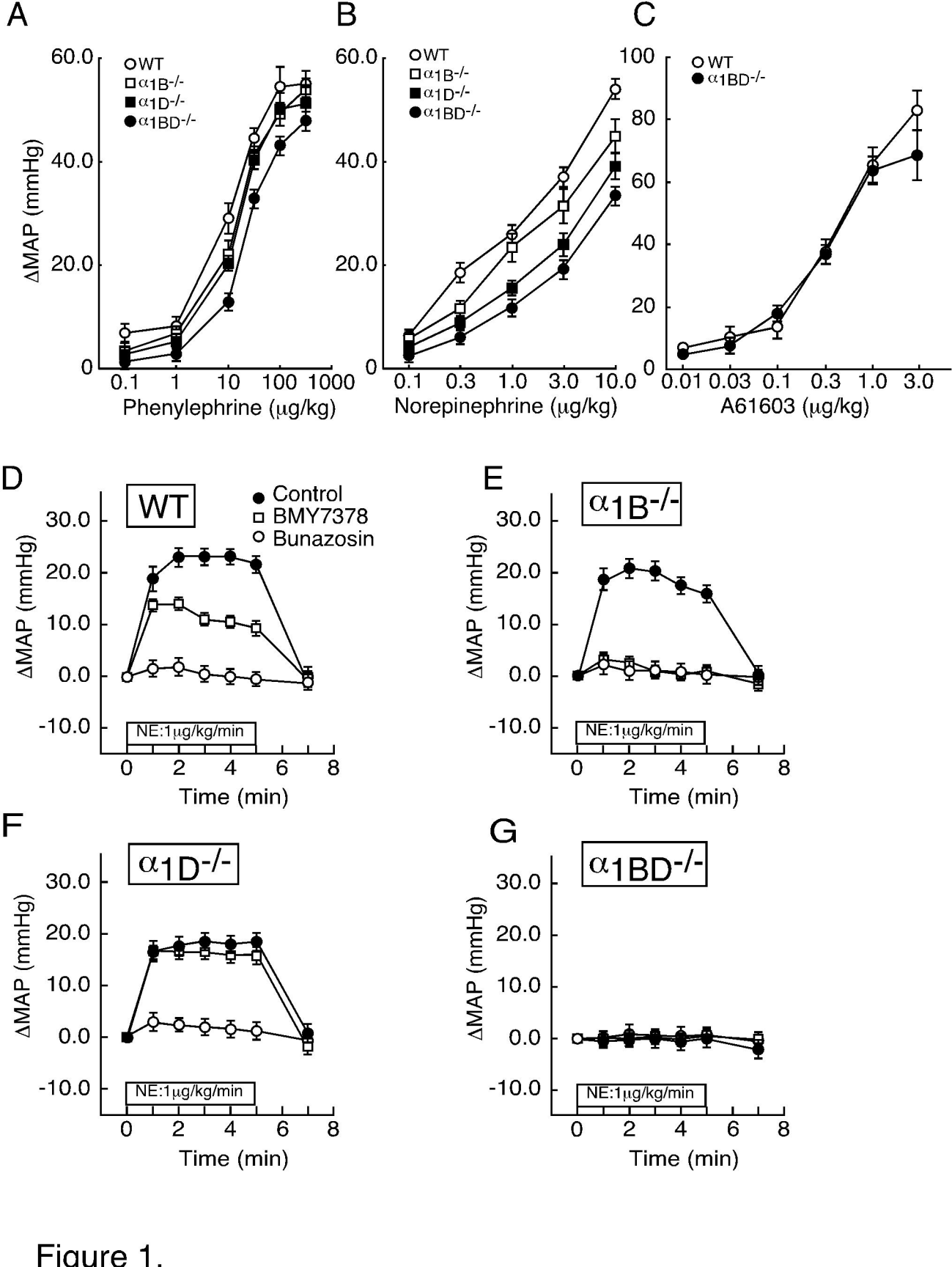
A
B
C
WT
α1B-/-
α1D-/-
α1BD-/-
ΔMAP (mmHg)
Phenylephrine (μg/kg)
Norepinephrine (μg/kg)
A61603 (μg/kg)
D
E
F
G
Control
BMY7378
Bunazosin
NE:1μg/kg/min
Time (min)

Figure 1.

