Studies on Vasoconstriction and Cardiac Hypertrophy Controlled by α1A-Adrenergic Receptor

January 2020

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A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
(Doctoral Program in Life Sciences and Bioengineering)

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Chapter I.

Preface

Cardiovascular diseases (CVDs) are the leading cause of death worldwide. In 2017, more than 17 million people died because of CVDs, accounting for 31% of all reported deaths (1). The World Health Organization (WHO) defines CVDs as a group of disorders of the heart and blood vessels, including coronary artery disease and heart failure. Many signaling molecules are involved in the pathogenesis of these diseases. Above all, G-protein coupled receptors (GPCRs) are key regulators of cardiovascular function. In this study, I have focused on the function of GPCRs in circulatory systems.

Blood vessels are tubular organs found throughout the body that play an essential role in circulatory systems by transporting blood and by regulating blood pressure. Since normal vasoconstriction helps transport blood, including dissolved oxygen, nutrients, and hormones into many tissues of the body in order to maintain whole-body homeostasis, impairment

of blood vessels frequently causes organ dysfunctions and results in lethal conditions.

A recent study reported that several GPCRs are expressed in the cardiovascular system and play roles in tissue homeostasis. Although GPCRs generally bind to their corresponding ligands and transmit extracellular signals into the cell interior by interacting with G proteins (2), recent studies suggested that multiple GPCRs form functional dimers that contribute to the progression of CVDs such as pre-eclampsia and age-related hypertension (3-6). However, there have been no reports concerning the relationship between GPCR dimerization and vascular contractility.

APJ, a GPCR specific for apelin, has been the subject of intensive research by our research group to date. We have investigated the roles played by APJ in the cardiovascular system and showed that APJ overexpression in cardiomyocytes causes phenotypes similar to those associated with peripartum cardiomyopathy (7). Although APJ in vascular endothelial cells (VECs) is known to promote NO synthesis and induce vasodilation in vessels (8,9), our endothelial dysfunction model mice exhibited apelin-induced vasoconstriction when treated with NOS inhibitors (10). These results

suggest that APJ in vascular smooth muscle cells (VSMCs) may constrict vessels by apelin.

Therefore, we generated VSMC-specific APJ-overexpressing (SMA-APJ) mice to demonstrate the vasoconstrictive effect *in vivo*. SMA-APJ mice displayed vascular constriction following apelin administration *ex vivo* (Fig. I-1B). We therefore discovered that APJ acts as a vasoconstrictor in VSMCs. Moreover, we noticed that APJ may functionally interact with α1A-adrenergic receptor (α1A-AR) in VSMCs. Adrenergic receptors, including nine functional subtypes, are mainly expressed in tissues of the heart, lung, kidney, and vessels, and play various roles in each tissue. In particular, the α1-subtype plays a major role in regulating blood vessel diameter, i.e. vascular tone (11). In Chapter II, I will describe my findings concerning the functional interaction between APJ and α1A-AR in vasoconstriction, by measuring vascular contractility in response to ligands *ex vivo* in SMA-APJ/α1A-AR-KO mice that I generated.

The incidence of CVDs differs between men and women (12), especially since pregnancy sometimes becomes a risk factor for CVDs in women. The pregnancy-associated risk arises because of hemodynamic

changes, including increases in heart rate and circulating blood volume. These alterations in normal pregnancy induce physiological cardiac hypertrophy (13,14), whereas hypertensive disorders of pregnancy (HDP) frequently develop into pathological cardiac hypertrophy, which is a hallmark of progression toward heart failure (15). Whereas there are only a few studies focused on cardiac hypertrophy under HDP conditions, our research group has previously generated pregnancy-associated hypertensive mice (PAH) that exhibit physiological cardiac hypertrophy during gestation (16) (Fig. I-2).

In summary, I have elucidated novel functions of α1A-AR in APJ-induced vasoconstriction. These findings provide new insights into α1A-AR in relation to CVD.

