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Vascular Response to Angiotensin II Is Exaggerated through an Upregulation of AT1 Receptor in AT2 Knockout Mice

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Blood pressure is elevated and pressor response to angiotensin II (Ang II) is exaggerated in AT2 null mice. The purpose of the present study was to elucidate the mechanism for the increased responsiveness to Ang II in the mice. The contraction of aortic strips generated by Ang II was significantly greater in the AT2 gene-deleted mice than the control, which was completely abolished by AT1 antagonist losartan. The aortic content of AT1 receptor was significantly increased ($P < 0.05$, $n = 5$) in the AT2 null mice (212 ± 58.2 fmol/mg protein) compared with the control (98.2 ± 55.9 fmol/mg protein). While both AT1 and AT2 mRNAs were expressed in the aorta of the control mice, only AT1 mRNA was expressed in the AT2 knockout mice. The expression of AT1 mRNA in the AT2 knockout mice was significantly higher (1.5-fold, $P < 0.05$, $n = 5$) than that in the control. The present study clearly demonstrated that the increased vascular reactivity to Ang II in AT2 knockout mice is at least partly due to an increased vascular AT1 receptor expression and suggested that AT2 counteracts AT1-mediated vascular action of Ang II through downregulation of AT1 receptor by a crosstalk between these receptors by some as yet unknown mechanisms.

Angiotensin II (Ang II), an effector peptide in the renin-angiotensin system (RAS), plays an integral role in the regulation of blood pressure and electrolyte and body fluid balance (1, 2). Recent pharmacological and molecular biological studies have identified two distinct subtypes of Ang II receptors, designated AT1 and AT2 (3–9). A large majority, if not all, of the physiological actions traditionally ascribed to Ang II, such as vasoconstriction and sodium and water retaining actions, are mediated by AT1 receptors (10, 11). Since AT2 mRNA was demonstrated to be expressed at high levels in various tissues of the fetus and precipitately drops to low levels after birth (8, 9), it is suggested that AT2 receptor is involved in the growth and development. However, increased blood pressure associated with exaggerated sensitivity to Ang II was found in the AT2 gene deficient mice (12), whereas Hein *et al.* observed increased sensitivity of blood pressure to Ang II infusion without elevated basal blood pressure (13). The results suggest a counterregulatory roles of AT2 and AT1 in the blood pressure control.

The purpose of the present study was to elucidate the mechanism for the increased responsiveness to Ang II in the AT2 knock out (AT2 KO) mice. We determined vascular contractility to Ang II *in vitro* using aortic strip, and expression of the AT1 and AT2 receptors in the aorta at the mRNA level by competitive reverse transcription-polymerase chain reaction (RT-PCR) and at the protein level by ligand binding studies.

MATERIALS AND METHODS

Animals. Male mice lacking the AT2 receptor generated as described previously (12) and C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) as genetic controls of the mutant mice were used. All the studies were performed at approximately 4 months of age.

Experiments of vascular contractility with aortic strip. Mice were killed by an overdose of pentobarbital anesthesia and the thoracic aorta was rapidly removed. The aorta was cleaned of adherent connective tissue and cut helically under a dissecting microscope. The

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Abbreviations used: Ang II, angiotensin II; AT1, angiotensin II type 1 (receptor); AT2, angiotensin II type 2 (receptor); NO, nitric oxide; RAS, renin angiotensin system; RIA, radioimmunoaasay; SHR-SP, stroke-prone spontaneously hypertensive rats; VSMC, vascular smooth muscle cells.

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strips were mounted under 0.5 g of resting tension in 20 ml organ baths containing a modified Krebs–Henseleit solution of the following composition (mM): NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$, $NaHCO_3$ 25.0, NaH_2PO_4 1.2 and glucose 11.5 at 37°C and bubbled with 95% O_2, 5% CO_2 (pH 7.4). Developed tension was recorded isometrically through force-displacement transducers. The endothelial cells were removed by rubbing them with filter paper (14), and their removal was confirmed by the loss of the relaxant response to acetylcholine.

Although our preliminary studies demonstrated that mouse aortic strips rapidly develop tachyphylaxis to Ang II, it was reversed by 60 min incubation in Ang II-free medium. To obtain concentration response curves, therefore, four doses of Ang II (0.1, 1, 3 and 10 nM) were sequentially challenged at 60-min intervals. To examine the involvement of AT1 receptor in this response, AT1 antagonist losartan (Dup 753, Dupont Merck Pharmaceutical Company, Wilmington, DE) was added to 1 μM 5 min before the stimulation with 10 nM of Ang II. The contractile response to KCl (60 mM) after removal of Ang II by two washes was served as control.

