APJ is a G-protein-coupled receptor with seven transmembrane domains, and its endogenous ligand, apelin, was identified recently. They are highly expressed in the cardiovascular system, suggesting that APJ is important in the regulation of blood pressure. To investigate the physiological functions of APJ, we have generated mice lacking the gene encoding APJ. The base-line blood pressure of APJ-deficient mice is equivalent to that of wild-type mice in the steady state. The administration of apelin transiently decreased the blood pressure of wild-type mice and a hypertensive model animal, a spontaneously hypertensive rat. On the other hand, this hypotensive response to apelin was abolished in APJ-deficient mice. This apelin-induced response was inhibited by pretreatment with a nitric-oxide synthase inhibitor, and apelin-induced phosphorylation of endothelial nitric-oxide synthase in lung endothelial cells from APJ-deficient mice disappeared. In addition, APJ-deficient mice showed an increased vasopressor response to the most potent vasoconstrictor, angiotensin II, and the base-line blood pressure of double mutant mice homozygous for both APJ and angiotensin-type 1a receptor was significantly elevated compared with that of angiotensin-type 1 receptor-deficient mice. The base-line blood pressure of APJ-deficient mice is highly conserved among species, suggesting its important physiological roles. Intravenous administration of apelin suggested a hypotensive effect in rat (5, 7–9). On the other hand, apelin potently contracts human saphenous vein smooth muscle cells in vitro (10), indicating that apelin is a potent vasoconstrictor. Thus, at this moment, the action of apelin in blood pressure regulation is controversial, and it is still unclear whether these actions of apelin are really through APJ because of the absence of specific receptor blocker to clarify the in vivo functions of APJ. Therefore, in this study, by using animal models such as APJ-deficient mice, APJ/AT1a double knock-out mice, and spontaneously hypertensive rat and by using endothelial cells from mice, we evaluated the functional importance of apelin-APJ signaling in the blood pressure regulation in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Gene Targeting and Generation of Mutant Mice—The genomic DNA containing the APJ locus were isolated from a phage library from C57BL/6J mice (11) with the human AT1 cDNA as a probe. To construct a targeting vector for the APJ gene, the 156-bp fragment of the mouse APJ gene between the NeoI site including the translation initiation codon of the gene and the Cap85I site was replaced with the nuclear localization signal-lucZ cassette. The neomycin phosphotransferase (neo) gene cassette derived from pMC1neoPolyA (Stratagene) was placed downstream of the nuclear localization signal-lucZ gene. The

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6.9-kb XhoI/NcoI fragment and the 1.6-kb Cep45I/Sau3AI fragment of the APJ gene were included upstream and downstream of these cassettes, respectively (Fig. 1A). Details of the negative selection with the diphtheria toxin-A cassette are described elsewhere (12). The TT2 ES cells were grown on embryonic fibroblast feeder cells as described previously (13). Homologous recombination in TT2 ES cells was detected by Southern blotting using probe a (687-bp BanIII-Sau3AI fragment). Chimeric mice were generated by injecting the ES cells into ICR 8-cell embryos (13, 14). AT1a-deficient mice were generated as described previously (11). Double knock-out mice for APJ and AT1a used in this study were generated from heterozygous mice after the crossing of single APJ-deficient and AT1a-deficient mice.

RNA Preparation and Northern Blot Analysis—Total RNA was isolated from liver, lung, heart, skeletal muscle, kidney, and brain with RNAeasy (Qiagen) using TRIzol (Invitrogen) and was further purified with ISOGEN (NipponGene) (15). Fifteen micrograms of RNA were denatured with glyoxal, separated by electrophoresis, and transferred to a nylon membrane. The 728-bp NcoI/NsiI fragment that corresponds to the coding regions of APJ was used as the APJ receptor-specific probe (probe b). Probes for mouse glyceraldehyde-3-phosphate dehydrogenase were described previously (16).

Measurement of Blood Pressure—The heart rate and systolic, mean, and diastolic blood pressures were measured by a programmable sphygmomanometer (BP-200, Softron, Japan) using the tail cuff method as described previously (17). Unanesthetized mice were introduced into a holder mounted in a thermostatically controlled warming plate and maintained at 37°C during measurement.

Intraperitoneal Injection of Apelin—Experiments were performed using 4-month-old male mice under the conscious and unrestrained conditions. [Pyr1]Apelin-13 (Peptide Institute 4361-v) was suspended in saline (0.9% NaCl in distilled water). After the measurement of the basal systolic blood pressure, [Pyr1]apelin-13 was administered by intraperitoneal injection at 285 μg/kg body weight and the systolic blood pressure was measured continuously. The data were calculated at 5-min intervals for 20 min after the administration of apelin.