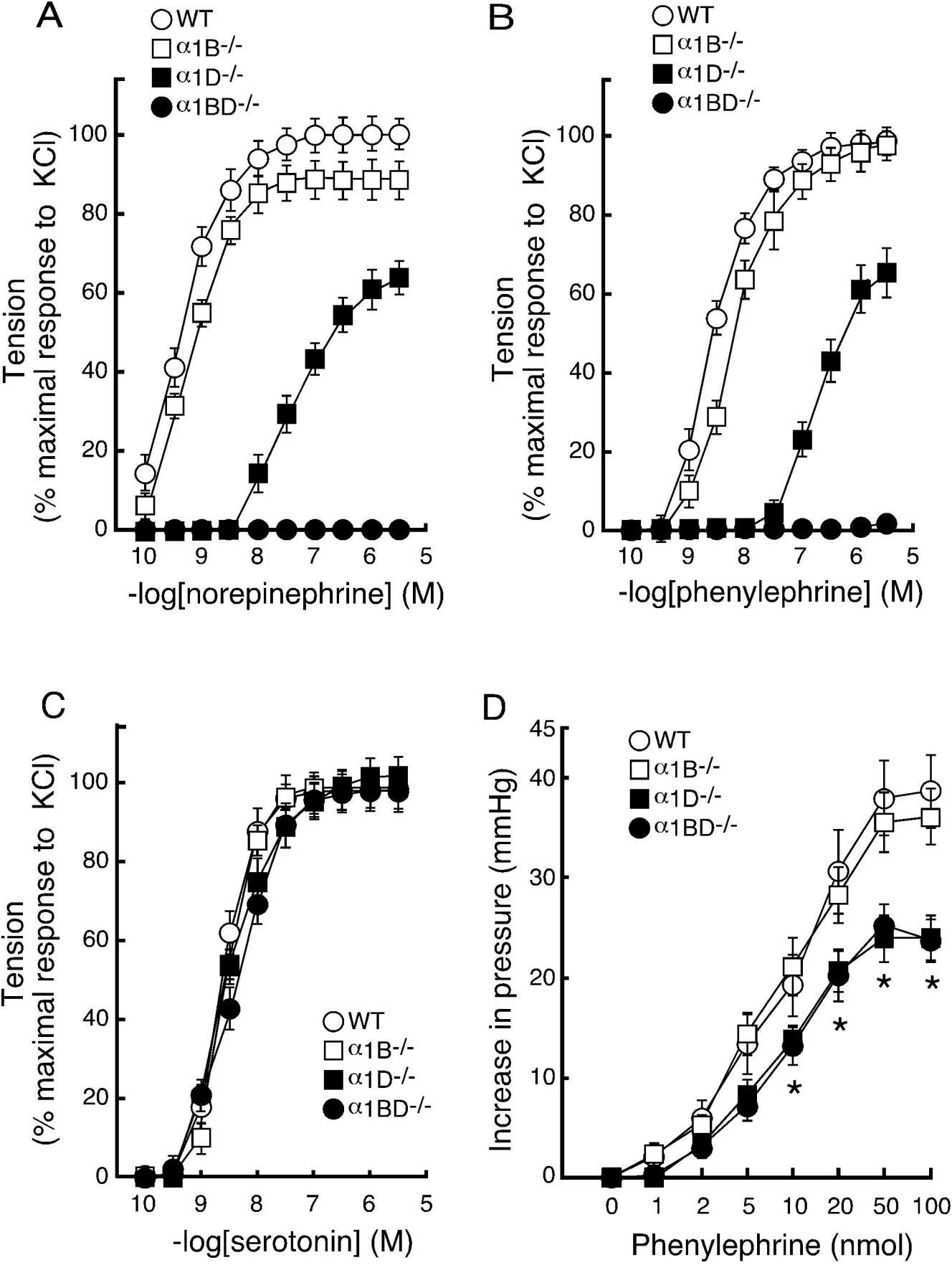
A
WT
α1B-/-
α1D-/-
α1BD-/-
Tension
(% maximal response to KCl)
-log[norepinephrine] (M)
B
-log[phenylephrine] (M)
C
-log[serotonin] (M)
D
Increase in pressure (mmHg)
Phenylephrine (nmol)

Figure 2.

