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Effect of novel GPCR ligands on blood pressure and vascular homeostasis

Zaira Palomino Jara^{*}, Khuraijam Dhanachandra Singh^{*}, Hamiyet Unal^{*}, Russell Desnoyer^{*}, Rodrigo Yokota[†], Jorge Luis Pesquero[‡], Dulce Elena Casarini[†], and Sadashiva S. Karnik^{*,1}

^{*} Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, United States

[†] Nephrology Division, Department of Medicine, Federal University of Sao Paulo, Sao Paulo, Brazil

[‡] Physiology and Biophysics Department, Biology and Science Institute, Federal University of Minas Gerais, Belo Horizonte, Brazil

Abstract

Maintenance of normal blood pressure under conditions of drug treatment is a measure of system-wide neuro-hormonal controls and electrolyte/fluid volume homeostasis in the body. With increased interest in designing and evaluating novel drugs that may functionally select or allosterically modulate specific GPCR signaling pathways, techniques that allow us to measure acute and long-term effects on blood pressure are very important. Therefore, this chapter describes techniques to measure acute and long-term impact of novel GPCR ligands on blood pressure regulation. We will use the angiotensin type 1 receptor, a powerful blood pressure regulating GPCR, in detailing the methodology. Normal blood pressure maintenance depends upon dynamic modulation of angiotensin type 1 receptor activity by the hormone peptide angiotensin II. Chronic activation of angiotensin type 1 receptor creates hypertension and related cardiovascular disease states which are treated with angiotensin type 1 receptor blockers (ARBs). Thus, a prototype for evaluation of blood pressure control under experimental evaluation of novel drugs.

1 INTRODUCTION

An abundant diversity of receptors exists in cells to perceive a wide spectrum of signaling molecules present in the immediate environment. The superfamily of G protein-coupled receptors (GPCRs) is the largest and most diverse group of seven-transmembrane (7TM) proteins. Versatile GPCR signaling is involved in a myriad of cellular processes, including cellular metabolism, growth, differentiation, embryonic development, wound healing, secretion, motility, pain sensation, cell death, memory, inflammatory or immune responses, and learning. The human genome contains ~826 unique GPCR genes. Many of the human GPCRs have orthologs in other eukaryotic species. Based on sequence similarity, the GPCR superfamily is divided into (i) rhodopsin family, (ii) secretin family, (iii) glutamate family, (iv) adhesion family, and (v) frizzled family of receptors in higher vertebrates. GPCRs share

¹ Corresponding author: karniks@ccf.org.

a common molecular architectural pattern that consists of a single polypeptide chain with an extracellular N-terminus, a 7TM alpha-helical bundle with three intracellular and three extracellular loops, and an intracellular C-terminus. However, amino acid identity of GPCRs vary widely, endowing each GPCR with the ability to sense a unique molecular pattern of ligands and prompt intracellular signaling that is appropriate for the ligand. GPCRs are thus molecular microprocessors that produce cellular responses to a wide variety of stimuli which include photons, odorants, and small organic molecules including drugs, peptide hormones, and proteins (Bockaert & Pin, 1999; Pierce, Premont, & Lefkowitz, 2002; Unal & Karnik, 2012). The orthosteric site of GPCRs binds natural endogenous ligands and competitive pharmacological ligands. Allosteric ligands bind outside the orthosteric site of a GPCR. Regardless of the site, ligand binding causes transduction of ligand-specific intracellular signals by a common principle applicable to all, i.e., by reducing intrinsic disorder which in turn increases the GPCR's affinity to couple to distinct intracellular molecules (Unal & Karnik, 2012). Thus, activated GPCRs can initiate diverse forms of intracellular signals that in turn regulate a variety of physiological processes including blood pressure in our body.

Interestingly, this superfamily consists of a large number of orphan GPCRs because neither endogenous ligands nor specific pharmacological ligands are known for them. In spite of this, pharmacologist's interest in the GPCR superfamily is due to the fact that >30–40% of the known ligands/drugs used to combat diseases target GPCRs. The ligands/drugs may act as an agonist, antagonist, inverse agonist, or biased agonist to modulate receptor activity and transduce their signals. Downstream effectors coupled to a GPCR are diverse and include heterotrimeric G proteins, G protein-coupled receptor kinases (GRKs), β -arrestins, ion channels, regulators of G protein signaling (RGS), scaffold signaling proteins, and many other proteins. Clinical pathology, genetic, and pharmacological studies demonstrate that human hypertension (as well as hypotension) is a multifactorial disease that involves a functional contribution from many GPCRs and/or their signaling components.

The control of blood pressure (BP) by GPCRs is essential for the maintenance of normal functions of the cardiovascular system. Improper GPCR activation can result in hypertension and vascular dysfunction (Karnik et al., 2015; Pierce et al., 2002). In fact, the master regulator of BP in our body, the renin-angiotensin system (RAS), deploys three different GPCRs, namely angiotensin type-1 (AT1R), angiotensin type-2 (AT2R), and MAS1 receptors. Of these, the AT1R plays a critical role in normal BP regulation. In conditions of very high or very low amounts of salt intake which alters the kidney fluid-pressure control system, activation of AT1R signaling contributes to the development of hypertension and end organ damage (Chen & Coffman, 2015; Guyton, 1991; Leenen, Blaustein, & Hamlyn, 2017). Three facets of BP regulatory mechanisms are predisposed to GPCR control. First, direct effects of GPCRs on vasoconstriction and relaxation through the generation of second messengers that alter the resistance of vessels to blood flow. Second, the proliferative and growth effects of GPCRs on cells of the vascular wall by increasing protein and DNA synthesis leading to remodeling of vascular intima and media as well as the myocardium. Third, effects of GPCRs on the indirect regulation of components of tissue RAS. In almost all tissues, most components of the RAS are expressed and produce local effects of RAS. Hence it is proposed that assessment of BP changes and related mechanisms may be used in the evaluation of future GPCR targeted therapeutic modalities. In this chapter, methods for

evaluating blood pressure regulation as a result of full and biased activation as well as inactivation of the AT1R are presented.