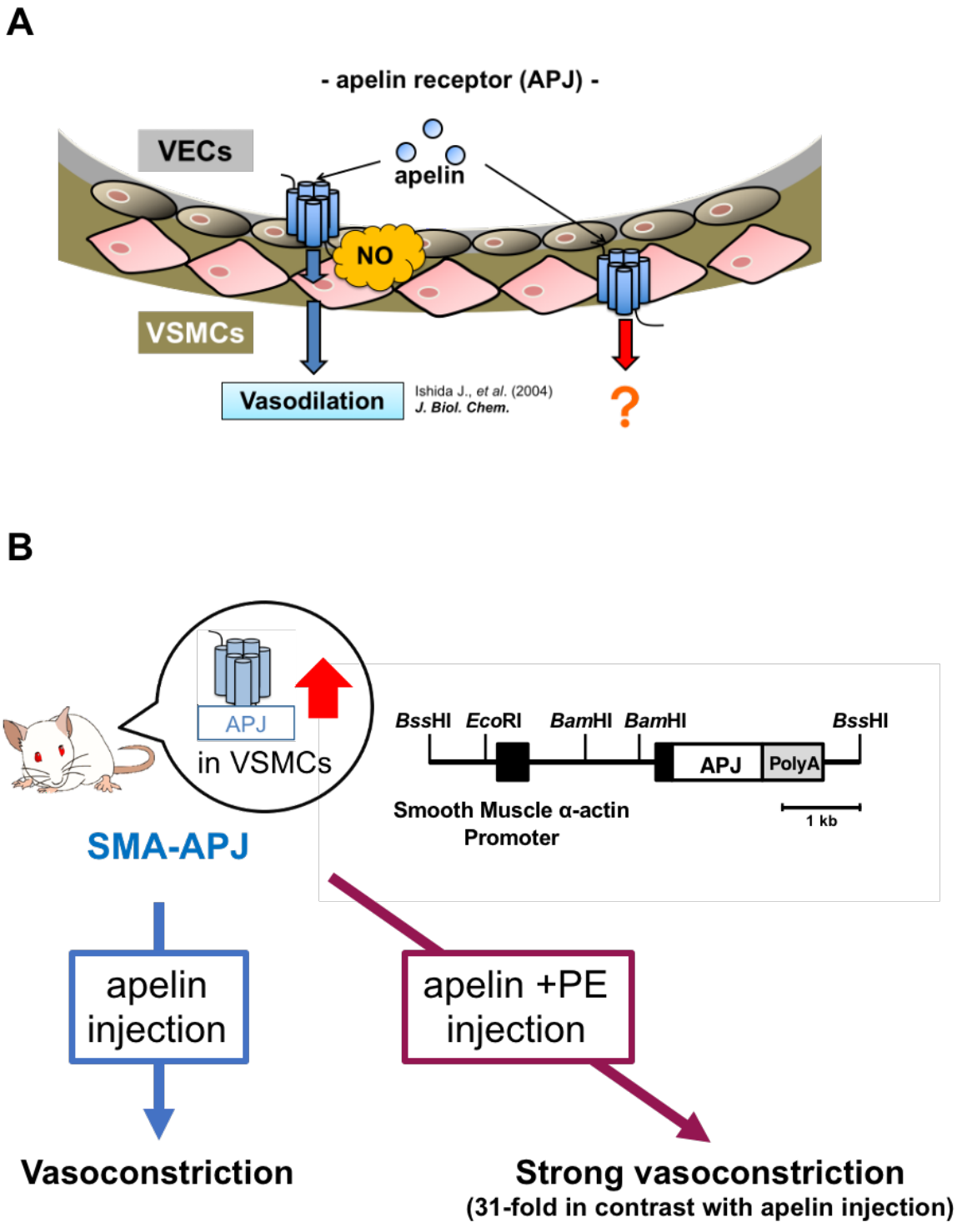

Figure I-1. VSMCs-specific APJ overexpressing mice (SMA-APJ)

(A) Diagrams indicating apelin receptor (APJ) expression on vascular tissues.
(B) Details of transgene and vascular response with ligands on SMA-APJ mice.

Chapter II.

The cooperative role played by α1A-AR in vascular smooth muscle cells during APJ-induced vasoconstriction

Summary

Vascular tone is regulated by the balance between constriction in vascular smooth muscle cells (VSMCs) and dilation in vascular endothelial cells (VECs). These responses are mainly induced by G-protein coupled receptors (GPCRs) expressed in vascular tissues. Our group has previously elucidated the *in vivo* functions of the apelin receptor (APJ) which is well-known for its vasodilatory effect in VECs. Additionally, although APJ is a unique receptor expressed in both VECs and VSMCs, there are only a few reports focused on its function in VSMCs.

Therefore, to elucidate the role of APJ in VSMCs, our group generated VSMC-specific APJ overexpressing (SMA-APJ) mice and discovered that APJ in VSMCs functions as a vasoconstrictor in blood pressure regulation.

Furthermore, isolated aortae from SMA-APJ mice demonstrated intense constriction following treatment with apelin and an α1-adrenergic receptor agonist. These results suggest that APJ and α1-subtypes (α1A, α1B, and α1D) induce a prolonged effect on vasoconstriction. However, it remains unclear which α1-subtype contributes to APJ-induced vasoconstriction.

