—Original—

A-Type Natriuretic Peptide Level in Angiotensin II Type 1a Receptor Knockout Mice

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Abstract: A-type (atrial) natriuretic peptide (ANP) levels in the heart and plasma were examined by immunohistochemistry, electron microscopy, and radioimmunoassay (RIA) in angiotensin II type 1a receptor knockout (*Agtr1a* KO) mice. Additionally, the ANP mRNA level in the heart was measured using a real-time polymerase chain reaction (PCR) assay. The blood pressure in *Agtr1a* KO mice was significantly lower than that in wild-type (WT) mice. The number of ANP granules and ANP immunoreactivity in the auricular cardiocytes were significantly lower in *Agtr1a* KO mice than in WT mice. Ultrastructurally, the ventricular cardiocytes in *Agtr1a* KO mice occasionally had ANP-like granules, which were not present in WT mice. The plasma, auricular, and ventricular ANP and plasma cyclic guanosine monophosphate (cGMP) concentrations were significantly higher in *Agtr1a* KO mice than in WT mice. The ANP mRNA levels of the auricular and ventricular cardiocytes in the *Agtr1a* KO mice were almost twice as large as those in WT mice. The present data suggest that a notable increase in the ANP biosynthesis and release in the heart of *Agtr1a* KO mice may account for the reduction in blood pressure together with the lack of an AGTR1A receptor in this model.

Key words: A-type natriuretic peptide, *Agtr1a* knockout mice, cardiocyte, hypotension

Introduction

A-type (atrial) natriuretic peptide (ANP) is a circulating hormone with a wide range of biological effects, including natriuresis, diuresis, and vasodilation, and it plays an important role in the regulation of blood pressure and volume homeostasis [24]. This peptide hormone is mainly produced in the cardiac atria, where it is stored within secretory granules as a large precursor (proANP), which is the major component of the granules [3, 5, 22]. ANP acts through guanylate cyclase A (GC-A) to lower blood pressure (BP) and dilate blood vessels in mammals

[16, 17, 23, 29], and to inhibit the growth of cardiac myocytes and fibroblasts in cultured cells [11].

The renin-angiotensin system (RAS), composed of renin, angiotensinogen, angiotensin, and angiotensin-converting enzyme, is believed to be one of the most important regulators of cardiovascular homeostasis. A potent vasoconstrictor, octapeptide angiotensin II (Ang II), the bioactive product of the RAS, exerts numerous physiological effects through its specific receptors located on the plasma membrane. At present, Ang II receptors are classified into two subtypes, Ang II type 1 (AGTR1) and type 2 (AGTR2), and in mice, the AGTR1

(Received 12 September 2011 / Accepted 9 November 2011)

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receptors are further subcategorized into AGTR1A and AGTR1B receptors [13, 16, 32]. Most of the well-known functions of Ang II in the cardiovascular system are mediated through the AGTR1 receptor [2, 27, 33], especially through the AGTR1A receptor in rodents [10], and Ang II plays a key role in the development of cardiac hypertrophy [12]. Recently, several reports have demonstrated that the hypertension and cardiac hypertrophy of GC-A-deficient mice [26] can be partially ascribed to augmented cardiac AGTR1A signaling and that GC-A inhibits AGTR1A signaling-mediated excessive remodeling [18, 19]. However, the biological mechanisms, especially those underlying the response to ANP release from the heart via the AGTR1A receptor pathway are still unknown.

The Ang II type 1a receptor knockout (*Agtr1a* KO) mouse, which exhibits homozygous and heterozygous mutations of the AGTR1A receptor gene that render it nonfunctional, was generated by gene targeting [14, 31]. *Agtr1a* KO mice show reduced blood pressure and a lack of responsiveness to Ang II [14, 31]. Recent evidence suggests that cardiovascular homeostasis is determined by the balance in the cross-talk between the natriuretic peptide system and the angiotensin system [18]. In a previous study, we demonstrated that ANP synthesis and release in heart tissues are stimulated by the cross-regulation between the cardiac ANP and RAS in an animal model of human essential hypertension (the Tsukuba hypertensive mouse) [20]. However, it is unclear whether the Ang II receptors are involved in the interaction between the ANP level and the RAS, and to our knowledge, no data have been available on the plasma and heart ANP levels of *Agtr1a* KO mice.