[^{125}I] [Sar^1, Ile^8] Ang II binding experiments. [Sar^1, Ile^8] Ang II (Peninsula Laboratories, Belmont, CA) was labeled with [^{125} I] iodine (New England Nuclear, Boston, MA), using lactoperoxidase (Sigma Chemicals, St. Louis, MO) (15) and purified by reverse-phase high performance liquid chromatography (16). Aortic membranes were prepared as described previously (17). Membranes (150 μg) were incubated with [^{125} I] [Sar^1, Ile^8] Ang II (0.5 nM) at 37°C for 2 h in the presence and absence of 1 μM Ang II antagonists in 10 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$ containing 1 mM EGTA, 0.5 mM PMSF, 20 μg/ml leupeptin and 10 μg/ml antipain. Free and bound radioligands were separated as described (17). Specific [^{125}I] [Sar^1, Ile^8] Ang II binding sensitive to 1 μM losartan and PD 123319 (AT2 antagonist, Park-Davis Pharmaceutical Company, Ann Arbor, MI) was estimated as AT1 receptor and AT2 receptor, respectively.

RT-PCR analyses of AT2 receptor. RT-PCR method was chosen to demonstrate AT2 mRNA because of its low level expression in the aorta. Total RNA was extracted from aorta by the acid guanidium thiocyanate–phenol–chloroform method. One microgram of total RNA was reverse-transcribed with an oligo-dT primer. One microliter of the resultant cDNA was subjected to PCR amplification using Taq DNA polymerase (Stratagene Inc., La Jolla, CA) with the following primers designed on the basis of the nucleotide sequence of mouse AT2 cDNA (18): 5′-GCTGAGTAAGCTGATTTATG-3′ as a sense primer (309–328) and 5′-TTAAGACACAAAGGTGTCCA-3′ as an antisense primer (2646–2665). The anticipated PCR product was about 1.2 kb and could be distinguished from the genomic amplification product because the primers were designed to span a 1182-bp genomic intron. The reaction was run for 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 2 min of polymerization at 72°C. One fifth of the reaction mixture (10 μl) was subjected to electrophoresis in 1% agarose gel and stained with ethidium bromide.

Competitive RT-PCR analysis of AT1 receptor mRNA. The AT1 receptor in rodents is situated as the two isoforms, termed AT1a and AT1b (5, 19). In this study, however, we detected the mRNAs for the both subtypes without distinction using the common PCR primers because they share the same signaling pathways.

A 1.6-kb *Hin*dIII fragment containing the entire coding region of the mouse AT1a gene was subcloned into the Hind III site of the pBluescript KS(+) (Stratagene Inc., La Jolla, CA). The resultant plasmid was cut with *Pma*CI (Boehringer-Mannheim) and Bsp MI (NEB), and self ligated to contain an insert lacking the *Pma*CI–*Bsp*MI fragment (85 bp). The deletion-mutated *Hin*dIII fragment lacking this 85 bp fragment was used as a competitor template for competitive RT-PCR to quantify AT1 mRNA. The following primers were used for PCR (20): 5′-GGTGGGAATATTGGAAACAG-3′ (5′-sense primer, 120–140) and 5′-AAGAAGAAAAGC ACAATCGCC-3′ (3′-antisense, 729–749). Total RNAs (2 μg) were reverse-transcribed using random primers and the resultant cDNA mixtures (1/20 volume of the mixture) were amplified by PCR using the same primers described above in the presence of various amounts of the competitor template with a trace amount of [α-^{32}P]dCTP (Amersham, Arlington Heights, IL) to quantify the PCR products. Denaturing, annealing and polymerase reaction for AT1 were performed 27 times at 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min, respectively. Native AT1 cDNA produces a 630-bp fragment, whereas the competitor template for AT1 generates a 545 bp fragment. No sequence divergence was present in the primers used here between the two AT1 subtypes, AT1a and AT1b. The PCR products were size fractionated on 5% polyacrylamide gels. The gels were dried and exposed to X-ray films (Kodak XAR-5, Eastman Kodak, Rochester, NY) between two intensifying screens for 12 h. The ratio of AT1 band to competitor band was used to estimate AT1 mRNA according to the published method (19).