To elucidate the contribution of α1A-AR for collaborative action with APJ in the murine aorta, I generated SMA-APJ/α1A-AR-KO mice by genome editing. I observed that α1A-AR deficiency significantly suppressed the intense contraction caused by apelin and a selective α1A-AR agonist (A-61603) in SMA-APJ mice. Taken together, this study indicates that α1A-AR regulates vascular tone by coordinating with APJ in VSMCs.

Introduction

Blood pressure control is important in managing health and disease. Thus, disruption of blood pressure regulation often causes various forms of tissue damage. In general, blood pressure regulation is determined by cardiac output, circulating blood volume, and vascular resistance. In particular, vascular resistance is a homeostatic response to acute blood pressure changes (17). Previous studies have shown that several endogenous GPCR ligands, such as angiotensin II, endothelin-1, adrenaline and histamine, mainly induce vasoconstriction *in vivo* by increasing the intracellular calcium concentration (18).

The apelin receptor (APJ) is a GPCR that is widely distributed in the body. APJ expression is especially high in cardiovascular tissues (19, 20). Apelin, an endogenous APJ ligand, was originally isolated from bovine stomach (21). Apelin induces cell proliferation and migration (22) and promotes hematopoiesis in human embryonic stem cells (23). Additionally, it was recently reported that apelin reverses age-associated sarcopenia (24). These functions indicate that apelin is involved in developmental processes

and in modulating various physiological functions.

As many studies to date have shown that apelin and APJ are highly expressed in vascular endothelial cells (VECs) (20, 25-27), a growing body of research has investigated the role of apelin/APJ-mediated action in endothelial function. Previously, we used APJ-deficient mice to demonstrate that APJ lowers blood pressure and induces nitric-4-oxide (NO) production in an apelin-dependent manner (8). 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 (28). Furthermore, apelin stimulates increased myosin light chain (MLC) phosphorylation in rat vascular smooth muscle cells (VSMCs); MLC phosphorylation is the rate-limiting event during vascular contraction (29).

Interestingly, apelin has been reported to exert a vasoconstrictor effect in mice under vascular endothelial dysfunction (10). Therefore, to clarify the *in vivo* function of APJ in VSMCs, our group established transgenic mice that overexpress APJ in VSMCs (SMA-APJ). SMA-APJ mice showed transient and intense elevation of blood pressure following apelin injection.

Moreover, we postulated that APJ in VSMCs may induce vasoconstriction requiring the α1A-adrenergic receptor (α1A-AR or ADRA1A), because pharmacological inhibition of α1A-AR suppressed the vascular contractile action induced by co-stimulation with apelin and an α1-AR-specific agonist in SMA-APJ mice (Fig. I-1B). However, an α1A-AR selective inhibitor (RS100329) showed some activity against the other α1 adrenergic subtypes (α1B-AR, α1D-AR). Therefore, it is unclear whether α1A-AR enhances APJ-induced vasoconstriction *in vivo*.

In this study, I aim to derive *ex vivo* evidence that APJ and α1A-AR work together to enhance vascular contractility in VSMCs by genetic ablation of the *Adra1a* gene that encodes α1A-AR in SMA-APJ mice.

Materials and Methods

Generation of genetically modified mice

α1A-AR deficiency was induced by a frameshift mutation close to the start codon of the *Adra1a* gene. The oligonucleotides were annealed and ligated into the *Bbs*I restriction site of the pX330 vector (#42230; Addgene, Watertown, MA, USA). The overhanging nucleotides are written as lowercase letters in the following sequences. For the α1A-AR knockout allele, the sequences were 5'-caccGCCGATGACAGGCCACCGAG-3' and 5'-aaacCTCGGTGGCCTGTCATCGGC-3'. The plasmids were microinjected into the pronuclei of fertilized oocytes from the C57BL/6J strain (Charles River Laboratories Japan, Yokohama, Japan). The founder offspring were screened by genotyping tail DNA. The WT allele was detected by PCR using the following primers:

(Forward primer) 5'-TTCCTCAGGCTCACGTTTCC-3'

(Reverse primer) 5'-AGGCCACCGAGAGGATCA-3'

Primers for detecting KO allele are:

(Forward primer) 5’-GGTGGCTTTCACAGCATGTC-3’

(Reverse primer) 5’-GAGTGCAGATGCCGATGATATTTAGG-3’.

These mice were backcrossed three times to the ICR strains (CLEA Japan, Tokyo, Japan), 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, at 22°C, 40–60% humidity, and a 12 h light–dark cycle, with food and tap water 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.