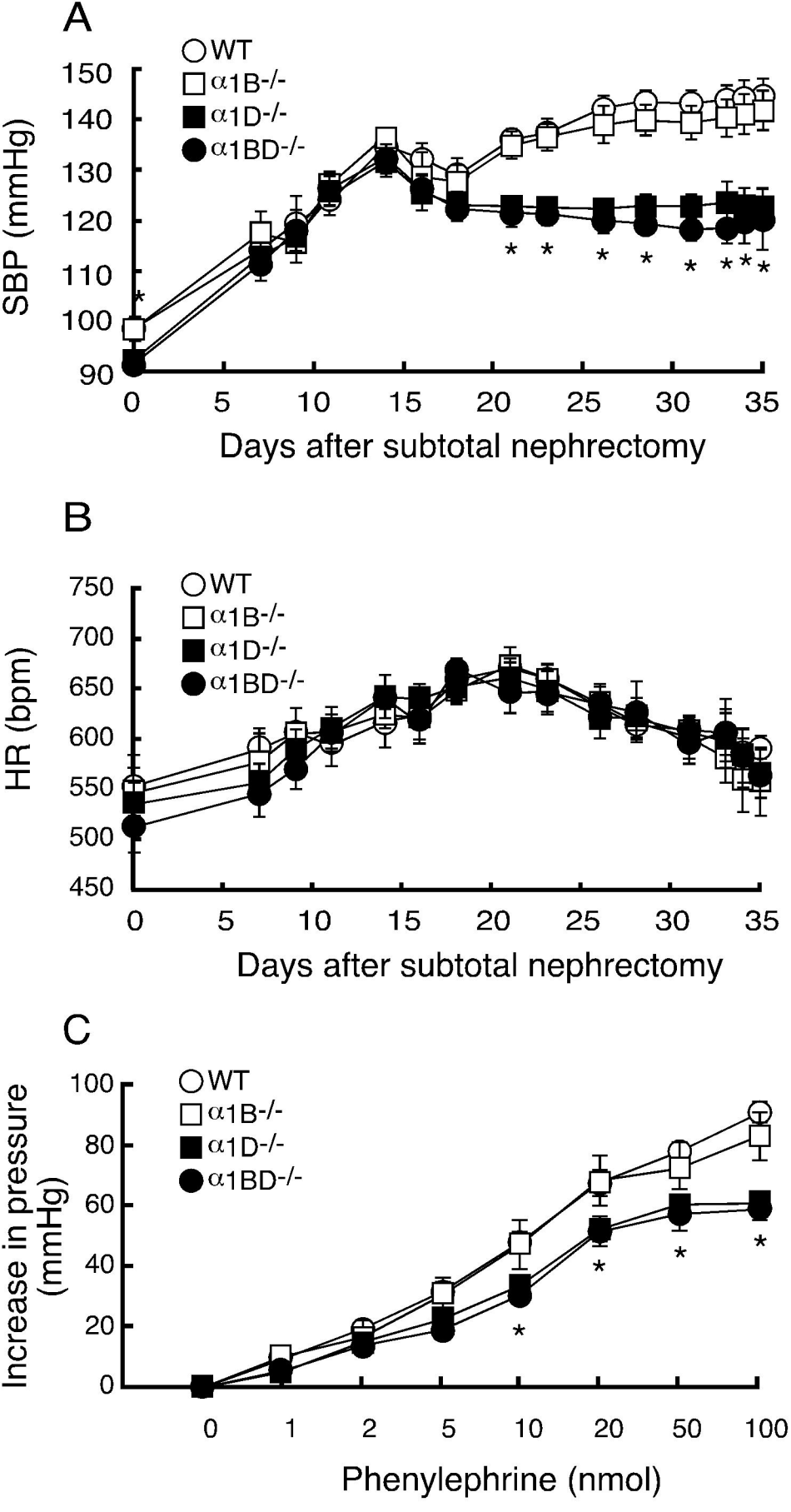
A
WT
α1B-/-
α1D-/-
α1BD-/-
SBP (mmHg)
150
140
130
120
110
100
90
0
5
10
15
20
25
30
35
Days after subtotal nephrectomy
B
HR (bpm)
750
700
650
600
550
500
450
Days after subtotal nephrectomy
C
Increase in pressure (mmHg)
100
80
60
40
20
0
0
1
2
5
10
20
50
100
Phenylephrine (nmol)

Figure 3.