Intravenous Injection of Apelin in Wistar-Kyoto (WKY) Rat and SHR—SHR and WKY rats at 12 weeks of age were anesthetized with sodium pentobarbital (35 mg/kg intraperitoneal). PE-10 catheters (Clay Adams) were inserted into the femoral and the right femoral arteries for measuring blood pressure and into the right femoral vein for allowing the administration of [Pyr1]apelin-13. The arterial catheter was connected to a pressure transducer (TP-200T, Nihon Kohden, Tokyo, Japan), and blood pressure was measured continuously. The anesthetic level was maintained by subcutaneous injection of 10 mg/kg pentobarbital every 40 min. [Pyr1]Apelin-13 dissolved in 0.1 ml of saline was administered through the vein catheter (2, 4, and 10 nmol/kg).

Pretreatment with Nω-Nitro-L-Arginine Methyl Ester (l-NANED) and Intraperitoneal Injection of Apelin in Wild-type and APJ-deficient Mice—Experiments were performed using 4-month-old male mice under the conscious and unrestrained conditions. l-NANED (Sigma) was suspended in saline (0.9% NaCl in distilled water). At 15 min after the administration of l-NANED, the additional administration of [Pyr1]apelin-13 (285 μg/kg body weight) or saline alone was performed by intraperitoneal injection and the systolic blood pressure was measured continuously. The maxima of systolic blood pressure responses to injections of apelin were calculated in a 0–5 min-post-injection of apelin.

Preparation of Endothelial Cells from Wild-type and APJ-deficient Mice—The lung of wild-type and APJ-deficient male mice at 11 weeks of age was perfused with 0.25% heparin/phosphate-buffered saline (−) and removed aseptically, rinsed in 0.25% heparin/phosphate-buffered saline (−1 × 2-mm squares), and digested in 20 ml of collagenase type I (4 mg/ml, Worthington) in serum-free DMEM containing antibiotic at 37°C for 60 min with shaking. The cellular digest was filtered through a sterile 40-μm nylon mesh and washed in 20 ml of serum-free DMEM, and the cell pellet was resuspended in 4 ml of serum-free DMEM. 2 ml of cell suspension were put into the tube containing 10% Percoll and centrifuged at 800 × g for 5 min. Contaminated vascular smooth muscle cells were stripped off physically as necessary. Confluent cells were passed routinely at a split ratio of 1–3 after trypsin/EDTA digestion and cultured under the same conditions. We ascertained the purity of endothelial cells by Western blotting with monoclonal anti-mouse CD-31 antibody (BD Biosciences) (data not shown).

Detection of Endothelial NO Synthase (eNOS) Phosphorylation—Isolated endothelial cells from wild-type and APJ-deficient mice were cultured in 6-well plates and stimulated by [Pyr1]apelin-13 (10 μM) or fetal bovine serum (10%) for 5 min, and the reaction was terminated by adding Laemmli buffer. The cell lysates were subjected to SDS/7.5% polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane (Millipore). After blocking with a blocking buffer containing 5% milk, the membrane was incubated with a polyclonal anti-human phospho-eNOS (Ser1177) antibody (Cell Signaling Technology) and bound antibody was detected by horseradish peroxidase-labeled anti-rabbit IgG (Amersham Biosciences) and chemiluminescence reagents (Perkin Elmer Life Sciences) to measure the phosphorylation of eNOS. After washing with a reprobing buffer of the composition (62.5 mM Tris-HCl (pH 6.7), 100 mM β-mercaptoethanol, and 2% SDS), the same membrane was subjected to Western blotting with a monoclonal anti-eNOS antibody (BD Transduction Laboratories) to detect the expression levels of eNOS as an internal control.

Treatment with Captopril and Intraperitoneal Injection of Angiotensin II—Systolic blood pressure was measured in conscious and unrestrained female mice at 4 months of age as mentioned above. The basal systolic blood pressure and the pressure responses against intraperitoneal injection of angiotensin II (10 and 30 μg/kg) were measured prior to the administration of captopril. After the administration of captopril (500 mg/liter in drinking water) for 1 week to inhibit the endogenous production of angiotensin II, the pressure responses to angiotensin II given from lower dose (3–30 μg/kg) were recorded continuously for 40 min after the administration of angiotensin II.

Statistical Analysis—The data were analyzed by Student’s t test for unpaired values. p < 0.05 was considered significant. Results are expressed as the means ± S.E.