2 ANGIOTENSIN TYPE 1 RECEPTOR AND BLOOD PRESSURE

This seven-transmembrane receptor is fully activated by the octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) hormone, angiotensin II (AngII). BP regulation is thus directly related to AngII concentration (see Fig. 1). Coordinating of AT1R activation in the vasculature, kidney, adrenal gland, heart, immune system, and both central and peripheral nervous systems is essential to maintain normal BP (Chen & Coffman, 2015). Acute regulation of BP by AngII binding to AT1R involves vasoconstriction at small arteries throughout the body (Guyton, 1991; Kopf, Phelps, Schupbach, Johnson, & Peuler, 2018). Interestingly, vasoconstriction caused by AT1R in the vasculature is the net result of opposing activities, constriction of vascular smooth muscle cells (VSMC), and dilation produced by endothelial cells (EC) (Ramchandran et al., 2006).

The mechanism for long-term BP regulation involves a number of entwined actions of AngII/AT1R on the vessel wall, sympathetic nervous stimulation, and volume homeostasis due to increased aldosterone production and renal natriuretic effect (Chen & Coffman, 2015; Guyton, 1991; Kopf et al., 2018). The renal AT1R plays an indispensable role in the volume homeostasis that affects the BP. Reduced urine flow and sodium excretion are observed in AngII-dependent hypertension via AT1R (Kuczeriszka, Kompanowska-Jezierska, Sadowski, Prieto, & Navar, 2018; Tamura et al., 2015; Zhuo et al., 2016). Activation of AT1R in brain areas that exert cardiovascular control plays an important role in controlling BP (Nakagawa & Sigmund, 2017).

Endogenous peptide ligands AngIII and AngIV cause partial activation of AT1R (Bosnyak et al., 2011). Mechanical stretch and autoantibodies produce ligand independent, constitutive activity of AT1R (Karnik et al., 2015). The hemodynamic effect of mechanical stretch and constitutive activity of AT1R have been reported (look up Unal and Karnik, Komuro papers), and AT1R-activating autoantibodies have been implicated in inducing the pathological state responsible for malignant hypertension in preeclampsia (Liles et al., 2015).

The heterotrimeric G proteins are the most important transducers of AT1R signals. Upon ligand binding, the AT1R undergoes specific conformational changes that promote coupling to different classes of G proteins which determines the signaling pathways that will be activated (Forrester et al., 2018; Karnik et al., 2015). The Gq/11 family G proteins coupled to AT1R mediate activation of phospholipase C (PLC) to produce two intracellular second messengers: IP3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP3 promotes intracellular calcium release from the endoplasmic reticulum stores, whereas DAG and calcium regulate the activation of classical Ser/Thr protein kinases which in turn activate MAP kinase cascades. Gq protein signaling can also regulate Rho-GEFs leading to the activation of the Rac or Rho/ROCK signaling pathways. AT1R coupled to Gi/o inhibits adenylyl cyclase and some Ca⁺⁺ channels and activates Rho kinase (Toth, Turu, Hunyady, & Balla, 2018). The Gβ2 subunit of the G protein interacted with specific nucleosome core histones and sequence motif of several transcription factors (Bhatnagar et al., 2013). G12

and G13 proteins activation by AT1R interact with multiple partners, including HSP90 (heat shock protein 90), AKAP (A-kinase-anchoring proteins), non-receptor tyrosine kinases, ras-GAP (GTPase-activating protein) cadherin, and Rho-GEFs. The crosstalk between heterotrimeric (G12/13 and Gq) and small (Rho, Rac, and Cdc42) G proteins explains diverse changes on cytoskeleton dynamics involved in cell shape, migration, secretion, polarization, survival, and adhesion (Bhattacharya, Babwah, & Ferguson, 2004).

The GRKs phosphorylate the AT1R activated by both AngII and its biased analogs to provide docking sites for β -arrestins which prevent G protein coupling and facilitate endocytosis. β -Arrestins also serve as docking platforms for ERKs leading to a different mode of MAPK signaling cascade involved mainly in cell proliferation and survival (Kim, Ahn, Rajagopal, & Lefkowitz, 2009). These events are called second wave AT1R signaling (Toth et al., 2018). The G protein-dependent and -independent signaling waves of AT1R occur in different time and space in a cell. This phenomenon was defined using a peptide analog of AngII, Sar1, Ile4, and Ile8-AngII (SII-AngII) (Noda et al., 1996; Violin & Lefkowitz, 2007; Wei et al., 2003). Survival and apoptosis are disproportionately regulated by activation of β -arrestins by AT1R. Thus, β -arrestin biased signaling at AT1R has redefined classical concepts in AngII pharmacology, offering possibilities for more effective therapeutics. In order to understand the physiological and pathophysiological consequences of β -arrestin biased ligands on the blood pressure, infusion of SII-AngII in vivo was necessary. However, SII-AngII was inadequate for use in an animal model due to its low affinity for AT1R ($K_d=0.3\mu\text{M}$). Thus, based on the well understood mechanism of activation of AT1R by AngII, novel β -arrestin biased ligands were created as detailed in Table 1.

Full activation of AT1R engages other scaffolding regulatory proteins such as ATRAP1, filamin, GIT1, CARMA3, SHP-2, as well as other signaling pathways such as reactive oxygen species signaling, non-receptor type tyrosine kinase signaling, and activation of small G proteins (Karnik et al., 2015). In some cases, the intensity of the signal is the difference between healthy and pathological states. For instance, in endothelial cells, a low amount of superoxide production by eNOS maintains a supply of NO, contributing to vasodilation and vascular health. In disease state, eNOS is uncoupled, leading to excessive superoxide that reacts with NO to form peroxynitrite, a toxic radical, which directly contributes to disease (Karnik et al., 2015). In hypertension, AT1R signaling is excessive. Drugs such as ARBs specifically bind to all forms of the receptor preventing all signaling conformations. Currently, ARBs are prescribed as first line treatment for reduction of sustained high blood pressure levels in patients.

3 ANGIOTENSIN TYPE 1 RECEPTOR LIGANDS VS BLOOD PRESSURE

We tested the effect of AT1R ligands on BP by using (a) wild-type mice, (b) tonin-overexpressing mice, and (c) apolipoprotein E null (ApoEKO) mice. The first represented normal blood pressure and a healthy vascular system. Tonin-overexpressing mice presented high endogenous levels of AngII, and ApoEKO presents higher risk to develop vascular disease.