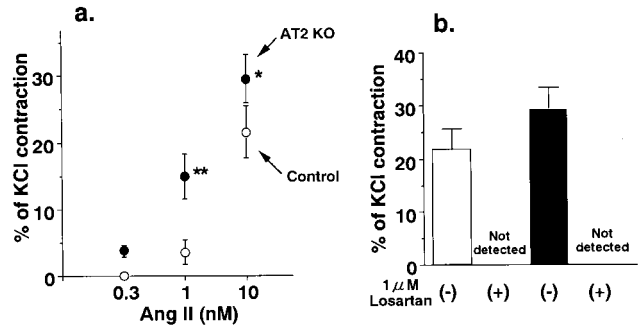

FIG. 1. Contractile response of the aortic strip from control (○, □) and AT2 KO (●, ■) mice. Dose dependent effect of Ang II (a) and the effect of 1 μM losartan on the 10 nM Ang II-induced contraction (b). The contractile response to 60 mM KCl is set as 100%. In the presence of 1 μM losartan, no contraction was detected. Vertical bars represent means ± SEM of five different experiments. $*P < 0.05$, $**P < 0.01$ vs control.

Detection of plasma Ang II and aldosterone concentration. Concentration of plasma Ang II and aldosterone were measured by RIA using commercially available kits (Ang II: American Laboratory Products, Windham, NH; aldosterone: Diagnostic Products, Los Angels, CA).

Statistical analysis. Results are presented as mean values ± SEM of the number (n) of observations. Statistical analysis was performed by Student's *t* test for nonpaired data and differences were considered to be significant when $P < 0.05$.

RESULTS

Ang II-induced contraction of aortic strip. Ang II (0.3, 1, 10 nM) induced a dose-dependent contractile response of the aortic strip from control mice (Fig. 1a). By contrast, contractile response of the aorta from AT2 KO mice was significantly greater than that of the control mice. Both of the contractile response in control and AT2 KO mice were completely abolished by the pretreatment with 1 μM AT1 antagonist losartan (Fig. 1b).

Binding studies of Ang II receptors. We compared the binding of the nonselective Ang II antagonist [^{125}I] [Sar^1, Ile^8] Ang II to aortic membranes prepared from the AT2 KO and control mice. The AT1 and AT2 receptors were distinguished by using their specific antago-

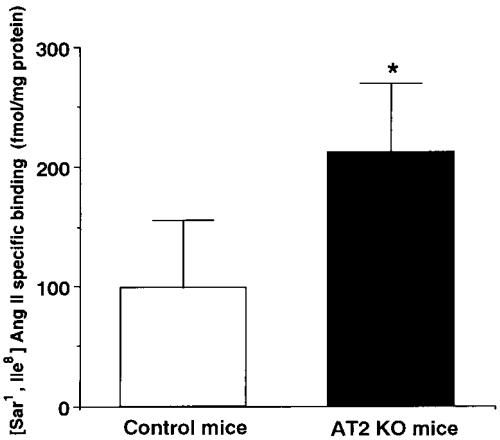

FIG. 2. AT1 specific binding in the aortic membranes from control and AT2 KO mice. Binding studies were performed in the presence of 1 μM PD 123319. Vertical bars represent means ± SEM of five different experiments. *$P < 0.05$ vs control.

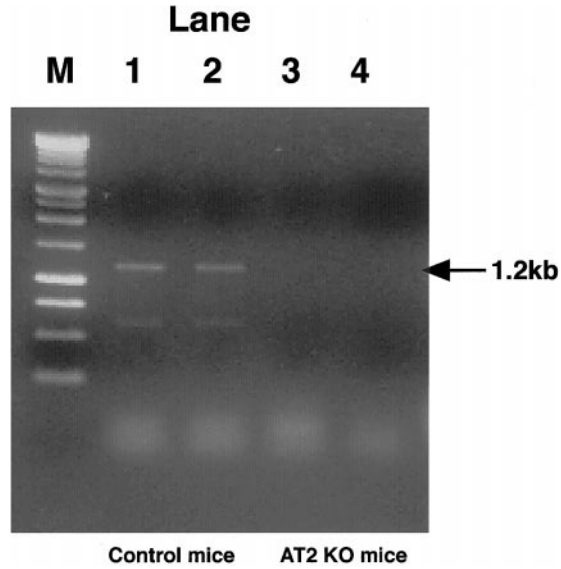

FIG. 3. Electrophoretic analysis of RT-PCR products of mouse AT2 receptor transcripts in the aorta from control (lanes 1 and 2) and AT2 KO (lanes 3 and 4) mice. 1 μg of total RNA was reverse-transcribed and amplified by the PCR method. Aorta from control mice, but not AT2 KO mice, expressed the products of AT2 receptor transcripts of about 1.2 kb. M, molecular weight marker.