RESULTS AND DISCUSSION

Generation of APJ-deficient Mice—To generate a null mutation at the mouse APJ gene locus, we designed a targeting vector that would replace a portion of the APJ coding region with the promoterless lacZ gene (Fig. 1A). After electroporation of TT2 cells with the targeting vector, homologous recombination was confirmed by Southern blotting (Fig. 1B, left panel). 10 independent cell lines of 195 G418-resistant cells had undergone homologous recombination at the mouse APJ locus. Eight clones were injected into ICR 8-cell embryos to generate chimeric mice, and two clones gave rise to germ line transmission by backcross mating with C57BL/6J mice. The heterozygous mice were intercrossed to produce homozygous offspring, and the mutation at APJ loci was detected by Southern analysis of tail DNA (Fig. 1B, right panel). Of the 396 offspring analyzed, 76 (19%) were homologous for the disrupted allele and 103 (26%) were wild type, indicating the normal embryonic development of the homozygous mutant mice. The histological sections of heart, lung, kidney, spleen, brain, ovary, skeletal muscle, liver, and white adipose did not reveal any differences in morphology between wild-type and heterozygous or homozygous mutant mice (data not shown). In the following study, to gain the equivalent effects of other gene backgrounds with the exception of for the APJ gene, we used these intercrossed littermates of heterozygous mice for further physiological experiments.

RNA Analysis—To determine whether APJ message was present in homozygous mutants, we performed Northern blot analysis of heart and lung RNA. Although heart and lung highly express the APJ gene in rodents (3), homozygous mutant mice had no detectable APJ message (Fig. 1C). A duplicate blot was analyzed with a glyceraldehyde-3-phosphate dehydrogenase probe to confirm that the RNA sample was intact. These results indicated that APJ transcripts were absent completely from homozygous mutant mice (APJ-deficient mice).

Measurement of Blood Pressure and Administration of Apelin—To ascertain whether APJ-mediated pathways participate in the regulation of the cardiovascular system, we measured the systolic blood pressure and heart rate under the steady
state. As shown in Fig. 2A, APJ-deficient mice and wild-type mice did not show any difference in the base-line systolic blood pressure (106.7 ± 2.0 and 105.9 ± 2.5 mm Hg, respectively) and the heart rate (632.0 ± 19.2 and 616.1 ± 32.8 cmap, respectively), suggesting that APJ is not essential for the maintenance of base-line blood pressure. Furthermore, it is reported that apelin is concerned in the regulation of drinking behavior (5, 7, 18), but the volume of water intake and the concentration of urinary electrolytes of APJ-deficient mice are not distinguishable from those of wild-type littermates when water is freely available (data not shown).

It has been reported that blood pressure was decreased transiently by the systemic administration of apelin, the endogenous ligand of APJ, in rat (5, 7–9). We administered apelin to APJ-deficient mice to ascertain whether these actions of apelin are really through APJ. Apelin is derived from a 77-amino acid precursor and processed to several isoforms by deleting the amino terminus (3, 4). The pyroglutamylated form of apelin-13, [pGlu]apelin-13, has been reported to have the effective activity at the receptor in vitro (3). Conscious male mice were intraperitoneally injected with [pGlu]apelin-13. The acute administration of apelin transiently and significantly decreased in the systolic blood pressure of wild-type mice (Fig. 2B). On the other hand, the apelin injection revealed no change in systolic blood pressure of APJ-deficient mice (Fig. 2C) without a change in heart rate as well as that of wild-type mice (data not shown). These results clearly demonstrate that the systemic administration of apelin lowers the blood pressure in wild-type but not in APJ-deficient mice and that APJ is really responsible for this action of apelin on the blood pressure regulation.