Mouse aorta preparation and total RNA extraction

Mouse aortae were flash frozen in liquid nitrogen after perivascular lipids were removed. Total RNA was extracted using the RNAgents Total RNA Isolation System kit (#Z5110; Promega, Madison, WI, USA). A Multi-beads Shocker (Yasui Kikai, Osaka, Japan) was used to crush the frozen aortae into a powder. The powder was suspended in denaturing solution and mixed with phenol-chloroform. Ethachinmate (#312-01791; Nippon Gene, Tokyo, Japan) was added to the supernatant and collected by centrifugation at 17,800 *g* and 4°C for 20 min. Finally, total RNA was precipitated with ethanol.

Gene expression analysis by quantitative RT-PCR

Approximately 1 μg of total RNA was reverse-transcribed using the QuantiTect Reverse Transcription kit (#205311; Qiagen, Hilden, Germany). Using a Thermal Cycler Dice and SYBR Premix Ex Taq II (#RR820S; TaKaRa Bio, Kusatsu, Japan), real-time quantitative PCR reactions were

performed. Target gene expression levels were normalized to *Gapdh* using the ΔΔCt method. Primer amplification efficiency was verified to be equal by using serial dilutions of cDNA for each target gene. The following primers were used for amplification:

Gapdh,

(Forward primer) 5'-TGTGTCCGT CGTGGA TCTGA-3'

(Reverse primer) 5'-TTGCTGTTGAAGTCGCAGGAG-3'

Adra1a (α1A-AR),

(Forward primer) 5'-GCGGTGGACGTCTTATGCT-3'

(Reverse primer) 5'-TCACACCAATGTATCGGTCGA-3'

Adra1b (α1B-AR),

(Forward primer) 5'-CCTGGTCATGTACTGCCGA-3'

(Reverse primer) 5'-GACTCCCGCCTCCAGATTC-3'

Adra1d (α1D-AR),

(Forward primer) 5’-TGCAGACGGTCACCAACTATTT-3’

(Reverse primer) 5’-GGCAACACAGCTGCACTCAG-3’.

Measurement of aortic isometric tension

Aortic rings were excised from mouse thoracic parts in ice-cold Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.1 mM glucose), after mice were anesthetized with isoflurane. Three millimeter sections of the dissected rings were mounted using an Easy Magnus System (Kishimoto Medical Instruments, Kyoto, Japan) as described previously. Ring sections mounted in the chamber were soaked and equilibrated for 1 h under a 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 with 60 mM KCl, and once with 80 mM KCl. The following agents were used to contract the rings: [Pyr1] apelin-13 (#4361-v; Peptide Institute, Tokyo, Japan), phenylephrine (#163-11791; FUJIFILM Wako Pure Chemical Co.,

Osaka, Japan) and A-61603 (#1052; TOCRIS, Ellisville, Mo, USA), with or without apelin.

Bimolecular fluorescence complementation (BiFC) assay

DNA fragments encoding the N-terminal (VN) and C-terminal (VC) fragments of Venus were cloned into the *Not*I/*Kpn*I restriction sites of a pEGFP-N1 vector (Clontech Laboratories, CA, USA) (*30*). DNA fragments encoding murine APJ, α1A-, α1B, and α1D-AR without the stop codon were amplified by PCR and cloned into the *Eco*RI/*Xho*I restriction sites of either the pVenus-N1, pVenus-VN, or pVenus-VC expression vectors. HEK293T cells were co-transfected with full-length Venus, VN, VC, or BiFC pairing VN and VC vectors. pmCherry was also used as a transfection control. Hoechst 33258 staining was performed to visualize cell nuclei. Cell images were captured using an FV10i, a confocal laser scanning microscope (Olympus Corporation, Shinjuku, Japan) equipped with an EGFP filter.

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 a post hoc test or Fisher LSD test was used to determine the significance of differences between groups, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

α1A-AR and APJ co-localize mainly in the cell membrane

Our group previously showed that mRNA expression of α1-subtypes in VEC-removing vessels were equivalent between WT and SMA-APJ mice. Meanwhile, α1D-AR expression was higher than α1A-AR and α1B-AR. Therefore, I determined the expression of the three subtypes in whole thoracic vessels. As a result, similar to the previous study, there were no differences between WT and SMA-APJ mice. On the other hand, α1A-AR was expressed at higher levels than α1B-AR and α1D-AR (Fig. II-1A). These findings suggest that the distribution of α1-subtypes may differ between VECs and VSMCs.

Additionally, I determined the subcellular localization of α1-subtypes in HEK293T cells. α1B-AR and α1D-AR were expressed in both the cytoplasm and cell membrane. In contrast, α1A-AR localized mainly to the plasma membrane (Fig. II-1B). Moreover, the Bi-FC assay showed that the combination of APJ and α1A-AR enhanced fluorescence signals compared with those of α1B-AR and α1D-AR (Fig. II-1C). These data indicate that

α1A-AR and APJ form heterodimers *in vitro*.