BP can be evaluated using two methods: method for direct BP measurement and method for indirect BP measurement. The method for direct BP measurement can be used to acutely evaluate the AT1R effect on baseline BP. In this chapter, the acute AT1R effect on physiological and pathological BP is presented. A wild-type mouse was used to represent a normotensive state, and tonin-overexpressing mouse was used to represent a sustain hypertensive state. Inactivation of AT1R with an ARB (single dose of 150 μ L from 10mg/mL) showed higher dipping on the wild-type BP than on the tonin-overexpressing BP. This method allows evaluation of the ligand/AT1R effect on baseline BP values accurately and has the advantage to measure the BP before other compensatory mechanisms take over returning the BP within the normal levels due to an integrated system BP controller system that exists in vivo.

The method for indirect BP measurement can be used for long-term BP evaluation, in this case the other controller BP mechanisms are acting on the BP. In this chapter, the long-term AT1R effects on physiological and pathological BP are presented. A wild-type mouse was used to represent a normotensive state and a healthy vascular system, and an ApoEKO mouse was used to represent a normotensive state with risk for vascular morbidity. The full agonist Ang II dose (1.4 μ mol/g for 28 days) caused hypertension and vascular damage in wild-type and ApoEKO strains. ApoEKO presented higher vascular morbidity correlated with mortality due to aortic diseases. β -Arrestin biased ligands, SFdA-AngII, and SYdA-AngII also called TRV, at matching concentration (1.4 μ mol/g for 28 days) did not cause an increased BP, nor vessel damage in both strains. Matching concentration of ARBs (1.4 μ mol/g for 28days) maintains the BP in normotensive levels without vessel damage.

The mechanisms underlying BP regulation through AT1R involves release of renin, aldosterone, and catecholamines as well as its effect on organ structures that are involved in the BP control as detailed in Fig. 2. In this chapter, methods for catecholamine measurement as well as for vascular structure evaluation using are presented.

3.1 METHODOLOGY OF BLOOD PRESSURE MEASUREMENT

Two methods are generally used for blood pressure evaluation, direct and indirect blood pressure measurements. There are advantages and disadvantages inherent in each. However, both are good techniques, the choice depends on the goals of your study. For acute evaluation of BP, direct blood pressure measurement is recommended, it allows evaluation of BP only once however with accuracy. For long-term evaluation of BP, indirect blood pressure measurement is recommended because it allows you to record the BP of the same animal many times over a defined period of time.

3.1.1 Method for direct blood pressure measurement using indwell catheter

3.1.1.1 Materials and instruments

- a. Catheter for mouse carotid artery (C10PU-MCA1513, Instech, USA) and jugular vein (C10PU-MJV1403, Instech, USA)
- b. Polyethylene tube (PE-100)
- c. Metal pins with diameter (20ga, 15mm)

- d. Sterile saline
- e. Heparin
- f. Surgical suture (4-0 and 6-0)
- g. Isoflurane (MAC%=1.38)
- h. Surgical microscope
- i. Inhalation anesthesia system (VetEquip, USA)
- j. Electromagnetic transducer (Statham, model P23Dd)
- k. Amplifier (General Purpose Amplifier, Stemtech, Inc., USA)
- l. 10-Bit analog-to-digital conversion board (CODAS, DataQ Instruments, USA)
- m. Windaq® program (DataQ Instruments, USA)

3.1.1.2 Catheter preparation

- a. In order to easily recognize the aortic catheter from the vein catheter, place a mark at the end of the catheter.
- b. Flush the catheters with heparinized saline (250IU heparin/mL saline) making certain there are no bubbles. Insert a metal pin (20ga, 15mm) at the end of the catheter to retain heparinized saline.
- c. Submerge the catheters in heparinized saline while preparing mice.

3.1.1.3 Catheter implantation

- a. Prepare a sterile workstation, using 70% alcohol to spray the workstation and instrument trays.
- b. Anesthetize the animal with 3–4% isoflurane mixed with oxygen in chamber. After the mouse is unconscious with the absence of pain, transfer the mouse onto a heated surgical table. Place its head into the nose and mouth cone that delivers 2–3% of isoflurane to maintain the animal under anesthesia.
- c. Shave fur from posterior and anterior areas of the neck. Clean the posterior area with three alternating cycles of betadine and 70% ethanol and make a 4mm incision. Reposition the mouse facing the anterior area toward the operator. Clean the shaved anterior area with three alternating cycles of betadine and 70% ethanol. Restrain the animal by its upper and lower extremities to maintain gentle steady posture during the surgery.
- d. Using a scalpel, make 1cm incision in the midline skin of the neck. Using forceps, separate muscle to locate the right jugular vein, and isolate the vessel from the surrounding tissues. Prevent dryness by adding drops of saline to the exposed vessel. Pass two silk suture threads (4-0), underneath the isolated vein leaving 8mm of distance (approximately) between the two suture threads and tie a loose knot on the vessel. Using a microsurgical scissor, nick the vessel wall.

Using forceps, gently insert the 1.2cm of the catheter toward the heart. Secure the catheter into the vessel by tightening the both knots.

- e. Using forceps, locate the left carotid artery, which lies in parallel with the vagus nerve. Gently separate the artery from the nerve and surrounding tissue. Prevent dryness by adding drops of saline to the exposed aorta. Pass two silk suture threads (4–0) underneath the isolated artery. Place 8mm of distance between the two suture threads. Tie a knot to completely occlude blood passage in the cranial end. Tie a retractable knot to temporally occlude the caudal end. Prevent dryness by adding drops of saline to the exposed aorta. Using a microsurgical scissor, nick the vessel wall in between the two knots. Using forceps, gently insert 1.1cm of the catheter toward the heart; when the catheter tip reaches retractable knot untie this knot and continue inserting the catheter until it reaches the silicon part of the catheter. Tie the catheter with the two silk threads.
- f. Using a hemostat, make a subcutaneous tunnel toward the dorsal incision. Place a tube in the subcutaneous tunnel, insert in the tube both catheters, and pass them through the dorsal incision. Close the anterior area of the neck with stainless wound clips, and suture the dorsal incision using 4–0 silk suture thread. Immediately post-surgery, apply antibiotic on the incision areas and inject buprenorphine (subcutaneously).

3.1.1.4 Animal recovery and inclusion in the study: Place the animal in a clean cage and use a heating lamp to keep the animal warm until its full recovery. Monitor the animals for 48h; if signs of decreased activity, reluctance to move, hunched posture, and ungroomed fur are present, withdraw the animal from the study.