nists losartan and PD 123319, respectively. As shown in Fig. 2, aortic membranes from AT2 KO mice (212 ± 58.2 fmol/mg protein) showed significantly ($P < 0.05$) higher specific binding to AT1 receptor than that from control mice (99.2 ± 55.9 fmol/mg protein). On the other hand, the AT2 receptor expression was below the detection level by this method even in the control mice (date not shown).

RT-PCR of AT2 receptor. The size of the predicted PCR product of cDNA is about 1.2 kb, whereas that of genomic DNA is about 2.3 kb. As shown in Fig. 3, 1.2 kb bands corresponding to AT2 mRNA were clearly detected in the aorta from control mice, whereas no such bands were seen in the aorta of the AT2 KO mice.

Competitive RT-PCR of AT1 receptor mRNA. Several different amounts of cDNA mixtures containing AT1 mRNA were amplified as shown in Fig. 4. The arbitrary units obtained from calculated AT1 mRNA levels correlated well with the input amounts of the cDNA mixtures, indicating the validity of the method.

Figure 5a showed representative chromatographic pattern of the competitive RT-PCR analyses of AT1 mRNA. The AT1 mRNA apparently increased in the aorta of AT2 KO mice compared with those of control mice. As shown in Fig. 5b, the AT1 mRNA levels in the AT2 KO mice were 1.5-fold higher than that in the control mice ($P < 0.05$, $n = 5$).

Plasma Ang II and aldosterone concentration. Plasma Ang II concentration in the control mice and AT2 KO mice was 19.2 ± 3.0 pg/ml and 18.6 ± 4.6 pg/ml, respectively. Plasma aldosterone concentration in the control mice and AT2 KO mice was 28.2 ± 8.5 ng/dl and 25.8 ± 11.9 ng/dl, respectively. There was no significant difference of plasma Ang II and aldosterone concentration between the two strains.

DISCUSSION

It has been reported that blood pressure is elevated and pressor response to Ang II is exaggerated in mice

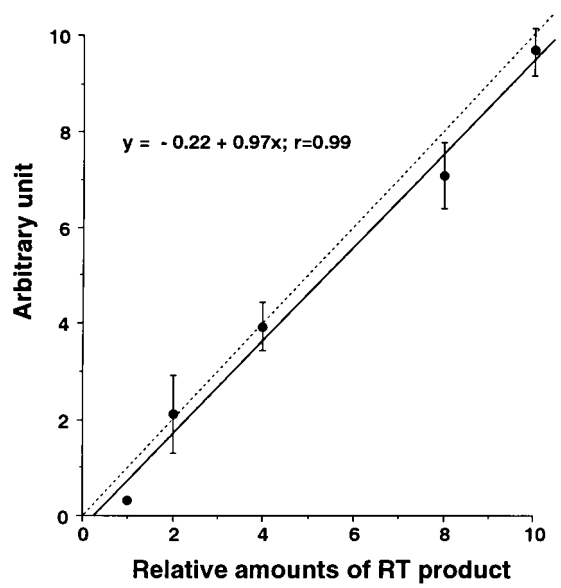

FIG. 4. Validity of the competitive RT-PCR analyses of AT1 mRNA level. One, two, four and ten volumes of the known amounts of cDNA mixtures were added to PCR mixtures with the increasing quantities of the AT1 competitor template (10, 25, 50, 75, 100, 250, 500 fg) and amplified in 27 cycles. One arbitrary unit indicates the value of reverse-transcribed target product obtained from competitive RT-PCR with one volume of the known amount of cDNA mixtures (solid line) and identical line (dotted line) are shown.