Generation of α1A-AR knockout mice using the CRISPR/Cas9 system

To determine the contribution of α1A-AR to cooperative vasoconstriction *in vivo*, I generated α1A-AR knockout mice by genome editing targeted at the *Adra1a* gene. α1A-AR deficiency was induced by introducing a frameshift mutation in *Adra1a*. Genotyping PCR was used to detect the indel mutation in transgenic mice (Fig. II-2A and 2B). In these strains, A-61603-induced temporary hypertension was suppressed (Fig. II-2C). Hence, *Adra1a* gene disruption in mutant mice was verified phenotypically by pharmacological tests.

Cooperative vasoconstriction by apelin and an α1A-selective agonist is abrogated in SMA-APJ/α1A-AR-KO mice

The isolated vessels of SMA-APJ mice demonstrated hypercontraction by apelin and PE *ex vivo*. The vasoconstrictive response with these ligands, however, was not suppressed in SMA-APJ/α1A-AR-KO mice (Fig. II-3A). This latter finding was unexpected. Therefore, I investigated whether an

α1A-AR agonist (A-61603) enhances apelin-induced vasoconstriction in SMA-APJ mice. Hence, A-61603 induced cooperative contraction like PE (Fig. II-3B). Moreover, this cooperative response with apelin and A-61603 was completely inhibited in SMA-APJ/α1A-AR-KO mice (Fig. II-3C). Taken together, I demonstrated a functional interaction between APJ and α1A-AR in abnormal vasoconstriction *ex vivo*.

Discussion

The molecular mechanisms involved in vascular regulation via the apelin/APJ system are not yet well understood. In this study, I gathered evidence that APJ in VSMCs plays a role in regulating vascular tone. In addition, I have demonstrated that the intense vasoconstriction in SMA-APJ aortae is induced by co-stimulation with apelin and α1A-AR ligands *ex vivo* (Fig. II-3B). Moreover, this cooperative constriction was suppressed in SMA-APJ/α1A-AR-KO mice (Fig. II-3C). Therefore, I showed the cooperative interaction between APJ and α1A-AR in isolated vessels.

α1A-AR-KO mice had previously been generated in 2002 (31). My transgenic mice displayed the same phenotypes as the previous study, such as lower SBP and suppression of PE-induced temporary hypertension. Additionally, our mutants showed a reduction of hypertension induced by an α1A-AR agonist (A-61603) (Fig. II-2). Furthermore, WT mice infused with A-61603 died after several days, whereas α1A-AR-KO mice survived (n = 5, data not shown). Although the cause of death is unknown, this result indicated that excessive α1A-AR stimulation might be dangerous for

biological homeostasis.

Unexpectedly, SMA-APJ/α1A-AR-KO mice did not suppress apelin/PE -induced abnormal vasoconstriction (Fig. II-1). On the other hand, apelin/A-61603-induced constriction was reduced (Fig. II-3). These results suggest not only the significance of α1A-AR to apelin-induced vasoconstriction, but also the possibility that other α1-ARs (α1B,α1D) interact with APJ.

Interestingly, SMA-APJ mice display temporary constriction of their coronary arteries (32). As coronary artery spasms can lead to angina, the synergistic action of APJ with α1A-AR in VSMCs may contribute to microvascular stenosis. Although how APJ and α1A-AR induce vascular contraction in coordination is still unknown, it is known that crosstalk between GPCRs plays diverse roles in regulating biological systems (33). For example, APJ reportedly forms heterodimers with other GPCRs including opioid receptors (34, 35) and neurotensin receptor 1 (36), and these interactions modulate receptor signaling. Moreover, the heterodimer formation between APJ and AT1, an angiotensin II receptor subtype, leads to reduced signaling and attenuated formation of atherosclerotic lesions,

suggesting that this interaction has protective effects (37). Accordingly, my finding provides new insights into the progression of vasospasm and GPCR dimerization.

In conclusion, my study demonstrates that the 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 (11,38), for example, in the lung, kidney, and brain, where both receptors are expected to interact and have tissue-specific functions. The findings described here may provide insights into the roles of APJ and α1A-AR in fine-tuning blood pressure and enable better understanding of potential mechanisms for regulating vascular tone by VSMCs and VECs, as well as for inducing pathological conditions such as vascular stenosis and vasospasm.