In order to elucidate the underlying mechanism responsible for the secretion of heart ANP in the *Agtr1a* KO mice, we studied the morphological changes in ANP granules in atrial cardiocytes and the plasma and heart ANP levels in these mice. Moreover, we examined the ANP mRNA levels in atrial and ventricular cardiocytes to determine whether the ANP producing system was upregulated in *Agtr1a* KO mice.

Materials and Methods

Animals

Ten males of 3-month-old *Agtr1a* KO mice and wild-type (WT) mice (Jcl: C57BL/6J, CLEA Japan, Inc., Osaka, Japan) from the same genetic background were used in the present study. The *Agtr1a* KO mice, which

had the same genetic background as described previously [31], were kindly provided by Tanabe Seiyaku Co., Ltd. (Osaka, Japan). All animals were kept in automatically controlled rooms (temperature of $24 \pm 1^\circ\text{C}$; humidity of $55 \pm 5\%$; automatic lighting from 7:00 a.m. to 7:00 p.m.) and fed CE-2 pellet diet (CLEA Japan, Inc.) and water *ad libitum*. These animals were sacrificed under sodium pentobarbital anesthesia (50 mg/kg, i.p.), and the heart and blood were removed. All experiments were undertaken in accordance with the *Guidelines for Animal Experimentation*, Kurume University.

Measurement of blood pressure (BP)

The BP was measured in all mice. The systolic BP was measured by the tail-cuff procedure (BP monitor MK-2000; Muromachi-Kikai Co., Ltd., Tokyo, Japan). The mice were placed in an equipped holder for 5–10 min until they became calm prior to monitoring their blood pressure with the tail-cuff method. The BP data of mice that could not keep quiet during measurement were excluded. Data with noise during pulse wave monitoring of blood pressure was also excluded. The BP values obtained from three consecutive measurements of the respective mice were averaged and recorded at each time point.

Immunohistochemistry

The right auricular tissue blocks were fixed in Zamboni's solution for 24 h at 4°C . After washing with 0.15 M phosphate-buffered saline (PBS) at pH 7.3, they were dehydrated, embedded in paraffin, and sectioned at a thickness of $5\ \mu\text{m}$. Prior to immunohistochemical staining, the deparaffinized sections were incubated in absolute methanol containing 3% H_2O_2 for 20 min. Immunohistochemical staining was performed according to the modified avidin-biotin-peroxidase complex (ABC) technique described previously [21]. Following incubation in normal swine serum, sections were incubated with primary antibody overnight at 4°C . For ANP immunohistochemical study, rabbit antiserum against synthesized human ANP 99–126 (Code: NAW160) was used as the primary antibody diluted 1:1,000 with PBS containing 0.02% Triton X-100. As the secondary antibody, biotinylated swine anti-rabbit immunoglobulin (1:500; DAKO A/S, Glostrup, Denmark) was used.

Electron microscopy

The right auricular and left ventricular tissues were

fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 and postfixed in 1% osmium tetroxide in the same buffer. They were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined with a JEM-2000 EX electron microscope.

Ultrastructural morphometry

The number of secretory granules of the right auricular cardiocytes was measured according to the modified morphometry method of Cantin *et al.* [3, 4]. Thin sections were viewed on a JEM-2000 EX electron microscope at a magnification of $\times 3,000$. Ten photographs were randomly chosen from sections of the right auricular cardiocytes of each mouse in accordance with the criteria proposed by Cantin *et al.* [3, 4]. The counting of ANP-granules was done on 25 cm $\times$ 30 cm prints at a final magnification of $\times 14,000$, corresponding to an area of 382.7 μm^2 . The mean ($\pm$ SD) counts were calculated from 50 photographs for 5 animals each of the knockout and WT mice.

Radioimmunoassay (RIA)

Blood was drawn from a common carotid artery into a syringe containing 1 mg of EDTA and 1,000 units of the kallikrein-inhibitor aprotinin (Bayer, Leverkusen, Germany). The plasma was rapidly frozen and stored at -80°C until measurement of the plasma ANP and cyclic guanosine monophosphate (cGMP) levels. The right auricular and left ventricular tissue samples were boiled for 5 min in 10 volumes of 0.1 M acetic acid to abolish intrinsic proteolytic activity and then homogenized with a POLYTRON homogenizer (Kinematica AG, Littau, Lucerne, Switzerland) at 25,000 rpm for 60 s. Each homogenate was centrifuged at 30,000 g for 30 min at 4°C , and the supernatants were stored at -80°C until RIA. The plasma and heart ANP levels were measured using an Atrial Natriuretic Factor (rat) RIA kit (Phoenix Pharmaceuticals, Inc., Mountain View, CA, USA). The plasma cyclic GMP concentration was measured by RIA (Amersham International, Little Chalfont, Bucks., UK).

