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Rapid Communication

Cooperative action of APJ and α1A-adrenergic receptor in vascular smooth muscle cells induces vasoconstriction

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The apelin receptor (APJ), a receptor for apelin and elabela/apela, induces vasodilation and vasoconstriction in blood vessels. However, the prolonged effects of increased APJ-mediated signalling, involving vasoconstriction, in smooth muscle cells have not been fully characterized. Here, we investigated the vasoactive effects of APJ gain of function under the control of the smooth muscle actin (SMA) gene promoter in mice. Transgenic overexpression of APJ (SMA-APJ) conferred sensitivity to blood pressure and vascular contraction induced by apelin administration *in vivo*. Interestingly, *ex vivo* experiments showed that apelin markedly increased the vasoconstriction of isolated aorta induced by noradrenaline (NA), an agonist for α- and β-adrenergic receptors, or phenylephrine, a specific agonist for α1-adrenergic receptor (α1-AR). In addition, intracellular calcium influx was augmented by apelin with NA in HEK293T cells expressing APJ and α1A-AR. To examine the cooperative action of APJ and α1A-AR in the regulation of vasoconstriction, we developed α1A-AR deficient mice using a genome-editing technique, and then established SMA-APJ/α1A-AR-KO mice. In the latter mouse line, aortic vasoconstriction induced by a specific agonist for α1A-AR, A-61603, were significantly less than in SMA-APJ mice. These results suggest that the APJ-enhanced response requires α1A-AR to contract vessels coordinately.

Keywords: apelin; APJ; α1A-AR; vasoconstriction; GPCRs.

Abbreviations: APJ, apelin receptor; BiFC, bimolecular fluorescence complementation; ET-1, endothelin-1; FDSS, Functional Drug Screening System; HR, heart rate; MAP, mean arterial pressure; MLC, myosin light chain; MLCP, MLC phosphatase; NA, noradrenaline; PE, phenylephrine; ROCK, RhoA kinase; SBP, systolic blood pressure; SMA, smooth muscle actin; SR, synchrotron radiation; VC, Venus-C terminal fragment; VEC, vascular endothelial cells; VN, Venus-N terminal fragment; VSMC, vascular smooth muscle cells.

The apelin receptor (APJ) is a G-protein-coupled receptor that is widely distributed in the body, with especially high expression in cardiovascular tissue (*1*, *2*). Apelin, an endogenous ligand of APJ, was identified from bovine stomach (*3*). Recently, a new ligand, elabela (also known as apela or toddler), was found (*4*). Apelin induces cell proliferation and migration (*5*), and promotes haematopoiesis from human embryonic stem cells (*6*). Additionally, it was recently reported that apelin reversed age-associated sarcopenia (*7*). These functions indicate that apelin is involved in developmental processes and modulation of various physiological functions.

A number of studies have mentioned the binding of elabela to APJ with physiological relevance in both similar and different manners to apelin (*8*–*10*). The loss of elabela in mice also causes low penetrance embryonic lethality and defects in early mesodermal derivatives (*11*). Elabela has been reported to regulate abnormally shallow placentation via APJ, and is thought to be a potential diagnostic biomarker of pre-eclampsia, a pregnancy-specific syndrome of gestational hypertension and a cause of maternal mortality and morbidity (*12*, *13*). These observations suggest that the apelin/elabela/APJ system regulates diverse signalling under normal and pathological conditions.

As many studies to date have shown that apelin and APJ are highly expressed on vascular endothelial cells (VECs) (*2*, *14–16*), a growing body of research has examined the role of apelin/APJ-mediated action on endothelium function. Previously, we used APJ-deficient mice to demonstrate that APJ lowered blood pressure and evoked nitric oxide (NO) production in an apelin-dependent manner (*17*). However, APJ is localized to the medial layer of human blood vessels, and apelin causes intense contractions in endothelial cell-denuded saphenous-vein smooth muscle, indicating that it acts as a potent vasoconstrictor (*18*). Furthermore, apelin stimulation increased myosin light chain (MLC) phosphorylation, the rate-limiting event for vascular contraction, in rat vascular smooth muscle cells (VSMCs) (*19*). Interestingly, we noted that apelin exerts vasoconstrictor effect under pathological conditions, contributing to vascular endothelial dysfunction in mice (*20*). Although these reports suggest that the apelin/APJ system mediates a vasoconstrictor action, the function of APJ in VSMCs has not been fully characterized *in vivo*.

In this study, we established the significance of APJ in the regulation of vascular contraction using transgenic mice that overexpress APJ in VSMCs (SMA-APJ). SMA-APJ mice showed transient and intense elevation of blood pressure as a consequence of apelin injection. Remarkably, this APJ function in VSMCs required α1A-adrenergic receptor (α1A-AR), because pharmacological and genetic ablation of α1A-AR eliminated the vascular contractile action induced by the co-stimulation with apelin and an α1A-AR-specific agonist in SMA-APJ mice. Based on the functional interaction of these two receptors, we provide evidence that APJ and α1A-AR play a concerted role in increasing vascular contractility in VSMCs.

Materials and Methods

Generation of genetically modified mice

A 4.7 kb DNA fragment of the human smooth muscle α-actin (SMαA) promoter (*21*) and murine APJ *(Agtrl1)* cDNA were amplified by PCR and cloned into the pBSKS (−) vector. The linearized 7.2 kb DNA fragment was microinjected into the pronuclei of fertilized oocytes (ICR strain). The heterozygous mice were intercrossed to produce homozygous offspring (SMA-APJ mice). α1A-AR deficiency was induced by a frameshift mutation close to the start codon of *adra1a* gene. For the α1A-AR knock-out allele, the oligonucleotides were amplified, annealed and ligated into the *Bbs*I restriction site of pX330 vector (#42230; Addgene, Watertown, MA, USA) using following primers: 5′-CACCGCCGATGACAGGCCACCG AG-3′ and 5′-AAACCTCGGTGGCCTGTCATCGGC-3′. The plasmids were microinjected into the pronuclei of fertilized oocytes of C57BL/6J strain (Charles River Laboratories Japan, Yokohama, Japan). The founder offspring was screened by PCR of tail genome for detecting the WT allele using the following primers: 5′-TTCCTC AGGCTCACGTTTCC-3′ and 5′-AGGCCACCGAGAGGATCA-3′; for detecting KO allele: 5′-GGTGGCTTTCACAGCATGTC-3′ and 5′-GAGTGCAGATGCCGATGATATTTAGG-3′. These mice were backcrossed to the ICR strains CLEA Japan, Tokyo, Japan three times, and mated to the SMA-APJ strains. Mice were maintained in the Life Science Center for Survival Dynamics, Tsukuba Advanced Research Alliance (TARA)-SPF space, in an environment of 22°C, humidity of 40–60%, light and dark cycle every 12 h, and food and tap water were provided *ad libitum*.