3.1.1.5 Catheter connection to the hemodynamic instrument and drug delivery device

- a. Carefully restrain the mouse. Using a rubber capped hemostat, secure the aortic catheter close to the metal pin and remove it. Loosen the hemostat and allow 0.1mL of volume to drain. This ensures that the catheter is not occluded. Connect the PE-100 filled with heparinized saline (250IU heparin/mL saline) without bubbles, remove the metal pin from the other end, and connect to an electromagnetic transducer (Statham, model P23Dd) which is connected to an amplifier (General Purpose Amplifier, Stemtech, USA).
- b. Following the same procedure connect 30cm PE-100 filled with heparinized saline (no bubbles) to the venous catheter (see note); however maintain the metal pin at the end of 30cm PE-100 catheter. Place the animal in the cage.

3.1.1.6 Hemodynamic recording

- a. Whit conscious animal that is free to move, record its BP for 1h or until it stabilizes. When the BP is stabilized record the baseline BP at 4000Hz frequency (mouse frequency in the hemodynamic system used).

- b. While recording the baseline BP, connect a syringe containing the ligand in 150 μ L, to the connector plug to the venous catheter. Inject the ligand, followed by saline injection (see Section 3.1.1.9). With the acquisition software, mark the starting point of ligand injection. Record the BP for 30min more after ligand injection (see Section 3.1.1.9).

3.1.1.7 Data analysis: The program automatically marks diastolic and systolic signals along the recorded beat-to-beat sampling; however, it is important to carefully verify whether the program marked at the right point the diastolic and systolic signal. Manually correct the mistaken marks. Lunch the numeric number and transfer to Excel.

3.1.1.8 Notes

- a. Making catheters is a key step for the success of BP measurement as well as drug injection. Insertion of the catheter is a challenge when the diameter of the catheter is too thick or when the catheter tip is too sharp or too dull. Catheters that are too thin present higher probability of clogging. If required a catheter can be fashioned using 4cm polyethylene tubing PE-10, with the internal diameter of 0.01mm connected to the 3cm PE-50 part, with an internal diameter of 0.05mm.
- b. The catheter must not reach the atria, this may yield not accurate results.
- c. It is important to know the volume held by the 30cm PE-100 catheter+the volume of the venous catheter.
- d. The quantity of saline injected is determined by the volume of the venous catheter+PE 100 connector. A automated drug delivery system (ADDS) to the venous catheter is available.
- e. Monitor the animal while recording to avoid twisting the connector which will interfere with the quality of the signal being recorded.

3.1.1.9 Result: The wild-type animal after recovery from the catheter implantation is subjected to hemodynamic evaluation. Fig. 3 depicts the direct blood pressure measurement in a wild-type mouse and in the tonin-overexpressing mouse (high AngII-levels). ARB decreased baseline BP in both animals, 150 μ L of 10mg/mL ARB injected caused a rapid dipping in both systolic and diastolic BP, and 1h after ARB injection, the baseline BP returned to normal. However, the decrease in the AngII-TG, a hypertensive model, was greater than that in the wild type. As a result of blocking AT1R, the baseline systolic blood pressure dropped 10mmHg in the wild-type mouse and dropped 20mmHg in the AngII-TG mouse.

This technique allows one to quantify the AT1R contribution to baseline BP accurately. The same recording may allow quantification of the AT1R impact on autonomic modulation of BP variability and heart rate variability. Both are used as a prognostic tool for organ damage as reported (Borrelli et al., 2018; Kurita et al., 2013; Moraes et al., 2018).

3.1.2 Method for indirect blood pressure measurement using a tail cuff—The key to getting good readings from mice using the tail cuff is twofold: consistency and

patience. Mouse behavior response varies widely to placement of a tail cuff. You can identify those in the first day. Mice do not respond in the same manner to the first day of handling. Some are fearless and some are very afraid. This different behavioral response does impact the BP level. They can also have the so-called “white coat hypertension.” Therefore, some mice require more time to calm down than others.

3.1.2.1 Material and instruments

- a. Tail blood pressure system
- b. Heating pad or platform
- c. Mouse holders
- d. Pocket infrared thermometer
- e. Quiet and warm room, away from high-traffic and/or noise

3.1.2.2 Mouse training and blood pressure measurement: Day 1: Handle each mouse for 10min. Mice at the animal facility housing are not being handled by people, they are used to being transferred from cage to cage. They need to get used to being handled.

Day 2: Handle each mouse for 10min and place into the warming restrainer for 10min. The mouse tail must be warm to get a good signal.

Day 3: Handle each mouse for 10min, place into the warming restrainer for 10min. Place occlusion cuff and pulse sensor on the tail. Using infrared thermometer verify the tail temperature, it should be 32°C. Inflate (no more than 80mmHg) and deflate the occlusion cuff for 5 cycles with 20s space between each cycle (see Section 3.1.2.3).

Day 4: Verify that the occlusion cuff does not leak air. Repeat procedure of day 3. Start the measurement using 200mmHg pressure.

3.1.2.3 Notes

- a. It is very important to make sure to train the mice in the same way the training session.
- b. The temperature of the tail is a key factor to be able to get the pulse and consequently good BP reading. However, only the tail temperature should reach 32°C, not the body temperature. Avoid allowing mouse body temperature to get too warm, that can very stressful and potentially kill the mouse.
- c. When inflating the occlusion cuff for training do it with a pressure lower than the systolic pressure to avoid tail tissue and tail vessel injury by applying too much pressure for repetitive cycles.

3.1.2.4 Results: Our goal with this experiment was to evaluate the long-term effect of β -arrestin-biased AT1R ligands on BP. Wild-type mice were infused for 28 days using osmotic minipumps at matching concentration (1.4 μ mol/g) of the AT1R full agonist, AngII. At this concentration AngII causes progressive BP rise and vascular damage. This technique allows

BP measurement every 7 days. Fig. 3 depicts that after 28 days of AngII infusion, BP raised progressively. Both AT1R-biased-agonists did not cause an increase and kept the BP at similar levels as ARB did.

Further, we evaluated the biased ligand effect on blood pressure of ApoEKO animals who have with higher risk to develop vascular morbidity. Animals were co-infused with both AngII plus TRV as aforementioned. Both ligands were infused at the concentration of 1.4 μ mol/g. This high concentration of AngII infused caused aortic dissection and aneurysm associated with high penetrance of mortality in ApoEKO animals. Co-infusion of the biased ligands (SYdA-AngII=TRV and SFdA-AngII) prevented vascular damage and maintained normal blood pressure levels in similar manner as ARB (Fig. 4).