Real-time polymerase chain reaction (PCR) assay

Right auricular and left ventricular tissues from both mice were subjected to acid guanidinium thiocyanate-phenol-chloroform RNA extraction, and the RNA was then stored at -80°C until examination. Total RNA was

isolated using a RNeasy kit (Qiagen, Valencia, CA, USA), and its concentration determined using UV spectrophotometry (DU[®] Series 65; Beckman, Fullerton, CA, USA). The quality and integrity of total RNA were assessed on 1% formaldehyde-agarose gels. First-strand cDNA was synthesized using 1 μg of total RNA (DNase-treated) in a 20 μg reverse transcriptase (RT) reaction mixture. The amounts of ANP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA transcripts were evaluated by real-time PCR, which was performed using the following synthetic oligonucleotides as primers: sense and antisense primers 5'-CTCCTTCTC-CATCACCAAGG-3' and 5'-CTCTGGGCTC-CAATCCTGTGTC-3' for ANP and sense and antisense primers 5'-CTTCACCACCATGGAGAAGGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3' for GAPDH, respectively. All real-time PCR reactions were performed in a 20 μl mixture containing a 1/20 volume of cDNA preparation (2 μl), 1 $\times$ SYBR Green buffer containing FastStart Taq DNA Polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 mM MgCl_2 , 0.2 μM of each primer, and 0.2 mM dNTP. Real-time measurements of the mRNA levels were performed using a LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany). The fluorescence threshold value was calculated using the LightCycler[™] V3 system software.

Statistical analysis

All tests were performed using SPSS 11.0J for windows (SPSS, Chicago, IL, USA), and all data are represented as means $\pm$ SD unless otherwise indicated. The unpaired Student's *t*-test was used for comparisons of values between WT and *Agtr1a* KO mice. A *P* value less than 0.05 was considered to be statistically significant.

Results

Blood pressure and heart weigh

As shown in Table 1, the systolic BP in *Agtr1a* KO mice was reduced compared with WT mice, indicating a significant ($P < 0.001$) difference between *Agtr1a* KO

Table 1. Profiles of the *Agtr1a* KO and WT mice

	WT	<i>Agtr1a</i> KO
Systolic blood pressure (mmHg)	118.6 $\pm$ 11.3	87.6 $\pm$ 9.1 ^{a)}
Heart weight/body weight (%)	0.449 $\pm$ 0.01	0.448 $\pm$ 0.07

Values are means $\pm$ SD. ^{a)}: Significant difference ($P < 0.001$).

Fig. 1. Immunohistochemical staining of ANP in the right auricular cardiocytes in WT (a) and *Agtr1a* KO (b) mice. ANP immunoreactivity (IR) is located primarily in the perinuclear region of the cardiocytes in both mice (arrowheads). In *Agtr1a* KO mice (a), ANP-IR is weaker than that in WT mice (b). Each bar represents 50 μ m.

Fig. 2. Electron micrographs of the right auricular cardiocytes in WT (a) and *Agtr1a* KO (b) mice and the left ventricular cardiocytes in WT (c) and *Agtr1a* KO (d) mice. In the auricular cardiocytes, ANP granules (arrowheads) are variable in size and number and are mainly located in the perinuclear region in association with the Golgi apparatus (a, b). The number of granules in *Agtr1a* KO (b) mice is less than that in WT (a) mice. The ventricular cardiocytes in *Agtr1a* KO (d) mice rarely have ANP-like granules (arrowheads), which are not observed in WT (c) mice. Each bar represents 1 μ m. Nucleus (N).

and WT mice. The ratio of heart weight to body weight was the same in both mice.

Immunohistochemistry

ANP immunoreactivity (ANP-IR) was found in the right auricular cardiocytes in both groups of mice. The ANP-IR was located primarily in the perinuclear region of the cardiocytes in *Agtr1a* KO and WT mice (Fig. 1). In *Agtr1a* KO mice, ANP-IR was weaker than that in

WT mice (Fig. 1a and 1b).