All animal experiments in this study were carried out humanely after approval from the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were performed in accordance with the Regulation of Animal Experiments of the University of Tsukuba, and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Preparation of mouse aorta and extraction of total RNA

Mouse aortae were quickly frozen in liquid nitrogen, after perivascular lipids were removed. Total RNA was extracted using the RNAgents Total RNA Isolation System kit (Promega, Madison, WI, USA). A Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) was used to crush frozen aortae to a powder. It was suspended in denaturing solution and mixed with phenol-chloroform. Ethachinmate (Nippon Gene, Tokyo, Japan) was added to the supernatant and it was collected by the centrifugation (17,800 *g* at 4°C for 20 min). Total RNA was finally precipitated by ethanol.

Gene expression analysis by quantitative RT-PCR

Approximately 1 μg of total RNA was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Using a Thermal Cycler Dice and SYBR Premix Ex Taq II (TaKaRa Bio, Kusatsu, Japan), real-time quantitative PCR reactions were performed. Expression levels of the target gene were normalized to *Gapdh* expression levels using the ΔΔCt method. The amplification efficiency of primers was checked to be equal by using serial dilutions of cDNA for each target gene. The following primers were used for amplification: *Gapdh*, 5′-TGTGTCCGT CGTGGATCTGA-3′ and 5′-TTGCTGTTGAAGTCGCAGGAG-3′; APJ, 5′-CCTTCTAGGT GTGCCTGTCATG-3′ and 5′-CACTGGATCTTGGTGCCATTT-3′; *Adra1a* (α1A-AR), 5′-GCGGTGGACGTCTTATGCT-3′ and 5′-TCACACCAATGTATCGGTCGA-3′; *Adra1b* (α1B-AR), 5′-CC TGGTCATGTACTGCCGA-3′ and 5′-GACTCCCGCCTCCAGA TTC-3′ and *Adra1d* (α1D-AR), 5′-TGCAGACGGTCACCAACT ATTT-3′ and 5′-GGCAACACAGCTGCACTCAG-3′.

Blood pressure and heart rate measurements before and after intraperitoneal injection of Apelin or A-61603, an α1A-AR ligand

Systolic blood pressure (SBP) and heart rate (HR) were measured using a programmable sphygmomanometer (BP98A, Softron, Tokyo, Japan) by the tail-cuff method, as described previously (*22*, *23*). [Pyr^1]apelin-13 (Peptide Institute, Tokyo, Japan) and A-61603 (#1052, TOCRIS, Ellisville, Mo, USA) were suspended in saline (Otsuka Pharmaceutical, Tokyo, Japan). After the basal SBP was measured, [Pyr^1]apelin-13 (296 μg/kg body weight) or A-61603 (19.7 μg/kg body weight) was intraperitoneally injected. SBP was measured continuously for approximately 5 min, and data were collected every 20 s.

In vivo imaging at the SPring-8 Synchrotron

SMA-APJ transgenic mice and sibling WT mice at 14 weeks of age were surgically prepared for coronary microangiography at beamline BL28B2 of the Japan Synchrotron Radiation Research Institute (SPring-8, Sayo-gun, Hyogo, Japan) as described previously (*24*). All imaging experiments were performed with the approval of the SPring-8 Animal Experiment Review Committee (2012A1631) and the National Cerebral and Cardiovascular Center Animal Experiment Committee. Mice were injected intraperitoneally with pentobarbital (1:10 diluted solution, 50 mg/kg) to induce general anaesthesia and supplementary doses were periodically administered to maintain the level of anaesthesia (~20 mg/kg). Subsequently, mice were intubated for artificial ventilation (40% oxygen, 6 μl/g tidal volume and ~170–190 breath/min; Harvard Hugo-Sachs Minivent, Germany) and the right jugular vein was cannulated with polyethylene tubing to enable a continuous infusion of sodium lactate Ringer's solution (Otsuka, Tokyo, Japan) for the maintenance of body fluids. With a polyurethane catheter (Instech-Solomon FunnelCath^{TM} PUFC-C30-10), the right carotid artery was cannulated for the monitoring of arterial blood pressure and HR throughout experiments. Body temperature was maintained at 37–38°C level with the use of rectal thermistor and thermostatically controlled heating pad. From the carotid arterial line, blood pressure was recorded using a disposable pressure transducer (MLR0699, AD Instruments, NSW, Australia). The analogue signal was digitized at 1,000 Hz and recorded with CHART software (version 5.4.1, AD Instruments, NSW, Australia) to obtain mean arterial pressure

and HR. During surgery and subsequent imaging, the mouse was taped securely on a thin acrylic board in a supine position.