Administration of Apelin to Spontaneously Hypertensive Rat—Given that the activation of the apelin-APJ signaling pathways lowers the blood pressure under the steady state in mice (Fig. 2B) and rats (5, 7–9), is the hypotensive effect also evoked in the hypertensive conditions? To address this question, we administered apelin intravenously to a chronic hypertensive model animal, SHR, and measured continuously the arterial blood pressure. WKY rats were used as a control. Before the administration of apelin, the baseline mean blood pressure of WKY rats and SHR was measured (77 ± 4 mm Hg, n = 10, and 117 ± 2 mm Hg, n = 9, respectively). When apelin was injected into the normotensive WKY rats, a dose-dependent and significant decrease in mean arterial blood pressure was elicited (Fig. 2D, closed bar) as reported previously (5, 7–9). The intravenous administration of apelin to SHR was found to significantly lower the mean arterial blood pressure in a dose-dependent manner (Fig. 2D, open bar). Thus, the hypotensive effect by the systemic administration of apelin was evoked in hypertensive animals, but the degree of decrement was less than that of WKY rats. The effects of apelin on blood pressure regulation in the hypertensive model animals have not been explored previously to date, although it has been recently reported that apelin-APJ signaling pathways were down-regulated in the mechanical stretch models in vitro, in the animal models of chronic ventricular pressure overload, and in patients with chronic heart failure in vivo (19–21). In addition, the angiotensin-converting enzyme-related carboxypeptidase (ACE2), a zinc metalloprotease whose closest homolog is the angiotensin I-converting enzyme, was identified as the breakdown enzyme for apelin peptides (22). The reduction of apelin-induced hypotensive effects in SHR compared with WKY rats might be attributed to the differences in the balance of the production and degradation of apelin and in the sensitivity of APJ-mediated intracellular signalings including receptor desensitization.

Effects of a Nitric Oxide Synthase Inhibitor on the Action of Apelin Administration—Tatemoto et al. (8) suggest that apelin causes vasodilatation via the activation of the nitric oxide (NO)/l-arginine system. NO generated by eNOS has a central role in the regulation of vascular tone. Therefore, we examined the effects of a nitric-oxide synthase inhibitor, l-NNAME, against the depressor response of apelin-APJ signaling observed in wild-type mice. After a single intraperitoneal bolus injection (10 mg/kg body weight) of l-NNAME, the systolic blood pressure increased similarly from 111.0 ± 2.2 to 142.3 ± 2.9 mm Hg and from 108.1 ± 2.4 to 142.4 ± 2.5 mm Hg in wild-type and APJ-deficient mice (n = 7–8/group), respectively. This increase in systolic blood pressure with the administration of l-NNAME was described previously in rat, and the systolic blood pressure of each group reached a plateau at around 10 min after administration (data not shown). Accordingly, we injected the apelin peptide at 15 min after l-NNAME administration. The injection of apelin induced an acute and transient decrease in systolic blood pressure in the non-treated wild-type mice. In contrast, the administration of the same dose of apelin caused almost no change in systolic blood pressure in wild-type mice pretreated...
with L-NAME (Fig. 3A, closed bar). The systolic blood pressure in APJ-deficient mice was not changed by the apelin peptide injection regardless of L-NAME administration (Fig. 3A, opened bar), suggesting that the suppressive action point of L-NAME against the hypotensive effect observed in wild-type mice by apelin injection exists under the APJ-mediated signalings. Tatemoto et al. (8) also report that the systemic administration of apelin significantly increased the plasma NOx concentration in rats, whereas the increase in the NOx concentration was not observed in the rats pretreated with L-NAME. These findings provided a possibility that the depressor action of apelin-APJ signaling is the result of stimulation of the NO production.

Apelin-mediated Ser1176 eNOS Phosphorylation in Endothelial Cells from Mice—eNOS has a crucial role in the regulation of vascular tone. It was reported that eNOS is activated by a variety of physiological and pathophysiological stimuli, including hormones and growth factors, and by mechanical stimuli. eNOS is activated by phosphorylation at the Ser1177 residue (based on the human eNOS sequence and is equivalent to bovine eNOS-Ser1179 and mouse eNOS-Ser1176), the best characterized eNOS phosphorylation site, that is phosphorylated by protein kinase Akt, a downstream mediator of phosphatidylinositol 3-kinase, and greatly contributes to the eNOS activation (23–25). To determine whether apelin-APJ signaling can actually activate eNOS, the endothelial cells derived from wild-type and APJ-deficient mice were stimulated with apelin and phosphorylation of eNOS at Ser1176 residue was assessed by using a phosphorylation state-specific eNOS antibody. As shown in Fig. 3B, eNOS in the endothelial cells from wild-type and APJ-deficient mice was activated significantly by serum stimulation (gray bar) compared with non-treated cells (open bar). Interestingly, eNOS Ser1176 phosphorylation by apelin stimulation was promoted in wild-type endothelial cells but not in APJ-deficient endothelial cells (closed bar). This is the first report that apelin can activate eNOS in endothelial cells and that APJ plays a critical role in apelin-induced phosphorylation of eNOS, which potentializes the hypotensive effect on blood pressure regulation.