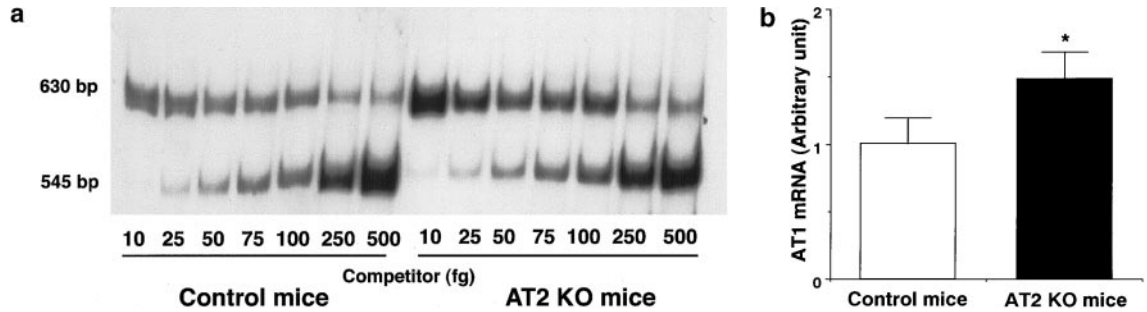

FIG. 5. Competitive RT-PCR analyses of AT1 mRNA level in the aorta. (a). Representative result of competitive RT-PCR for aortic AT1 mRNA in the control and AT2 KO mice. Aortic total RNA (2 μg) was reverse-transcribed and a part of the resultant cDNA mixtures (1/20 volume of the mixture) was subjected to PCR in the presence of the indicated amounts of the competitor template. (b). Calculated expression level of the AT1 mRNA. The value of the AT1 mRNA level in control mice was normalized to 1 arbitrary unit for quantitative comparisons. Vertical bars represent means ± SEM of five different mice, each determined in triplicate manner. *$P < 0.05$ vs control.

lacking the AT2 receptor (12). While both AT1 and AT2 mRNAs were expressed in the aorta of control mice, only AT1 mRNA was expressed in the AT2 KO mice. Since AT2 receptor is reported to generate nitric oxide (NO) and NO produces vasodilation by activating guanylate cyclase in vascular smooth muscle cells (VSMC) (21, 22), the absence of AT2 receptor may explain, at least in part, the change of blood pressure and vascular contractility.

However, the present study also demonstrated that the AT1 receptor in the aorta is increased both at the level of mRNA expression and binding capacity in AT2 KO mice. In both control and AT2 KO mice, aortic strips generated tension with the addition of Ang II in a dose-dependent manner and the response was completely abolished by the pretreatment with AT1 specific antagonist losartan. The results clearly indicate that Ang II induces vasoconstriction through the AT1 receptor. In addition, it was also shown that vascular response to Ang II is greater in AT2 KO mice than in control mice. These observations provided evidence that the increased vascular reactivity to Ang II is due to an increase in AT1 receptor expression in AT2 KO mice. It is, therefore, suggested that increased vascular response *in vivo* in AT2 KO mice is attributed to the exaggerated vascular contractility to Ang II through AT1 receptor.

The mechanism of the increased AT1 receptor mRNA in AT2 KO mice is not known. Since it is reported that AT1 receptor expression is up-regulated by the stimulation of RAS (23, 24), we determined plasma Ang II and aldosterone concentrations as a marker of the RAS in AT2 KO mice. However, there were no significant differences in plasma Ang II and aldosterone concentrations between the two strains.

Recent studies have elucidated the existence of the crosstalk between AT1 and AT2 receptors. In rat coronary endothelial cells, AT2 has antiproliferative effect which antagonizes the growth promoting effect of AT1 (25). In cultured neurons from rat hypothalamus and brainstem, AT1 activates and AT2 inactivates mitogen-activated protein kinases (26). Therefore, it is reasonable to consider that there exists such a crosstalk between AT1 and AT2 in our system. It is recently reported that AT2 receptor produces NO in rat kidney (27) and in aorta of SHR-SP (28). Since NO down-regulates the AT1 receptor gene transcription in rat VSMC (29), it is suggested that the AT2 receptor may suppress AT1 receptor expression through NO production and, by contrast, elimination of AT2 receptor may lead to up-regulation of AT1 receptor. It is fascinating to hypothesize that AT1 and AT2 counter-regulates at the level of gene expression as well as their vascular actions. Further investigation is required to identify the precise mechanisms of AT1 receptor up-regulation in AT2 KO mice.

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