Cine-angiography

Imaging was performed with monochromatic synchrotron X-ray radiation at 33.2 keV (energy bandwidth 20–30 eV), just above the iodine K-edge to produce maximal absorption contrast of the iodinated medium in the vessel lumen 1. A high X-ray flux $\sim 10^{12}$ photons/mm^2/s passed through the mouse chest to permit recording of cine-frames (1–2 ms shutter open times) with a resolution of 10-bit at 30 ms intervals utilizing the SATICON detector system (Hitachi Denshi Techno-System Ltd., Tokyo, Japan). High-resolution images were stored in a digital frame memory system with $1{,}024 \times 1{,}024$ pixel format with a 9.9 μm pixel size and a field of view of 10 mm^2. Between recordings the X-ray beam was blocked with filters. After verification of the catheter tip placement in front of the aortic valve, by using a syringe pump (PHD-2000, Harvard Apparatus, Holliston, MA, USA), contrast agent was injected remotely through the carotid catheter as a bolus (100–200 μl over 2 s; Iomeron 350; Bracco-Eisai, Tokyo, Japan). Image acquisition was initiated right before iodine contrast injection and ~100 frames were recorded for each scan. Contrast angiograms of the mouse hearts were made during a vehicle infusion (sodium lactate Ringer's solution) for baseline, followed by repeated imaging 12 min after an intraperitoneal injection of apelin (296 μg/kg body weight). Subsequently mice were euthanized by pentobarbital overdose (100 mg/kg i.v). Coronary arterial vessel internal diameter and the number of vessels visualized in the same field of view was determined.

Measurement of aortic isometric tension

Aorta rings were excised from mouse thoracic parts in ice-cold Krebs-Henseleit buffer (NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2$, 1.8 mM; NaH_2PO_4, 1.8 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM and glucose, 11.1 mM) and were anaesthetized with isoflurane. About 3-mm sections of dissected rings were mounted using an Easy Magnus System (Kishimoto Medical Instruments, Kyoto, Japan) as described previously, and these rings mounted to the chamber were soaked and equilibrated for 1 h under passive tension of 35 mN in Krebs-Henseleit buffer gassed with 95% O_2/5% CO_2 at 37°C. To optimize constriction, the resting tension was stimulated three times by 60 mM KCl, and once with 80 mM KCl. Following agents were utilized to contract the rings with noradrenaline (NA, #489350, Merck, Darmstadt, Germany), phenylephrine (PE, #163-11791, FUJIFILM Wako Pure Chemical Co., Osaka, Japan), endothelin (#4198-S, Peptide Institute, Tokyo, Japan), angiotensin II (#4001, Peptide Institute, Tokyo, Japan) and A-61603 (#1052, TOCRIS, Ellisville, Mo, USA) with or without apelin. RS100219 (#1325, TOCRIS, Ellisville, Mo, USA) and BMY7379 (#1006, TOCRIS, Ellisville, Mo, USA) were used for the inhibition of α1A-AR and α1D-AR, respectively.

Intracellular calcium influx measurement

Plasmids of various APJ and α1-AR subtypes were transfected into HEK293T cells, and transfected cells were cultured for 48 h. Adherent cells in culture were harvested with 0.02% EDTA/PBS and washed twice with HEPES-Hank's buffer. Collected cells were suspended in 0.1% BSA/HEPES-Hank's buffer and counted using a TC10^{TM} automatic cell counter (Bio-Rad, Hercules, CA, USA). To the cell suspensions, Fura2-AM (1 mM) was added and incubated for 40 min at 37°C with gentle shaking. After loading with Fura2-AM, cells were washed and suspended in 0.1% BSA/HEPES-Hank's buffer, and Fura2-loaded cells and ligands were plated in 96-well plates. Fura2 fluorescence was detected and quantified using a Functional Drug Screening System (FDSS) (Hamamatsu Photonics, Hamamatsu, Japan).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 for Mac (GraphPad Software Co, San Diego, USA). Student's t-test, the Mann-Whitney U test or one-way ANOVA followed by post hoc test or Fisher's Least Significant Difference (LSD) test was used to evaluate the significance of differences between groups, as appropriate. Results with $P < 0.05$ were considered statistically significant.

Results

Blood pressure elevation induced by apelin administration in SMA-APJ mice

To evaluate the enhanced input of APJ in VSMCs, we generated a transgenic mouse line in which APJ was expressed under the control of the human smooth-muscle α-actin (SMαA) promoter, called SMA-APJ mice (Fig. 1A). To determine APJ gene expression levels, we performed quantitative RT-PCR using mRNA extracts from the aorta of SMA-APJ, and found that the APJ gene expression was markedly increased in SMA-APJ compared with that of wild type (WT) mice (Fig. 1B). This result indicated that this line of transgenic mice overexpresses APJ in VSMCs. We next investigated the changes in the circulatory system following apelin administration. First, we measured SBP by the tail-cuff method. To activate APJ signalling *in vivo*, we intraperitoneally injected apelin into WT and SMA-APJ mice. SBP in SMA-APJ mice was slightly but statistically lower than that in WT mice during basal conditions before apelin injection (Supplementary Figure S1A). Although WT mice exhibited a hypotensive response after apelin administration, SBP was transiently and significantly increased in SMA-APJ mice (Fig. 1C). HR in SMA-APJ mice was not different from that in WT mice before apelin injection (Supplementary Figure S1B). After apelin administration, HR in WT mice was not changed, but it was significantly decreased in SMA-APJ mice (Fig. 1D). These results suggested that enhanced APJ in VSMCs induced sustained vasoconstriction leading to blood pressure elevation, and depressed HR.

In vivo imaging of changes in vascular contraction in SMA-APJ mice

To examine whether blood vessels in SMA-APJ mice contract after apelin stimulation in the body, we investigated the changes in vascular contraction in SMA-APJ mice, when apelin was administered, using high-intensity synchrotron radiation (SR) at SPring-8 (*24*, *25*). In contrast-enhanced images of the coronary vessels, the vessels were clearly visible in WT mice, while those in SMA-APJ mice showed pronounced constriction and increased resistance to contrast entry at 12 min after apelin administration (Fig. 2A). There were no differences in the internal diameter of vessels between WT and SMA-APJ mice before apelin administration (Fig. 2B). The relative changes in calibre and vessel number were significantly decreased in SMA-APJ mice across the coronary vasculature from large arteries to coronary microvessels (Fig. 2C and D). These results demonstrated that apelin contracts the blood vessels of SMA-APJ mice *in vivo*.