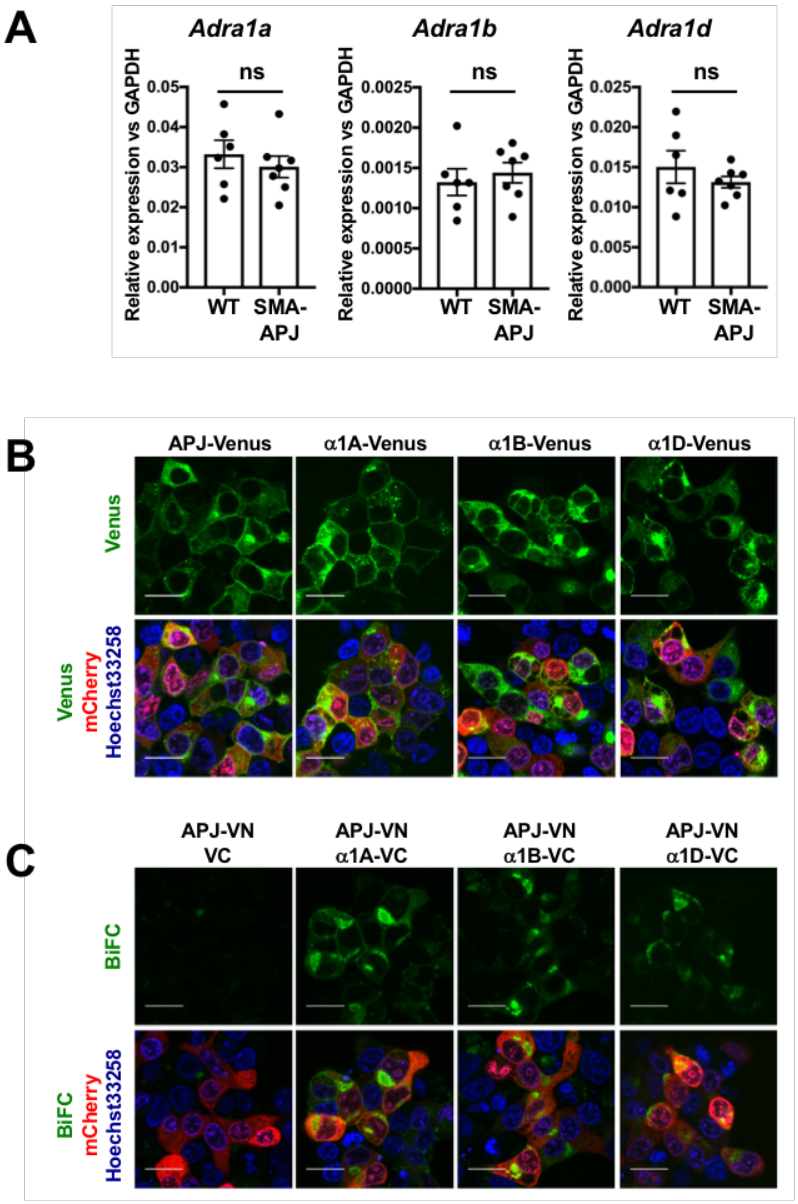

Figure II-1. Intracellular localization of α1-subtypes and APJ

(A) 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). **(B)** Localization of APJ-, α1A-, α1B-, or α1D-AR-Venus in HEK293T cells. Cells were co-transfected with mCherry vector as a transfection control and full-length Venus vectors. Hoechst 33258 staining was performed to visualize cell nuclei. **(C)** BiFC experiments in HEK293T cells show intracellular localization of dimer formation of APJ with α1A-, α1B-, and α1D-ARs. Cells were co-transfected with BiFC pairing vectors. mCherry vector was also transfected as transfection control. Scale bars, 20 μm.