Ultrastructure

In *Agtr1a* KO and WT mice, the right auricular cardiocytes contained a centrally located nucleus, numerous mitochondria, myofibrils, a small amount of rough endoplasmic reticulum, a Golgi apparatus and electron-dense granules (Fig. 2a and 2b). The granules were variable in size and number and were mainly located in the

perinuclear region in association with the Golgi apparatus. The ventricular cardiocytes in *Agtr1a* KO mice occasionally had ANP-like granules, which were not observed in WT mice (Fig. 2c and 2d). The number of granules in the right auricular cardiocytes is shown in Fig. 3. In *Agtr1a* KO mice (59.1 ± 19.9), the number of granules was significantly ($P < 0.05$) lower than that in WT mice (76.4 ± 24.5).

Fig. 3. Number of ANP granules in the right auricular cardiocytes in *Agtr1a* KO ($n=10$) and WT mice ($n=10$). Data are presented as means $\pm$ SD. *Significant difference ($P < 0.05$).

ANP and cGMP concentrations

The plasma, right auricular, and left ventricular ANP and plasma cGMP concentrations are shown in Table 2. The plasma ANP concentration in *Agtr1a* KO mice was significantly ($P < 0.001$) and approximately 3-fold higher than that in WT mice. The auricular and ventricular ANP concentrations in *Agtr1a* KO mice were also significantly ($P < 0.05$) higher and almost double or triple those in WT mice. The plasma cGMP concentration was approximately 10-fold higher in *Agtr1a* KO mice than that in WT mice ($P < 0.001$).

Real-time PCR assay

The ANP mRNA expression indices of the auricle and ventricle in *Agtr1a* KO mice (auricle, 2.49 ± 1.04 ; ventricle, 1.84 ± 0.74) were significantly ($P < 0.05$) higher than those in WT mice (auricle, 1.11 ± 0.58 ; ventricle, 1.05 ± 0.49), respectively. The levels in *Agtr1a* KO mice were almost double those in WT mice (Fig. 4a and 4b). The PCR products were electrophoresed in agarose gel, and their sizes were confirmed (ANP, 417 bp; GAPDH, 238 bp) (Fig. 4c).

Table 2. ANP and cGMP levels in *Agtr1a* KO and WT mice

	WT	<i>Agtr1a</i> KO
Plasma ANP concentration (pg/ml)	192.4 ± 48.3	577.8 ± 84.4^b
Auricular ANP concentration ($\mu\text{g/g}$ wet tissue)	58.2 ± 11.7	153.9 ± 17.2^b
Ventricular ANP concentration (ng/g wet tissue)	23.8 ± 7.5	46.8 ± 19.7^a
Plasma cGMP concentration (pmol/ml)	8.6 ± 1.9	74.3 ± 22.3^b

Values are means $\pm$ SD. ^{a)} and ^{b)}: Significant difference ($P < 0.05$ and $P < 0.001$, respectively).

Fig. 4. ANP mRNA levels of the right auricle (a) and left ventricle (b) in *Agtr1a* KO ($n=5$) and WT mice ($n=5$). Data are presented as means $\pm$ SD. *Significant difference ($P < 0.05$). (c) PCR products: right auricle in WT (lane 1) and *Agtr1a* KO (lane 2) mice and left ventricle in WT (lane 3) and *Agtr1a* KO (lane 4) mice. M: size markers.

Discussion

Generally, *Agtr1a* KO mice exhibit a reduced blood pressure and a lack of responsiveness to Ang II [14, 31], thereby providing an animal model to elucidate the role of AGTR1A or other receptors in the RAS. The *Agtr1a* KO mice in this study showed hypotension without a concomitant increase in heart weight, in good agreement with the previous reports [1, 14, 31].

The major findings of the present study are that the number of ANP granules and the ANP immunoreactivity in the auricular cardiocytes in *Agtr1a* KO mice were significantly smaller than in WT mice in spite of the higher levels of auricular ANP concentration and mRNA expression in the *Agtr1a* KO mice. In addition, ANP-like granules of the ventricular cardiocytes were only detectable in the *Agtr1a* KO mice, and the ventricular ANP concentrations and mRNA expressions in the *Agtr1a* KO mice were higher than those in WT mice. These findings indicate that the biosynthesis of ANP in the auricular and ventricular cardiocytes was augmented in *Agtr1a* KO mice.