Intense vascular contraction following sustained stimulation with apelin and α1-adrenergic receptor agonist

In order to understand the detailed mechanisms of blood pressure elevation and vascular contraction by apelin administration in SMA-APJ mice, we used an *ex vivo* assay for examining the involvement of APJ

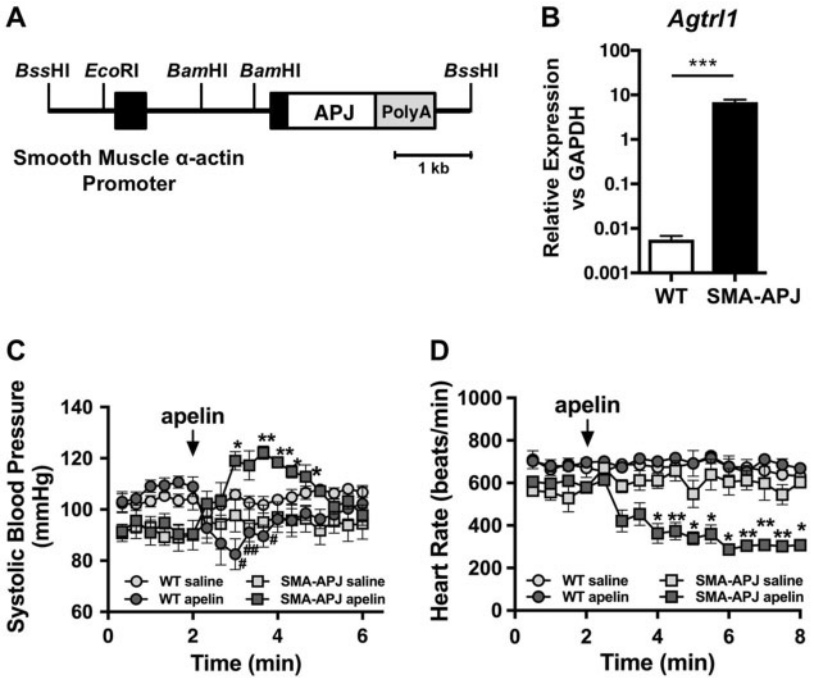

Fig. 1 Generation of a transgenic mouse line that overexpresses APJ under the control of the human smooth muscle α action promoter. (**A**) Structure of the transgene and partial restriction map (Bs, *Bss*HII; B, *Bam*HI; E, *Eco*RI). (**B**) mRNA expression of the APJ gene (*Agtrl1*) in aortae from WT and SMA-APJ mice ($n = 11$–13). Statistical differences were determined using Mann-Whitney U test (***$P < 0.001$ as compared with WT mice). (**C**) Changes in SBP in WT and SMA-APJ mice after saline or apelin administration ($n = 4$–9). Statistical differences were determined using Mann-Whitney U test ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$ as compared with saline-treated WT mice, and $*P < 0.05$; $**P < 0.01$ as compared with saline-treated SMA-APJ mice). (**D**) Changes in HR in WT and SMA-APJ mice after saline or apelin administration ($n = 4$–9). Statistical differences were determined using Mann-Whitney U test ($*P < 0.05$; $**P < 0.01$ as compared with saline-treated SMA-APJ mice).

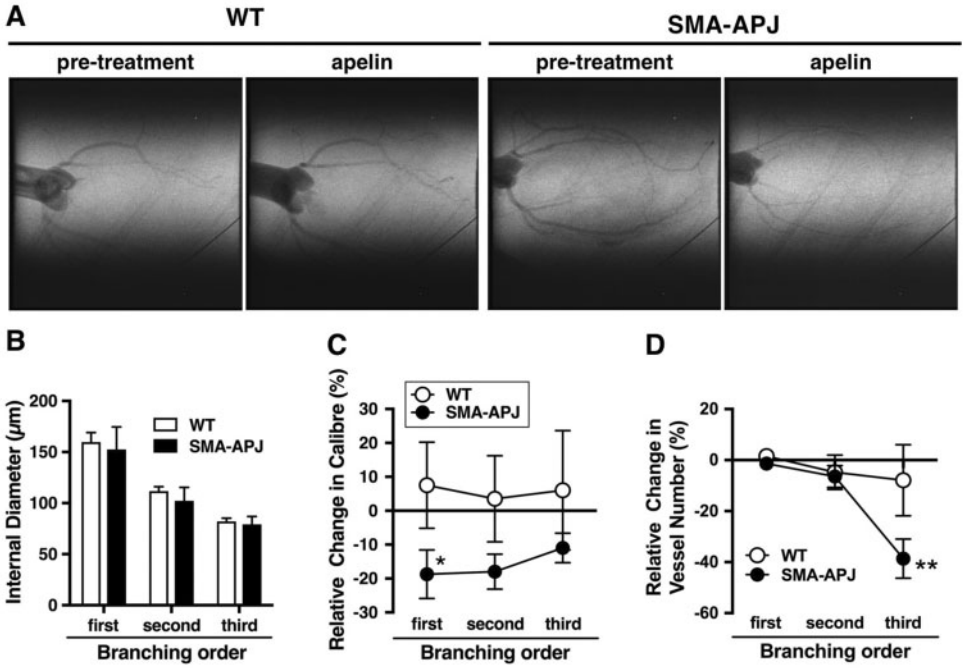

Fig. 2 Coronary artery angiography in WT and SMA-APJ mice using the high intensity SR at SPring-8. (**A**) Representative angiographic images from WT and SMA-APJ mice. A catheter was inserted into the mouse carotid artery for apelin administration. Images were obtained at baseline (pre-treatment) and at 12 min after apelin injection. Arterial vessel internal diameters at baseline across the first four branching orders of vessels from large arteries to arterioles (**B**), the relative change in internal diameter at 12 min post-apelin administration (**C**) and relative changes in vessel number (**D**) in coronary artery in WT and SMA-APJ mice ($n = 4$). Statistical differences were determined using one-way ANOVA followed by post hoc test or Fisher LSD test ($*P < 0.05$; $**P < 0.01$ as compared with WT mice). A 50 μm tungsten wire used for vessel internal diameter calibration is indicated in the bottom right corner of each image.