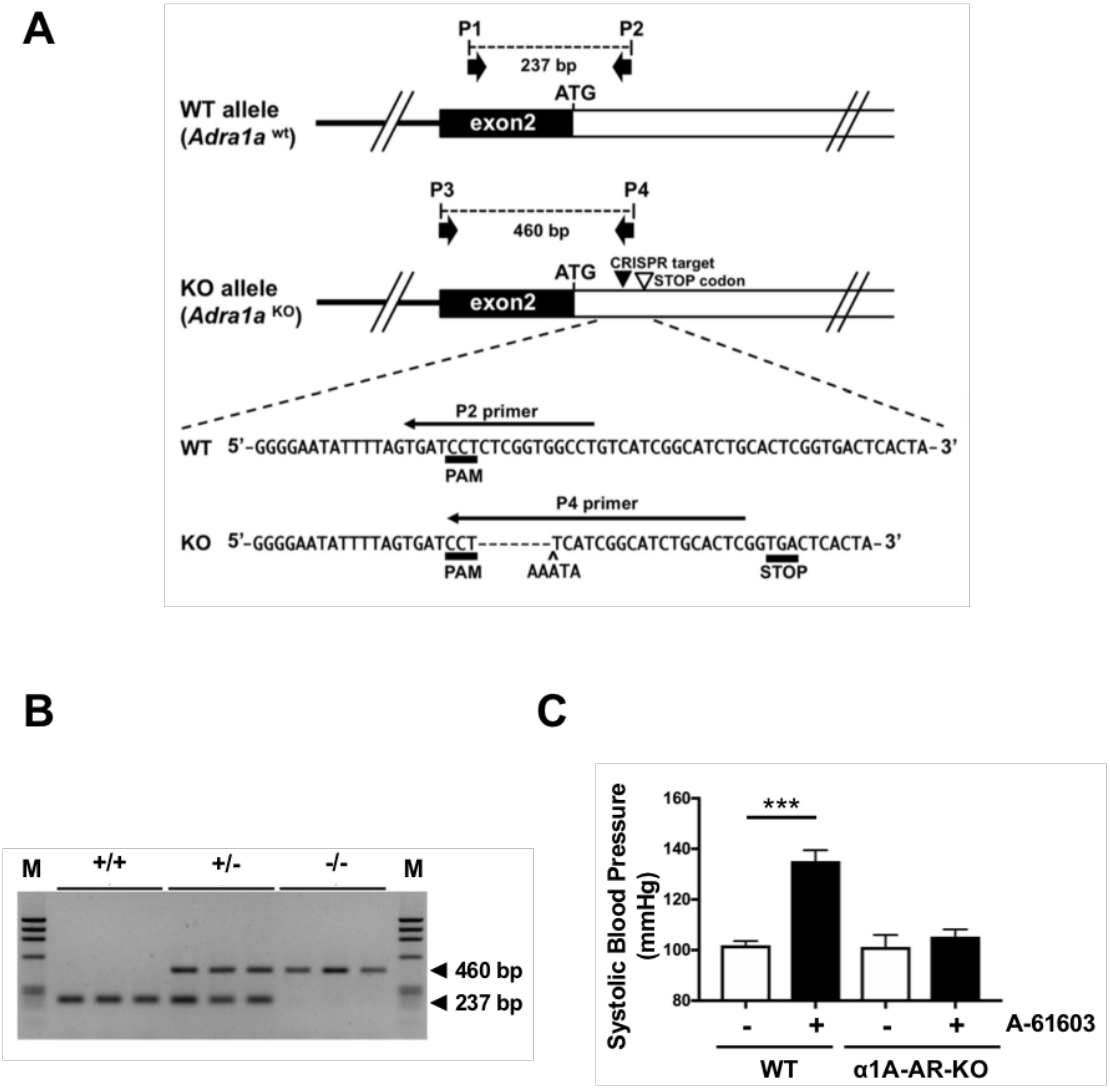

Figure II-2. Generation of α1A-AR deficient mice by genome editing

(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).