3.2 METHODOLOGIES FOR CATECHOLAMINE RELEASE AND VASCULAR STRUCTURE EVALUATION

3.2.1 Method for Isolation of the aorta and adrenal gland—To assess target organ function and structural integrity at the end of long-term ligand infusion, desired target organs are harvested from euthanized mice for evaluation of the secretory functions and tissue pathology. This type of analysis will reveal whether the observed BP change is based on tissue remodeling and endocrine reprogramming of BP control systems in the body. Such changes may be responsible for lifelong changes and sometimes could be irreversible as well.

3.2.1.1 Material and instruments

- a. Dissecting microscope
- b. Microscissor with 2mm cutting edge and 0.5mm tip diameter
- c. Standard scissor
- d. Forceps, tip straight with 0.05 $\times$ 0.01mm² dimensions

3.2.1.2 Procedure

- a. For catecholamine quantification of adrenal gland, decapitate mice without anesthesia.
- b. For aortic newly synthesized protein and DNA, euthanize mice with an overdose of isoflurane anesthesia followed by cervical dislocation.
- c. Place the animal on a surgical tray in the supine position. Restrain the animal by its upper and lower extremities.
- d. Using forceps lift the abdominal skin and muscle straight up at the middle line of the pelvic region (approximately 1cm from the rectum). Using scissors cut both tissues longitudinally toward the cranial region passing the diaphragm up to the height of the thyroid gland (cut avoiding laceration of any internal organ). Starting at the caudal end of the longitudinal incision, cut the skin and muscle transversally toward the back legs. Starting at the cranial end of the longitudinal incision, cut the skin, muscle, and rib bones at pectoral area transversally toward

the front legs. At the diaphragm level, cut transversally the skin, muscle, and rib bone. These three cuts allow opening the mouse to expose all the pectoral, abdominal, and pelvic organs.

- e. Using a 10cc syringe connected to butterfly needle and filled with 10mL of sterile cold phosphate buffer solution (1×), perfuse the heart and aorta. Insert 2mm of the needle through bottom tip of the heart into the left ventricle. Cut the right atrium, place sterile gauze, and start injecting the PBS over 1–2min. When the perfusion is completed, remove the gauze that absorbed fluids within the thoracic cavity.
- f. *Aorta isolation*: Remove the stomach, intestines, liver, pancreas, spleen, and lungs. Leave, behind the aorta, heart, and kidneys. Under a dissecting microscope or magnifying glass and using forceps, gently lift the heart, find the aortic arch, gently lift up the arch (beginning of the aorta). Using a microscissor, cut the carotids and brachiocephalic arteries and start to separate the aorta from the spine, moving toward the renal arteries until the iliac bifurcation. It is important to note that the aorta runs parallel to the spine cord in the thoracic region, while in the abdominal area, it becomes superficial reaching the renal artery until the iliac bifurcation. Flash freeze the removed aorta and store at -80°C .
- g. *Adrenal gland collection*: Remove the stomach and intestines and localize the kidneys. On top of each kidney an adrenal gland is located. Remove the adrenal glands, flash freeze, and store at -80°C .

3.2.2 Method for quantification of catecholamines

3.2.2.1 Materials and instruments

- a. Norepinephrine (1mM)
- b. Epinephrine (1mM)
- c. Dopamine (3-hydroxytyramine) (1mM)
- d. L-Dopamine (L-3,4-dihydroxyphenylalanine) (1mM)
- e. DHBA (3,4-dihydroxybenzylamine) (internal standard) (1mM)
- f. Sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$)
- g. Perchloric acid
- h. Tris (tris-hidroximetil) aminomethane
- i. Monobasic sodium phosphate
- j. Sodium citrate
- k. N-heptane sulfonic acid sodium salt
- l. Ethylenediaminetetracetic acid (EDTA)
- m. Methanol (HPLC grade)

- n. Centrifuge
- o. Tissue grinder
- p. 0.22 μm filter
- q. Reverse phase high-performance liquid chromatography (HPLC) system with electrochemical (0.75V) detection (SIL-20AC, Shimadzu, Japan) coupled with sphere-5 ODS, 5 μM , 250 $\times$ 4.6mm² (Brownlee) column and RP18, 7 μM , 15 $\times$ 3.2mm² (Brownlee).

3.2.2.2 Catecholamine extraction

- a. On the day of catecholamines extraction: thaw samples on ice, weigh adrenal glands, and place into tube containing 7mL of cold buffer containing 0.1M of perchloric acid, 0.02% sodium metabisulfite, and 1mM dihydroxybenzylamine.
- b. Pipette 10 μL from 1mM DHBA.
- c. Homogenize samples using a tissue grinder making sure to keep the buffer cold while the tissue is being homogenized. Keep the homogenates at 4°C for 12h or overnight. Next, centrifuge samples at 10,000 $\times$ g 4°C for 50min.
- d. Carefully separate supernatant and proceed to filter supernatants using 0.22 μm .
- e. Perfuse the HPLC system with mobile phase (0.02M sodium phosphate, 0.02 sodium citrate, 0.12mM EDTA, 566mg/mL n-heptane sulfonic acid sodium salt, and 10% methanol, pH 2.53). To equilibrate the system and column, perform 10 runs with isocratic flow of 0.8mL/min for 25min.
- f. Inject blank (100 μL of mobile phase).
- g. Inject 100 μL of the pool of standards (norepinephrine, epinephrine, dopamine, and L-dopamine at 1 μM) plus internal standard DHBA (1 μM).
- h. Inject 100 μL of each sample.
- i. Results are based on the peak area, in general, HPLC software calculate it based on the standards area.

3.2.2.3 Notes

- a. The storage temperature of the samples is a key step. Temperature of -80°C or below is recommended to avoid loss of catecholamines.
- b. Before injecting samples into the HLPC, run the isocratic program and verify that baseline does not present any peak.
- c. An electrochemical HPLC system is very sensitive to temperature, thus temperature fluctuation may cause variation in the retention time of the amines. In order to overcome this difficulty, a suggestion is to inject a duplicate of the same sample, one pure sample and another with a spike of standards (including the internal standard). Comparison between the duplicates may indicate the exact retention time.

3.2.2.4 Result: Release of catecholamines is one of the long-term mechanisms of blood pressure control by AT1R. AT1R activation in the adrenal gland releases these amines to the circulation where norepinephrine and epinephrine bind to the alpha-adrenergic receptor causing vasoconstriction. Fig. 5 depicts catecholamine levels in two strains of wild-type mice and tonin-overexpression mice.