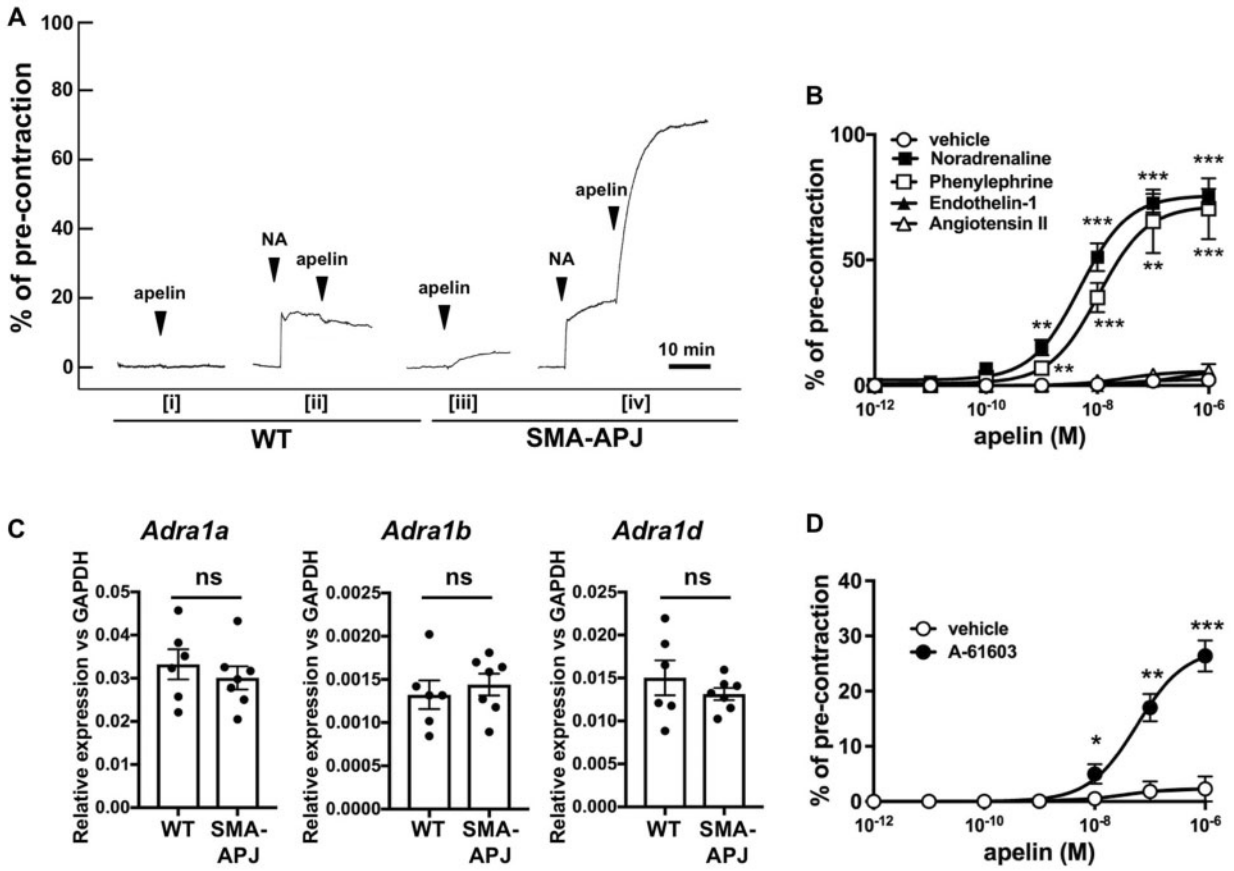

Fig. 3 Intense vascular contraction following continuous stimulation with apelin and α1 adrenergic receptor agonist *ex vivo*. (**A**) Changes in vascular contraction in the aortae from WT and SMA-APJ mice in response to apelin (10^{-7} M) (i and iii) or to NA (10^{-7} M) and apelin (ii and iv). (**B**) Changes in vascular contractile responses of aortae from SMA-APJ mice for vasoactive ligands (10^{-7} M) with apelin (n = 5–9). Statistical differences were determined using Student's t-test (**$P < 0.01$; ***$P < 0.001$ as compared with vehicle). (**C**) mRNA expression of the α1A-, α1B- and α1D-AR genes in aortae from WT and SMA-APJ mice (n = 6–7). Statistical differences were determined using Student's t-test (ns: not significant as compared with WT mice). (**D**) Changes in vascular contractile responses of aortae from SMA-APJ mice for A-61603 (10^{-5} M) with apelin (n = 5). Statistical differences were determined using Student's t-test or Mann-Whitney U test (*$P < 0.05$ **$P < 0.01$; ***$P < 0.001$ as compared with vehicle).

signalling in vascular contraction. First, we measured vascular contraction following addition of apelin, and observed that aortae of SMA-APJ mice contracted (Fig. 3A, iii) whereas those of the WT did not (Fig. 3A, i). Interestingly, we discovered that co-stimulation with apelin and NA-induced intense vascular contraction in aortae from SMA-APJ mice (Fig. 3A, iv), while this response was undetectable in aortae from WT mice (Fig. 3A, ii).

Next, to determine whether other vasoconstrictors interact with apelin and induce this signature reaction in aortae from SMA-APJ mice, we attempted to simultaneously stimulate aortic rings with apelin and several typical vasoconstrictors: NA, PE, endothelin-1 (ET-1) and angiotensin II (Ang II). Neither Ang II nor ET-1 induced potentiated contraction, but PE was as effective as NA (Fig. 3B). Because NA and PE are both agonists of α1-adrenergic receptors (α1-ARs), we hypothesized that the expression levels of α1-ARs might differ between WT and SMA-APJ mice. To test this, we analysed the gene expression of all subtypes of α1-ARs (α1A, α1B and α1D) in aortic tissues from mice, and confirmed no differences between WT and SMA-APJ mice in the expression levels of any α1-AR subtype (Fig. 3C). In addition, co-treatment with apelin and A-61603, a specific agonist for α1A-AR, induced dose-dependent intense vascular contraction in aortae from SMA-APJ mice (Fig. 3D). These results indicated that α1A-AR is a key molecule mediating vascular contraction in a coordinated manner with APJ on VSMCs.