Usually, ANP in the auricle is believed to be a vasodilating and natriuretic hormone that is stored in secretory granules and secreted by a "regulated pathway." In this pathway, newly synthesized proteins destined for secretion are stored at high concentrations in secretory granules until the cell receives an appropriate stimulus [15] such as distension of the atrial wall [9]. Alternatively, during constitutive secretion, the secretory products are hard to find in electron micrographs of the cytoplasm. If the proteins are secreted as fast as they are synthesized, this would suggest that the ventricular ANP is rapidly released by a "constitutive pathway" [15]. Our results, along with the proposition of Kelly [15], raise the possibility that ANP is more rapidly secreted from the ventricles by a "constitutive pathway" in *Agtr1a* KO mice than from the ventricles in WT mice. In support of this hypothesis, we noted that the ventricular ANP mRNA levels as well as the ventricular ANP concentrations in the *Agtr1a* KO mice were approximately 2-fold higher than those in the WT mice. At the same time, it is likely that decreased ANP granules and increased ANP concentrations and mRNA levels in the auricles of *Agtr1a* KO mice are attributable to an increased ANP biosynthesis and turnover in the auricles of these mice. As ANP was more rapidly secreted from the ventricles by a "constitutive pathway" in *Agtr1a* KO mice with the

existence of a small amount of ANP granules in the ventricular cardiocytes, we propose that ANP may be secreted from the auricle by both regulated and constitutive pathways in response to the demand of ANP and that ANP in the auricular cardiocytes may be widely distributed in the cytoplasm rather than stored as secretory granules in the *Agtr1a* KO mice.

Notably, the plasma cGMP level was markedly increased in *Agtr1a* KO mice compared with WT mice, and it was involved in the enhanced ANP synthesis and release in the heart of *Agtr1a* KO mice. Natriuretic peptides exert potent diuretic, natriuretic and vasorelaxant effects through the activation of their common receptor, GC-A [also called natriuretic peptide receptor (NPR)-A] [16, 23]. GC-A is a prototype plasma membrane-bound guanylate cyclase (GC), and all GCs serve as receptors that produce cGMP in response to ligand binding [8]. ANP acts through GC-A to lower the BP and dilate blood vessels [17, 29]. Thus, the enhanced plasma cGMP level induced by augmented ANP synthesis and release in the heart tissues can be interpreted as the acceleration of ANP effects through the binding to GC-A, which results in a decrease in the BP and hypotension in the *Agtr1a* KO mice.

It has been well reported that there is cross-regulation between the cardiac ANP system and RAS [6, 25], and we have previously reported this using Tsukuba hypertensive mice that exhibit overproduction of angiotensin I and II [20]. An ACE inhibitor and Ang II receptor blocker had direct effects on the induction of ANP secretion, thus suggesting that ANP secretion is regulated by the RAS, which plays a key role in lowering the blood pressure or preserving the myocardium [30]. Ang II has been reported to increase ANP secretion in isolated hearts [7]. The localization of immunoreactive Ang II in the secretory granules of ventricular myocytes, and the release of Ang II by acute stretching of the cardiac myocytes [28] suggest that the autocrine and paracrine functions of Ang II are involved in regulating the stretch-induced secretion of ANP. Whether or not the augmented ANP synthesis and release in the *Agtr1a* KO mice are attributed to Ang II activation itself or some indirect mechanisms will be elucidated in a future study.

An alternative explanation for the enhanced cardiac ANP synthesis and release in the *Agtr1a* KO mice in the present study is that the chronic absence of AGTR1A affects the AGTR2 signaling pathways, thereby increasing the ANP activity. Li *et al.* [19] demonstrated that

the enhanced plasma ANP level in GC-A-deficient mice caused by the effect of AGTR2 receptor signaling in the heart suggested that AGTR2 signaling is also an important factor controlling BP. Our results together with this previous report suggest that ANP synthesis and release in the heart tissues were stimulated by AGTR1A blockade in combination with AGTR2 acceleration. These observations are consistent with the idea that cardiovascular homeostasis is determined by the balance in the cross-talk between the natriuretic peptide receptor and angiotensin receptors [18, 19].

In conclusion, our present data, which showed the heart and plasma ANP levels to increase in *Agtr1a* KO mice, account for the reduction in blood pressure observed in this model together with the lack of an AGTR1A receptor. Meanwhile, the *Agtr1a* KO mice described here provide a genetically well-defined animal model that may be of considerable value for better understanding the chronic regulation of blood pressure by the balance in the cross-talk between the ANP system and the RAS.

Acknowledgments

We thank Dr. Takeshi Sugaya (Department of Internal Medicine, Division of Nephrology, Juntendo University School of Medicine, Tokyo, Japan) for providing *Agtr1a* KO mice from Tanabe Seiyaku Co., Ltd. The current work was supported in part by a Japanese Grant-in-Aid for Scientific Research (C) (No. 15591790).

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