Association of Mouse APJ with Pressor Action of Angiotensin II—In blood pressure regulation, the vasoconstriction and vasorelaxation systems constantly antagonized each other to maintain normal blood pressure. To test whether APJ plays a role in blood pressure regulation as a counterregulatory component against vasopressor actions, we performed the systemic angiotensin II administration and measured the systolic blood pressure of wild-type and APJ-deficient mice. The intraperitoneal injection of pharmacological doses of angiotensin II (10 and 30 μg/kg) resulted in a similar increase in systolic blood pressure in both groups (Fig. 3A), suggesting that the pressor response mediated by AT1 in the steady state was not affected by the disruption of the APJ gene.

We next used a different protocol for angiotensin II administration to clarify the APJ function in blood pressure regulation. Hein et al. (26) previously evaluated the role of angioten-
sin-type 2 receptor (AT2) in blood pressure regulation as a counterregulatory component against vasopressor actions by administrating captopril to AT2-deficient mice. We pretreated mice with captopril, the inhibitor of angiotensin-converting enzyme, for 1 week to block the production of endogenous angiotensin II and then continuously injected lower and more physiological doses of angiotensin II. The captopril-treated wild-type mice revealed an increased systolic blood pressure by the intraperitoneal injection of angiotensin II (above 100/92 mmHg). On the contrary, the captopril-treated APJ-deficient mice revealed a significant increased sensitivity to low dose angiotensin II (3 and 10 μg/kg) compared with wild-type littermates (Fig. 3A, captopril). In making another attempt to clarify a role of APJ in blood pressure regulations, we generated double knock-out mice for APJ and AT1a by crossing APJ-deficient and AT1a-deficient mice. As shown in Fig. 4B, AT1a-deficient mice displayed marked hypotension compared with that of wild-type mice as described previously (11). Under the attenuated conditions to the vasopressor actions on the AT1a-deficient background, the base-line blood pressure of double mutant mice homozygous for both APJ and AT1a was elevated significantly compared with that of AT1a-deficient mice (81.6 ±

**Fig. 3.** Effect of L-NAME pretreatment on apelin-induced blood pressure responses and activation of eNOS in endothelial cells from mice. A, apelin was injected to the wild-type mice (filled bar) and APJ-deficient mice (open bar) with or without pretreatment of L-NAME (n = 7–8/group). Change in SBP, change in systolic blood pressure. Data are means ± S.E. *, p < 0.05 by unpaired Student’s t test. B, apelin stimulates phosphorylation of eNOS at the Ser^1176 residue. Isolated endothelial cells from wild-type and APJ-deficient mice were treated with apelin or fetal bovine serum (FBS) for 5 min. Cell lysates were prepared and analyzed by Western blotting. The membrane was first probed with anti-phosphorylated eNOS antibody (eNOS-p) and then successively with anti-eNOS antibody after stripping (eNOS). The ratio of the intensity of phosphorylated eNOS to that of eNOS (eNOS-p/eNOS) was determined using NIH image, and the ratio of the sample with no stimulation was taken as 1.0. Representative results are shown for three independent experiments. Data are means ± S.E. *, p < 0.05 compared with control.

**Fig. 4.** Association of mouse APJ with pressor action of angiotensin II. A, systolic blood pressure responses of wild-type and APJ-deficient mice by the administration of angiotensin II. Systolic blood pressures in the 5-min postinjection of angiotensin II were indicated. Filled bars, wild-type (+/+) mice; open bars, APJ-deficient (−/−) mice; Captopril, the administration of an angiotensin-converting enzyme inhibitor to prevent endogenous production of angiotensin II; Basal, systolic blood pressure prior to the administration of captopril. B, base-line systolic blood pressure in 3-month-old wild-type (closed bar), AT1a-deficient (open bar), and double mutant mice homozygous for both APJ and AT1a (grayed bar) under conscious conditions (n = 12–17/group). Data are means ± S.E. *, p < 0.05; **, p < 0.01 by unpaired Student’s t test.
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4.0 and 75.8 ± 2.3 mm Hg, respectively; p < 0.05). These data indicated that APJ has a potential ability to lower the blood pressure. Previously, it was demonstrated that AT2-deficient mice exhibited the increase in sensitivity to low dose angiotensin II-induced vasopressor action (26, 27). Likewise, APJ may function as a member of the vasorelaxation system in blood pressure regulation against the vasoconstriction system including angiotensin II-induced AT1 signaling.

In conclusion, the inactivation of the APJ gene in mice by gene targeting is responsible for the hypotensive effect of apelin in adult mice in vivo and plays a counterregulatory role against vasopressor stimulation. In addition, by using primary endothelial cells derived from mice, it is suggested that the hypotensive effect induced by apelin-APJ signaling is mediated through the NO/L-arginine system in vivo.

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