Intense vascular contraction mediated primarily through calcium signalling in response to APJ

It is known that apelin and NA both elevate the level of intracellular calcium (*26*, *27*), a main mechanism for vasoconstriction in VSMCs. In addition, reversible phosphorylation of MLC by MLC phosphatase (MLCP) and by Ca^{2+}-dependent MLC kinase are key events in the molecular mechanism of vascular contraction. RhoA, a small G-protein that belongs to the small G-protein family, subsequently activates RhoA kinase (ROCK), and these signals negatively control MLCP phosphatase activity. To investigate which signal is preferred in vascular contraction induced by co-stimulation of apelin and NA, we

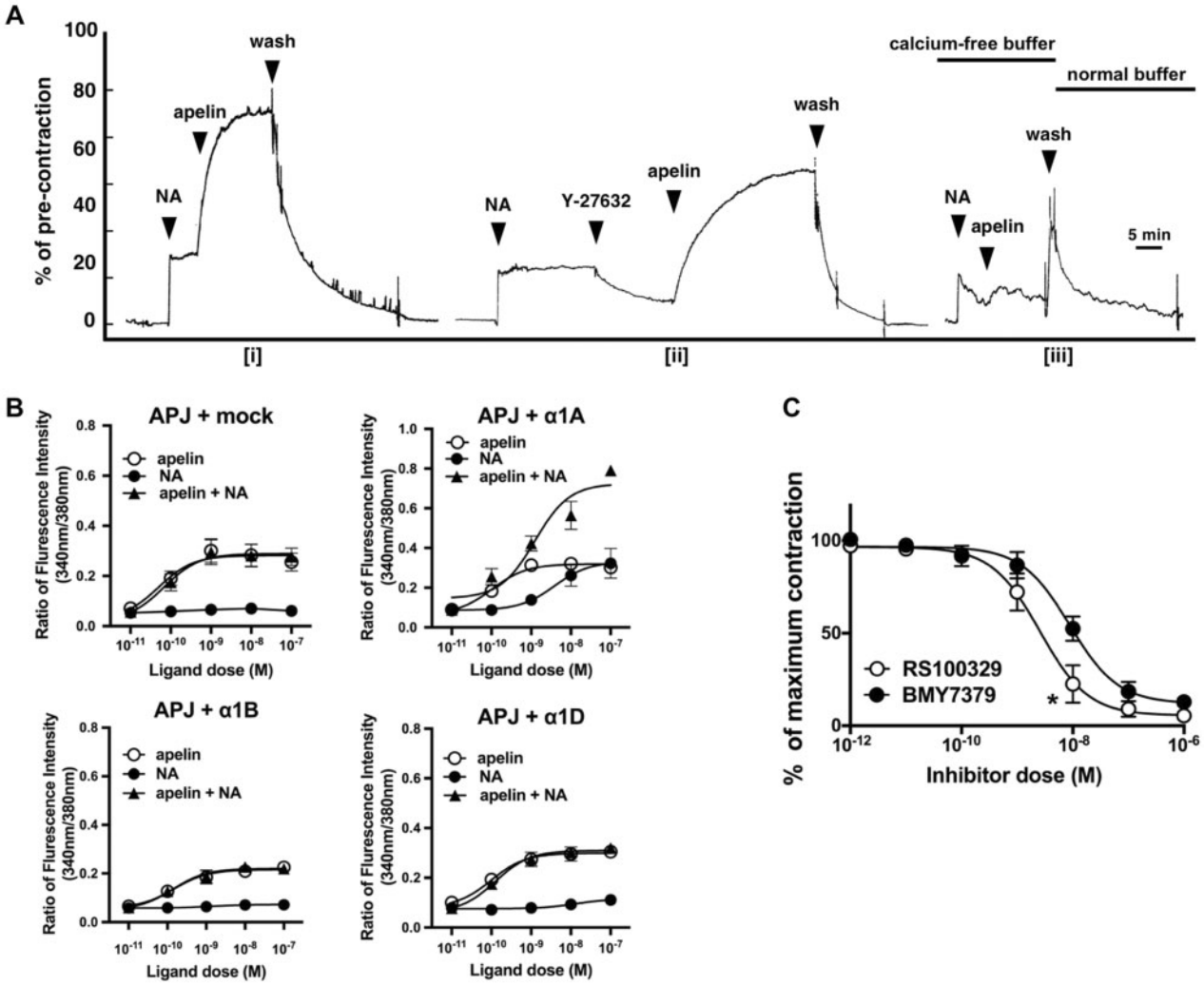

Fig. 4 Effect of the inhibition of calcium and Rho/ROCK signals on vascular contraction responses to apelin and NA in aortae from SMA-APJ mice. (**A**) Changes in vascular contraction by apelin (10^{-7} M) and NA (10^{-7} M) (i), with Y-27632 (10^{-7} M) treatment (ii), and under the condition with calcium-free buffer (iii). (**B**) Changes in intracellular calcium influx in cells co-expressing APJ and each α1-AR subtype. Fura2-AM, a calcium-dependent fluorophore, was loaded in co-expressing cells, and fluorescence was measured by FDSS ($n = 4$). (**C**) Changes in maximum contraction induced by apelin (10^{-7} M) and NA (10^{-7} M) simultaneous with RS100329 or BMY7378 in aortae from SMA-APJ mice ($n = 6$–7). Statistical differences were determined using Student's t-test (*$P < 0.05$ as compared with BMY7378 treatment).

performed further *ex vivo* assays. In the first assessment, we used a ROCK inhibitor Y-27632 to determine whether intense vasoconstriction via APJ and α1-ARs signalling is mediated by Rho/ROCK signal. Y-27632 inhibited vascular contraction induced by NA but did not suppress the intense contraction occurring under continuous apelin administration (Fig. 4A, i and ii). Next, to verify the influence of Ca^{2+} on this vascular activity, we used Krebs' buffer from which Ca^{2+} had been removed by chelation with 10 mM EGTA. Potent vascular contraction was completely inhibited in the Ca^{2+}-free condition (Fig. 4A, iii). These results suggested that the intense vascular contraction in aortae of SMA-APJ mice induced by co-stimulation with apelin and NA was mainly mediated through the Ca^{2+} signalling pathway.