3.2.3 Method to monitor vascular protein synthesis in vivo by surface sensing of translation (SUnSET)—Evaluation of vasculature is important because of the direct relationship between the vasculature structure and blood pressure regulation. Compromised vasculature is observed in the hypertension state due to the vascular hypertrophy process that underlies vascular functional, structural, and mechanical changes in hypertension (Touyz et al., 2018). The method of surface sensing of translation (SUnSET) previously applied to in vitro (Schmidt, Clavarino, Ceppi, & Pierre, 2009) and in vivo (skeletal muscle) (Goodman & Hornberger, 2013) evaluation of protein synthesis can be used to monitor vasculature protein synthesis.

Puromycin, an aminonucleoside antibiotic produced by *Streptomyces alboniger*, can be used in the SunSET method. Puromycin, due to its analog structure of aminoacyl tRNAs, is incorporated into the nascent polypeptide chain of newly synthesized proteins thus reflecting the rate of mRNA translation in vivo (Goodman & Hornberger, 2013).

3.2.3.1 Material and instruments

- a. Puromycin (10mg/mL)
- b. Sterile saline
- c. Syringe (10cc) with butterfly needle
- d. Cold PBS
- e. Protease and phosphatase inhibitors
- f. Cold PBS plus protease and phosphatase inhibitors
- g. Petri dish
- h. Lysis buffer
- i. Tissue grinder
- j. Western blot equipment
- k. 12D10 mouse monoclonal antibody (catalog # MABE343, Millipore Sigma)
- l. IRDye 800CW Donkey anti-Mouse IgG (Licor)

3.2.3.2 Puromycin injection

- a. Weigh the animal and prepare 0.04μmol puromycin per gram of body mass in 150μL of sterile saline solution.
- b. Administer the specific dose/body mass via intraperitoneal injection.

- c. Exactly 30min post-injection, proceed to isolate the aorta as previously described.
- d. Cleaning of the aorta. Thaw the aorta into a 100mm petri dish plate filled with cold PBS containing protease and phosphatase inhibitors (at the concentration recommended by the manufacturer). Place the petri dish on ice to maintain the cold PBS temperature while cleaning the aorta. Under the microscope and using forceps, remove the fat tissue surrounding the aorta.
- e. Place 1cm of aorta into 100 μ L of cold lysis buffer containing protease and phosphatase inhibitors. Using a tissue grinder proceed to homogenize the aorta. Centrifuge homogenates at 10,000 $\times$ g for 10min, separate the supernatant from the pellet, quantify protein concentration.
- f. Load 15 μ g/sample on a gel, run, transfer protein, and block membrane using the standard western blot protocol. For puromycin detection, incubate the membrane overnight at 4°C with 1:10,000 puromycin antibody. Wash 3 $\times$ with 0.1% TBST or PBST. Incubate the membrane for 1h at room temperature with 1:20,000 anti-mouse antibody. Wash 3 $\times$ with 0.1% TBST or PBST and develop the membrane.

3.2.3.3 Notes

- a. Post-injection time is a key step. Perform this experiment in exactly the same way for every animal.
- b. It is recommended to perform the euthanasia, perfusion, and aorta collection as fast as possible and spending the same amount of time on every animal.

3.2.3.4 Result: Fig. 6 depicts vascular puromycin incorporation in the newly synthesized proteins in the aortic vessel of mice infused with AngII, and co-infused either with ARB or TRV.

3.2.4 Method to monitor vascular cell proliferation in vivo by incorporation of 5-ethynyl-2'-deoxyurine (EdU)—This method can be used to verify whether vascular cell proliferation is the underlying mechanism of vascular hypertrophy. Initially, this method was developed for in vivo evaluation of cell proliferation in the small intestines (Salic & Mitchison, 2008). Further, it was used to evaluate skeletal tissue (Mead & Lefebvre, 2014). In this chapter, 5-ethynyl-2-deoxyuridine (EdU) incorporation is described for in vivo evaluation of the vasculature cell proliferation. EdU is a thymidine analog which is incorporated into cellular DNA during replication. In this nucleoside analog, a terminal alkyne group replaces the thymidine methyl group in the fifth position. The detection method is a click reaction, in which copper catalyzes a covalent reaction between an azide and an alkyne group.

3.2.4.1 Material and instruments

- a. Click-iTTM EdU colorimetric IHC detection kit (catalog number: C10644, ThermoFisher)

- b. Embedding medium for frozen tissue specimens (O.C.T. compound) (Sakura Finetek)
- c. Histo-choice or paraformaldehyde 4%
- d. 1× phosphate-buffered saline (1× PBS)
- e. 3% BSA in 1× PBS
- f. Superfrost™ plus microscope slides
- g. Glass cover slips
- h. Vectashield mounting media
- i. Immunostaining pen (optional)

3.2.4.2 EdU injection

- a. Dissolve 5mg EdU in 1mL sterile PBS.
- b. Weigh the animal and prepare 0.099μmol EdU per gram of body mass in 150μL of sterile saline solution.
- c. Administer the specific dose/body mass via intraperitoneal injection.
- d. Exactly 24h post-injection, proceed to isolate the aorta as previously described; however instead of flash freezing the sample, place it in Histo-Choice or 4% of PFA overnight at 4°C, with the ratio of 1:10 of tissue to PFA.
- e. Cleaning of the aorta: Place the aorta into a petri dish containing 1× PBS. Under the microscope and using forceps, remove the fat tissue surrounding the aorta.
- f. Cut 10mm of the fixed aorta and embed it in OCT embedded frozen blocks; proceed to cut sections 10μm thick and generate four cross section/slide.
- g. Perform EdU detection, as recommended by the manufacturer's protocol.

4 SUMMARY AND FUTURE PERSPECTIVES

In humans walking into clinics, measurement of blood pressure is as routine as recording the individual's given name and body temperature at the time of visit. Similarly, the evaluation of BP as part of in vivo ligand evaluation is a concept in GPCR biology where consequence of infusing a novel ligand targeting a GPCR on the overall physiology of the animal is evaluated. Methods discussed in this chapter provide approaches to BP analyzes and further to investigate organ, tissue, and cellular mechanisms that govern BP homeostasis. In the case of the AngII/AT1R, our labs previously demonstrated that BP regulatory mechanisms may directly alter contractile state or alter the remodeling of the vasculature. Prolonged action of AT1R-selective ligands (such as TRV) might favor cellular growth responses. As illustrated in Figs. 1 and 2, almost all GPCR ligands may influence BP regulation either robustly or not at all. Further investigation may shed light on the molecular mechanisms of specific effects of ligands.