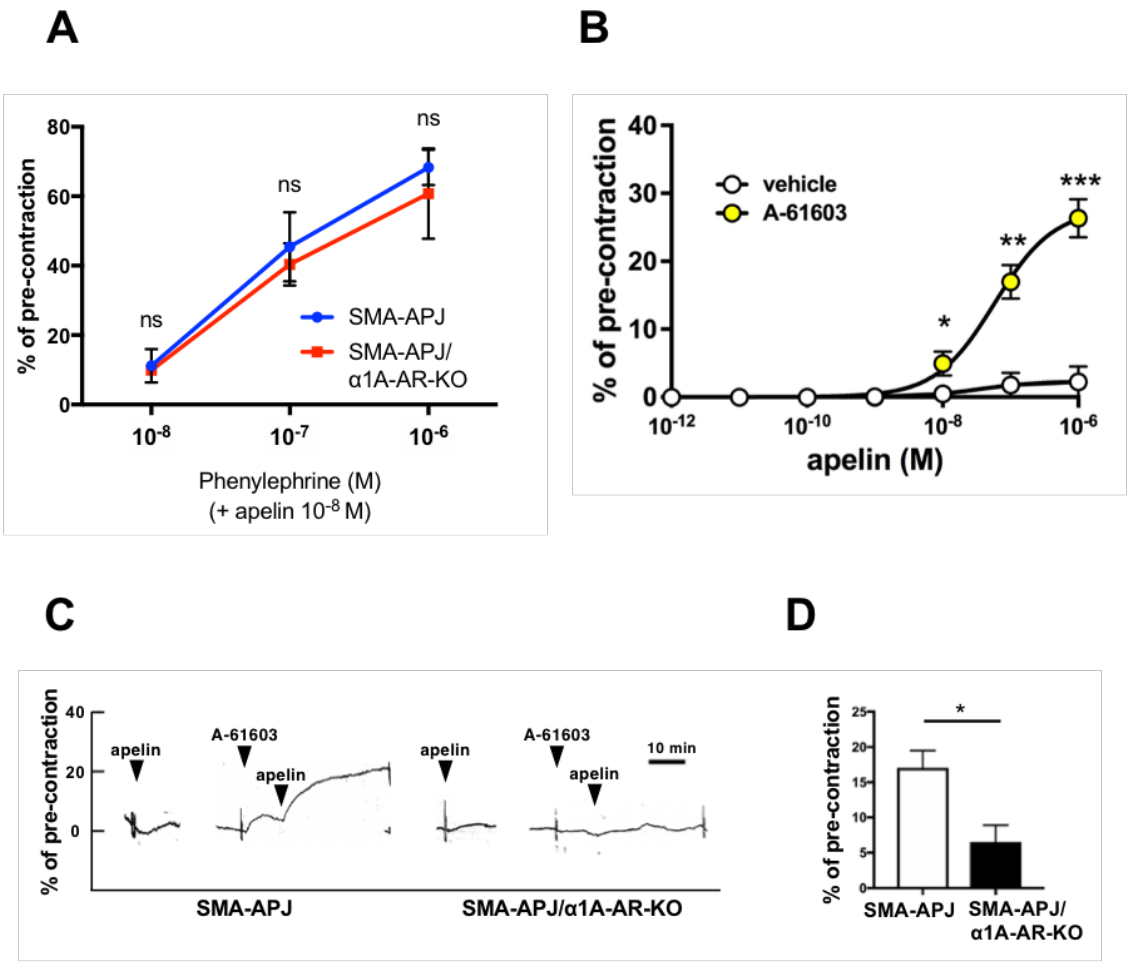

Figure II-3. Apelin and α1-ligands stimulation in SMA-APJ/α1A-AR-KO vessels

(A) Vascular constriction of aortae with apelin(10^{-8}M) and PE from SMA-APJ and SMA-APJ/α1A-AR-KO mice (n=4). **(B)** Changes in vascular contractile responses of aortae from SMA-APJ mice for A-61603 (10^{-5} M) with apelin (n = 5). **(C)** Changes in vascular contraction responses for apelin with A-61603 of aortae from SMA-APJ and SMA-APJ/α1A-AR-KO mice. **(D)** 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).

Chapter IV.

Concluding Remarks

GPCRs make up the largest family of cell surface receptors which respond to ligands and transduces various extracellular signals (18, 55); indeed, previous studies have shown correlations between diseases and GPCRs. Moreover, approximately 35% of approved drugs target a GPCR (64). Therefore, studies of GPCR function in disease model animals can potentially contribute to the development of novel therapeutics.

As explained in Chapter I, cardiovascular diseases, which are defined as a group of diseases that develops in hearts and vessels, are the leading cause of death around the world (1). Although many studies have elucidated the pathogenesis of CVDs, there are several diseases whose detailed mechanisms are still unknown. For example, the pathogenesis of coronary artery vasospasm, which is the major cause of angina, remains unclear. In general, Japanese people have a higher incidence of angina than Caucasians (65). Hence, studies of GPCR dimerization during abnormal

vasoconstriction may be useful for patients in our country.

Our previous studies suggested that the apelin receptor (APJ) may play a cooperative role with α1A-AR in vascular constriction. In Chapter II, I elucidated the functional interaction between APJ and α1A-AR by using isolated aortae from SMA-APJ/α1A-AR-KO mice. This is the first report of dimerization occurring between the two proteins. Moreover, because the cooperative action by apelin and α1-agonist (PE) treatment was not diminished in SMA-APJ/α1A-AR-KO mice, I hypothesized that other α1-subtypes (α1B, α1D) may also have important functions in APJ-induced vasoconstriction. Thus, I propose that α1-AR functions in APJ-induced vasoconstriction via *in vivo* GPCR dimerization.

In conclusion, my findings concerning α1A-AR provide new insights into the pathogenesis of several CVDs, such as angina from coronary artery vasospasm.

Acknowledgement

I would like to express my deep gratitude to all those who provided me guidance, support and encouragement during the preparation of this dissertation. Most of all, I would like to express my sincere thanks to Professor Akiyoshi Fukamizu for all his support and guidance throughout my research work. I am deeply indebted to Dr. Jun-Dal Kim, Dr. Junji Ishida, Professor Keiji Tanimoto, Dr. Keiji Kimura, Dr. Kazuya Murata and Dr. Yoshito Yamashiro for their teaching about the helpful discussions or experimental techniques. In addition, I would like to give my thanks all members of Fukamizu Laboratory for their kind support.

Finally, I appreciate greatly the helps of my parents and friends.

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