To investigate whether APJ and α1-AR work together, we measured intracellular calcium influx in HEK293T cells co-transfected with APJ and α1-ARs using Fura2-AM. Although all types of α1-ARs evoked intracellular calcium influx during apelin stimulation, cells co-transfected with APJ and α1A-AR were most responsive (Fig. 4B). We also observed that vascular contraction induced by apelin and NA was suppressed more efficiently by the α1A-AR inhibitor, RS100329, than by the α1D-AR inhibitor, BMY7379, in aortae from SMA-APJ mice (Fig. 4C). These results suggested that α1A-AR is the functionally important partner for APJ.

Attenuation of intense vasoconstriction induced by apelin and α1A-AR specific agonist in aortae of SMA-APJ mice due to α1A-AR deficiency

To validate the contribution of α1A-AR in the apelin-induced vascular contraction in SMA-APJ mice, we deleted α1A-AR in mice using CRISPR/Cas9 genome editing, referred to here as α1A-AR-KO mice (Fig. 5A and B). In α1A-AR-KO mice, elevation of blood pressure induction by A-61603 administration was not observed (Fig. 5C). We next generated a mouse line

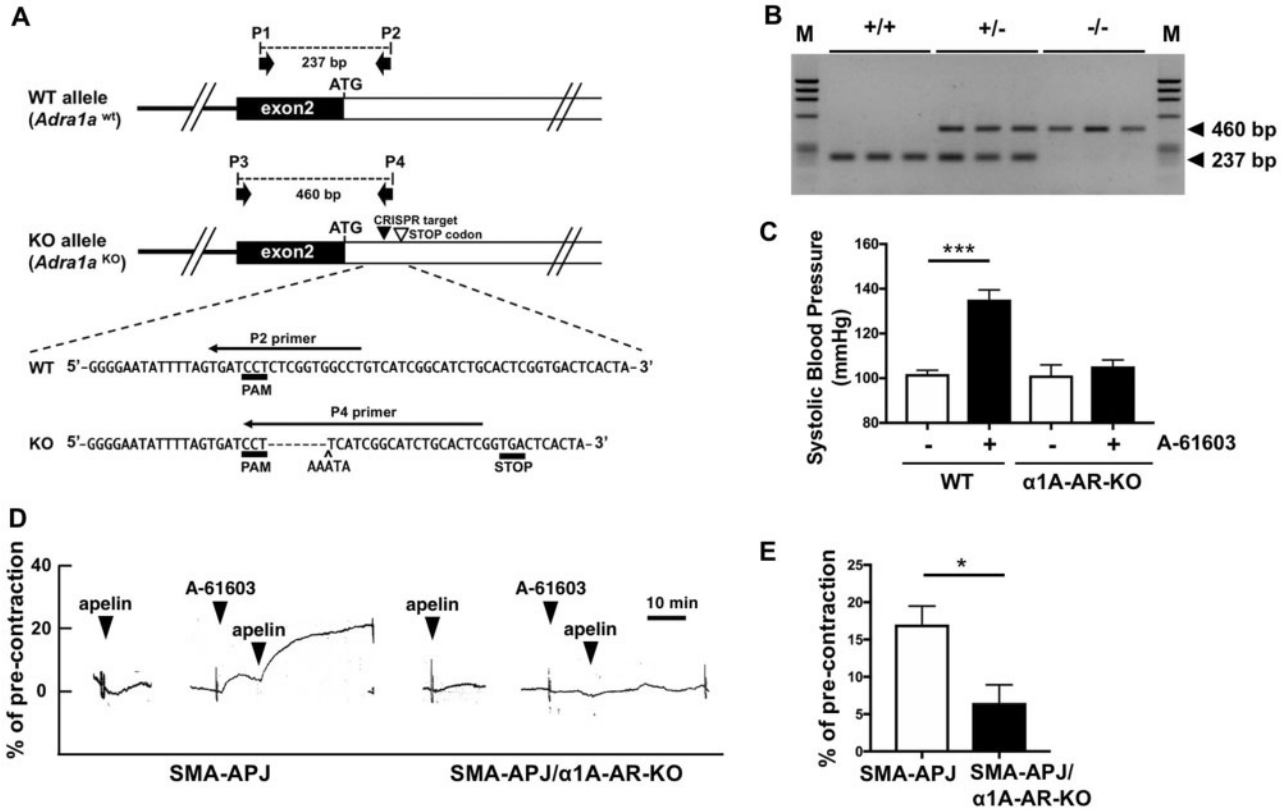

Fig. 5 Genetic deficiency of α1A-AR in SMA-APJ mice eliminated the intense vasoconstriction response to apelin and α1A adrenergic agonist. (**A**) Schematic diagram of the α1A-AR gene targeting using genome editing with CRISPR/Cas9 system. Genotyping sequence of wild type (WT) and mutated (KO) mice in target region. There were 29 pups born after transplantation, of which a pup showed induced indel mutation on *Adra1a* target sequence, introducing a frameshift mutation that abolishes *Adra1a* gene function. (**B**) Allele-specific PCR analysis for genotyping the α1A-AR-KO mouse line with tail genomic DNA. By using the two sets of primers, the products of 237 and 460 base pairs (bp) (as described in Materials and Methods) were generated from the α1A-AR-WT (+/+) and the α1A-AR-KO (−/−) alleles, respectively. The heterozygous allele (+/−) showed both PCR products. M: PhiX174 DNA-*Hae*III digest markers. (**C**) Changes in SBP in WT and SMA-APJ/α1A-AR-KO mice with A-61603 treatment (19.7 μg/kg BW, $n = 5$). Statistical differences were determined using Student's t-test (***$P < 0.001$ as compared with non-treated group). (**D**) Changes in vascular contraction responses for apelin with A-61603 of aortae from SMA-APJ and SMA-APJ/α1A-AR-KO mice ($n = 5$). (**E**) Vascular contraction of aortae from SMA-APJ and SMA-APJ/α1A-AR-KO mice in response to treatment with A-61603 (10^{-5} M) and apelin (10^{-7} M) ($n = 4$–5). Statistical differences were determined using Student's t-test (*$P < 0.05$ as compared with SMA-APJ mice).