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REFERENCES

- Bhatnagar A, Unal H, Jagannathan R, Kaveti S, Duan ZH, Yong S, et al. (2013). Interaction of G-protein betagamma complex with chromatin modulates GPCR-dependent gene regulation. *PLoS One*, 8(1), e52689. [PubMed: 23326349]
- Bhattacharya M, Babwah AV, & Ferguson SS (2004). Small GTP-binding protein-coupled receptors. *Biochemical Society Transactions*, 32(Pt. 6), 1040–1044. [PubMed: 15506958]
- Bockaert J, & Pin JP (1999). Molecular tinkering of G protein-coupled receptors: An evolutionary success. *The EMBO Journal*, 18(7), 1723–1729. [PubMed: 10202136]
- Borrelli S, Garofalo C, Mallamaci F, Tripepi G, Stanzione G, Provenzano M, et al. (2018). Short-term blood pressure variability in nondialysis chronic kidney disease patients: Correlates and prognostic role on the progression of renal disease. *Journal of Hypertension*, 1 10.1097/HJH.0000000000001825.
- Bosnyak S, Jones ES, Christopoulos A, Aguilar MI, Thomas WG, & Widdop RE (2011). Relative affinity of angiotensin peptides and novel ligands at AT1 and AT2 receptors. *Clinical Science (London, England)*, 121(7), 297–303.
- Chen D, & Coffman TM (2015). AT1 angiotensin receptors-vascular and renal epithelial pathways for blood pressure regulation. *Current Opinion in Pharmacology*, 21, 122–126. [PubMed: 25687370]
- Forrester SJ, Booz GW, Sigmund CD, Coffman TM, Kawai T, Rizzo V, et al. (2018). Angiotensin II signal transduction: An update on mechanisms of physiology and pathophysiology. *Physiological Reviews*, 98(3), 1627–1738. [PubMed: 29873596]
- Goodman CA, & Hornberger TA (2013). Measuring protein synthesis with SUNSET: A valid alternative to traditional techniques? *Exercise and Sport Sciences Reviews*, 41(2), 107–115. [PubMed: 23089927]
- Guyton AC (1991). Blood pressure control—Special role of the kidneys and body fluids. *Science*, 252(5014), 1813–1816. [PubMed: 2063193]
- Karnik SS, Unal H, Kemp JR, Tirupula KC, Eguchi S, Vanderheyden PM, et al. (2015). International Union of Basic and Clinical Pharmacology. XCIX. Angiotensin receptors: Interpreters of pathophysiological angiotensinergic stimuli [corrected]. *Pharmacological Reviews*, 67(4), 754–819. [PubMed: 26315714]
- Kim J, Ahn S, Rajagopal K, & Lefkowitz RJ (2009). Independent beta-arrestin2 and Gq/protein kinase Czeta pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. *The Journal of Biological Chemistry*, 284(18), 11953–11962. [PubMed: 19254952]
- Kopf PG, Phelps LE, Schupbach CD, Johnson AK, & Peuler JD (2018). Differential effects of long-term slow-pressor and subpressor angiotensin II on contractile and relaxant reactivity of resistance versus conductance arteries. *Physiological Reports*, 6(5), e13623 (1–11 pp).
- Kuczeriszka M, Kompanowska-Jezierska E, Sadowski J, Prieto MC, & Navar LG (2018). Modulating role of Ang1–7 in control of blood pressure and renal function in AngII-infused hypertensive rats. *American Journal of Hypertension*, 31(4), 504–511. [PubMed: 29329358]
- Kurita A, Takase B, Kodani E, Iwahara S, Kusama Y, & Atarashi H (2013). Prognostic value of heart rate variability in comparison with annual health examinations in very elderly subjects. *Journal of Nippon Medical School*, 80(6), 420–425. [PubMed: 24419712]
- Leenen FHH, Blaustein MP, & Hamlyn JM (2017). Update on angiotensin II: New endocrine connections between the brain, adrenal glands and the cardiovascular system. *Endocrine Connections*, 6(7), R131–R145. [PubMed: 28855243]

- Liles C, Li H, Veitla V, Liles JT, Murphy TA, Cunningham MW, et al. (2015). AT2R autoantibodies block angiotensin II and AT1R autoantibody-induced vasoconstriction. *Hypertension*, 66(4), 830–835. [PubMed: 26259590]
- Mead TJ, & Lefebvre V (2014). Proliferation assays (BrdU and EdU) on skeletal tissue sections. *Methods in Molecular Biology*, 1130, 233–243. [PubMed: 24482177]
- Moraes OA, Flues K, Scapini KB, Mostarda C, Evangelista FS, Rodrigues B, et al. (2018). ACE gene dosage determines additional autonomic dysfunction and increases renal angiotensin II levels in diabetic mice. *Clinics (São Paulo, Brazil)*, 73, e246.
- Nakagawa P, & Sigmund CD (2017). How is the brain renin-angiotensin system regulated? *Hypertension*, 70(1), 10–18. [PubMed: 28559391]
- Noda K, Feng YH, Liu XP, Saad Y, Husain A, & Karnik SS (1996). The active state of the AT1 angiotensin receptor is generated by angiotensin II induction. *Biochemistry*, 35(51), 16435–16442. [PubMed: 8987975]
- Pierce KL, Premont RT, & Lefkowitz RJ (2002). Seven-transmembrane receptors. *Nature Reviews. Molecular Cell Biology*, 3(9), 639–650. [PubMed: 12209124]
- Ramchandran R, Takezako T, Saad Y, Stull L, Fink B, Yamada H, et al. (2006). Angiotensinergic stimulation of vascular endothelium in mice causes hypotension, bradycardia, and attenuated angiotensin response. *Proceedings of the National Academy of Sciences of the United States of America*, 103(50), 19087–19092. [PubMed: 17148616]
- Salic A, & Mitchison TJ (2008). A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 105(7), 2415–2420. [PubMed: 18272492]
- Schmidt EK, Clavarino G, Ceppi M, & Pierre P (2009). SUnSET, a nonradioactive method to monitor protein synthesis. *Nature Methods*, 6(4), 275–277. [PubMed: 19305406]
- Tamura K, Wakui H, Azushima K, Uneda K, Haku S, Kobayashi R, et al. (2015). Angiotensin II type 1 receptor binding molecule ATRAP as a possible modulator of renal sodium handling and blood pressure in pathophysiology. *Current Medicinal Chemistry*, 22(28), 3210–3216. [PubMed: 26295465]
- Toth AD, Turu G, Hunyady L, & Balla A (2018). Novel mechanisms of G-protein-coupled receptors functions: AT1 angiotensin receptor acts as a signaling hub and focal point of receptor cross-talk. *Best Practice & Research. Clinical Endocrinology & Metabolism*, 32(2), 69–82. [PubMed: 29678287]
- Touyz RM, Alves-Lopes R, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A, et al. (2018). Vascular smooth muscle contraction in hypertension. *Cardiovascular Research*, 114(4), 529–539. [PubMed: 29394331]
- Unal H, & Karnik SS (2012). Domain coupling in GPCRs: The engine for induced conformational changes. *Trends in Pharmacological Sciences*, 33(2), 79–88. [PubMed: 22037017]
- Violin JD, & Lefkowitz RJ (2007). Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends in Pharmacological Sciences*, 28(8), 416–422. [PubMed: 17644195]
- Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, et al. (2003). Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America*, 100(19), 10782–10787. [PubMed: 12949261]
- Zhuo JL, Kobori H, Li XC, Satou R, Katsurada A, & Navar LG (2016). Augmentation of angiotensinogen expression in the proximal tubule by intracellular angiotensin II via AT1a/ MAPK/NF-small ka, CyrillicB signaling pathways. *American Journal of Physiology. Renal Physiology*, 310(10), F1103–F1112. [PubMed: 26864937]

FIG. 1.

AT1R ligands and intracellular signaling: endogenous activators are: (A) peptide ligands such as full activator Ang II, partial activators AngIII and AngIV and (B) ligand-independent stimuli such as autoantibodies, mechanical stretch, and constitutive activity. AT1R can be selectively activated by synthetic peptides such as SII-AngII, SFdA-AngII, and SYdA-AngII=TRV120027 (β -arrestin biased ligands). Specific AT1R inactivators are the angiotensin receptor blockers (ARBs).

FIG. 2.

Blood pressure is affected by the organ structure and endogenous hormones: measurement of renin is an indirect measurement of AngII and related peptides content. AngII is the most potent full agonist of the renin-angiotensin system. Activation of the AT1R on the adrenal gland releases both aldosterone and catecholamines. Aldosterone affects fluid homeostasis and catecholamines affects vasoconstriction. Specific organ–signaling pathways are involved for healthy structure as well as for hormone production.

FIG. 3.

Angiotensin type 1 receptor contribution to baseline BP in wild-type and AngII-TG mice: this figure depicts 10min of recorded systolic BP. Even though the direct BP was recorded for 2h, in this graph only 10min is depicted 4min before ARB injection, and 6min after ARB injection (150 μ L from 10mg/mL).

FIG. 4.

AT1R ligand impact on blood pressure using indirect blood pressure measurement: (A) mean blood pressure of male and female wild-type mice after 28 days of infusion with saline, ARB (candesartan), b-arrestin biased ligands (SYdA-AngII=TRV and SFdA-AngII). (B) Individual values of the groups depicted in (A); and (C) ApoEKO systolic, diastolic, and mean pressure after 28 days of saline, ARB, and TRV infusion.

FIG. 5.

Chromatographic profile of the catecholamines of the three strains: In the top chromatogram, the green trace represents the four catecholamines and the internal standard and the black trace represents the blank sample. In the bottom chromatogram, the green and brown traces represent catecholamines of the wild-type mice, and the blue traced in the transgenic mice that overexpress AngII through overexpression of tonin, an angiotensin II forming enzyme.

FIG. 6.

Puromycin incorporation in aorta after 30min of injection: different pattern of protein synthesis is observed after full agonist, antagonist, and biased agonist. On the left is the ponceau stained membrane and on the right is the developed membrane after incubation with puromycin (primary antibody) and anti-mouse (secondary antibody).

Pharmacological Properties of AT1R Ligands

Table 1

Sidechain Modification	Ligand	Affinity ^a Kd (nM)	<i>b</i> GTP-S Binding (%)	<i>c</i> Receptor Phosphorylation (%)	<i>c</i> Internalization (%)	<i>c</i> pERK1/2 Total (%)	<i>c</i> pERK1/2β-arrestin2 siRNA (%)	Pharmacological Properties
None	AngII	2.0 ± 0.02	100	100	100	100	65	Full agonist
[Sar ¹ Ile ⁴ Ile ⁸]	SII-AngII	300 ± 18	0	57	80	40	5	Biased agonist
[Sar ¹ Phe ⁴ dAla ⁸]	SFdA-AngII	1.0 ± 0.02	0	55	82	42	4	Biased agonist
[Sar ¹ Tyr ⁴ dAla ⁸]	SYdA-AngII	0.8 ± 0.01	0	58	88	55	5	Biased agonist
Sar ¹ Ile ⁸	SI-AngII	0.8 ± 0.01	15	41	95	21	14	Partial agonist
Non-peptide	Candesartan	1.0 ± 0.01	0	0	0	0	0	Inverse agonist

AngII: Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8; Sar: sarcosine; dAla: D-alanine; SYdA-AngII is also called as TRV120027.

^aSpecific ligand binding was analyzed using total membranes prepared from mouse aorta. Competition-binding assays were performed under equilibrium conditions, with 1nM 125I-Sar1-AngII and various concentrations of the specified ligands ranging between 0.04 and 10,000nM and 10μM PD123319. Specific binding kinetics were analyzed by non-linear curve-fitting program GraphPad Prism 5. The mean±SD from n=3 experiments is shown.

^b³⁵S-GTP-S binding analysis was performed using total membrane preparation from mouse aorta. Specific binding induced by 1μM AngII was set as 100% and compared to binding induced by specific ligands. Average of three replicates represented.

^cParameters measured in rat aortic smooth muscle cells (RASM) permanently expressing HA-AT1R, each response for 1μM AngII was set as 100% for calculating relative efficacy of specified ligand. β-Arrestin2 siRNA treated for 36h before ligand stimulation and pERK1/2 was measured. Note that the affinity of ligands is not significantly different in RASM.