that has both the SMA-APJ transgene and the α1A-AR deletion, called SMA-APJ/α1A-AR-KO mice. Although cooperative stimulation with A-61603 and apelin induced intense vascular contraction in aortae from SMA-APJ mice, this response was significantly reduced in the aortae of SMA-APJ/α1A-AR-KO mice (Fig. 5D and E). These results indicated that α1A-AR is required for APJ-mediated contraction of VSMCs in SMA-APJ mice.

Discussion

The molecular mechanisms and factors involved in vascular regulation *via* the apelin/APJ system are not yet well understood. In this study, we provided evidence that APJ in VSMCs functions as a regulator of vascular tone by cooperative action with α1-AR using SMA-APJ, a transgenic mouse line that overexpressed APJ in VSMCs under the control of the SMA gene promoter (Fig. 1A). Although basal SBP was slightly but statistically lowered in SMA-APJ mice than that in WT mice (Supplemental Figure S1A), why it is decreased has not yet been elucidated. One would mention that blood vessels are contracted by circulating apelin in blood because of the enhanced expression of APJ in VSMCs of SMA-APJ mice, and this might secrete vascular dilation factors including NO and adenosine in some peripheral blood vessels.

In response to apelin administered to SMA-APJ mice, we found acute elevation of blood pressure (Fig. 1C) and vascular contraction of coronary vessels (Fig. 2) by means of *in vivo* imaging with high intensity SR, a powerful experimental tool for micrometer-scale visualization of microcirculation (*24*, *25*). Although, at the same time, we observed a rapid and sustained decrease in HR in SMA-APJ mice after apelin treatment (Fig. 1D), this may be mediated initially by the arterial baroreceptor reflex (*28*, *29*). However, as arterial pressure declined to baseline within the same period in the SMA-APJ mice while HR remained depressed suggest that other unknown factors also contributed to apelin-induced bradycardia. To date this subacute phase of the HR response has not been reported and requires future investigation.

In addition, we demonstrated the intense vasoconstriction of the aorta from SMA-APJ mice by co-stimulation with apelin and α1A-AR ligands *ex vivo* (Fig. 3B and D). As it is recognized that microvascular angina is a risk for adverse cardiac events (*30*), the synergistic action of APJ with α1A-AR in VSMCs may contribute to microvascular stenosis. Although how APJ and α1A-AR induce the coordinated vascular contraction is still unknown, it is known that the crosstalk of G-protein-coupled receptors plays diverse roles in the regulation of biological systems (*31*). For example, APJ was reported to form heterodimers with other G-protein-coupled receptors including opioid receptors (*32*, *33*) and neurotensin receptor 1 (*34*), and these interactions modulate receptor signalling. Moreover, the heterodimer formation of APJ with AT1, an angiotensin II receptor subtype, is known to lead to reduced signalling and attenuated formation of atherosclerotic lesions, suggesting protective effects of this interaction (*35*).

One of our key findings was that intense vascular contraction in aortae from SMA-APJ mice (Figs 3B, D, and 4B) was induced by stimulation with APJ and α1A-AR, a new partner of APJ revealed in this study, but the functional cross-talk between APJ and α1A-AR has not been elucidated. We confirmed that the Venus fused to APJ or α1-ARs in HEK293T cells was localized in cytosol and plasma membrane (Supplemental Figure S2A), and next examined whether APJ and α1-ARs form heterodimer to use a bimolecular fluorescence complementation (BiFC) assay (*36*, *37*) which directly visualizes protein-protein interactions in living cells. A combination of the APJ fused to the Venus-N terminal fragment (VN) with the α1-AR subtypes connected to the Venus-C terminal fragment (VC) showed emitted fluorescence, whereas BiFC fluorescence was mainly localized to the plasma membrane when paired with the APJ-VN and the α1A-AR-VC (Supplemental Figure S2B). These observations might support the functional interaction between APJ/α1A-AR that cause augmented vascular contraction and calcium influx (Fig. 4A and B).

Vascular tone is known to be regulated by the balance of vasodilation in VECs and vasoconstriction in VSMCs, where APJ and α1A-AR are expressed (*2*, *16*, *38*, *39*). We and others have shown that systemic apelin administration releases vasodilatory substances, including nitric oxide (NO), and lowers blood pressure (*17*, *40*). By contrast, it has been reported that apelin contracts endothelial cell-denuded human saphenous vein (*18*). We also demonstrated that WT mice with endothelial dysfunction caused by L-NAME, an inhibitor of NO synthase, exhibited elevated blood pressure following apelin injection (*20*), suggesting the role of the apelin/APJ system in vasoconstriction in VSMCs. In this sense, the SMA-APJ mouse line is a useful tool to evaluate the potential significance of regulating the balance of action between VSMCs and VECs in the regulation of vascular tone in the absence of endothelial dysfunction.

In conclusion, our study revealed that enhanced action of APJ in VSMCs induces intense vascular contraction via cooperative action with α1-AR, especially with the α1A-AR subtype. In addition to VSMCs, it is known that APJ and α1A-AR are co-distributed in various tissues (*41*, *42*), for example, lung, kidney and brain, where both receptors are expected to potentially interact and have tissue-specific functions. The findings described here may provide insights into the roles of APJ and α1A-AR on the fine-tuning of blood pressure and enable a deeper understanding of the potential mechanisms for regulating vascular tone by VSMCs and VECs and for inducing pathological conditions such as vascular stenosis and vasospasm.

Supplementary data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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