

Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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Hypertension 1998;32;223-227

Hypertension is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 75214

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Effect of Genetic Deficiency of Angiotensinogen on the Renin-Angiotensin System

Kouichi Tamura, Satoshi Umemura, Yoichi Sumida, Nobuo Nyui, Shun-ichi Kobayashi, Tomoaki Ishigami, Minoru Kihara, Takeshi Sugaya, Akiyoshi Fukamizu, Hitoshi Miyazaki, Kazuo Murakami, Masao Ishii

Abstract—This study examined expression of renin-angiotensin system (RAS) component mRNAs in angiotensinogen gene knockout (*Atg*^{-/-}) mice. Wild-type (*Atg*^{+/+}) and *Atg*^{-/-} mice were fed a normal-salt (0.3% NaCl) or high-salt (4% NaCl) diet for 2 weeks. Angiotensinogen, renin, angiotensin-converting enzyme (ACE), angiotensin II type 1a receptor (AT_{1A}), and angiotensin II type 2 receptor (AT₂) mRNA levels were measured by Northern blot analysis. In *Atg*^{+/+} mice, activities of circulating RAS and renal angiotensinogen mRNA level were decreased by salt loading, whereas levels of renal and cardiac ACE; renal, brain, and cardiac AT_{1A}; and brain and cardiac AT₂ mRNA were increased by salt loading. Although activities of circulating RAS were not detected in *Atg*^{-/-} mice, salt loading increased blood pressure in *Atg*^{-/-} mice. In *Atg*^{-/-} mice, renal renin mRNA level was decreased by salt loading; in contrast, salt loading increased renal AT_{1A} and cardiac AT₂ mRNA levels in *Atg*^{-/-} mice, and these activated levels in *Atg*^{-/-} mice were higher than those in *Atg*^{+/+} mice fed the high-salt diet. Thus, expression of each component of the RAS is regulated in a tissue-specific manner that is distinct from other components of systemic and local RAS and that appears to be mediated by a mechanism other than changes in the circulating or tissue levels of angiotensin peptides. (*Hypertension*. 1998;32:223-227.)

Key Words: renin-angiotensin system ■ angiotensinogen ■ angiotensin-converting enzyme ■ receptors, angiotensin ■ RNA, messenger ■ sodium, dietary

The RAS plays a critical role in maintaining blood pressure and fluid electrolyte balance. The RAS historically has been viewed as a circulatory system. The various components of the RAS are produced by different organs and are delivered to their site of action by the bloodstream. However, accumulated evidence derived from biochemical and molecular studies of the physiological properties of angiotensin suggests that distinct local RAS with different regulatory mechanisms exist and function in the brain, heart, adrenal gland, kidney, vessel wall, and adipose tissue. Although it is controversial whether all components of the RAS are physiologically relevant and the exact pathophysiological role of the local RAS remains elusive, it is interesting to speculate that the local RAS may enhance the actions of Ang II in a specific physiological process of a given tissue system. In addition, previous studies showed that a variety of stimuli, including blood pressure, sodium intake, inflammation, and sympathetic nerve activity, modulate the expression of the tissue RAS genes in physiological and pathophysiological states.¹⁻³ Furthermore, several studies using antagonists of Ang II receptor subtypes suggest that Ang II exerts various effects on the expression of major component genes of the RAS by positive or negative feedback mechanisms.⁴⁻⁷

Recently, we and others generated angiotensinogen-deficient mice by gene targeting.⁸⁻¹⁰ Homozygous mutant (*Atg*^{-/-}) mice have no detectable plasma angiotensinogen or angiotensin peptides; they therefore lack a functional RAS and exhibit chronic hypotension. The aim of the present study was to examine whether dietary salt loading modulates the expression of major component genes of the RAS in *Atg*^{-/-} mice without affecting angiotensin formation, as a first step to analyze a feedback mechanism by which angiotensin peptides exert influences on expression of the RAS gene.

Methods

Animals

Atg^{-/-} mice were generated by gene targeting as described previously.⁸ *Atg*^{-/-} (n=12) and *Atg*^{+/+} (n=12) mice, aged 6 weeks, were used in this study. The mice were housed under a 12/12-hour day/night cycle at a temperature of 25°C and fed a normal-salt (0.3% NaCl) diet for 2 weeks. At 8 weeks of age, *Atg*^{-/-} and *Atg*^{+/+} mice were divided into 2 groups, were placed on either a high-salt (4% NaCl) or normal-salt (0.3% NaCl) diet, and were kept in metabolic cages for determination of daily urine output and levels of urinary aldosterone and electrolytes. Tap water was provided ad libitum. At 10 weeks of age, SBP and BW were measured. SBP was measured by tail-cuff plethysmography. Mice were killed by decap-

Received March 10, 1998; first decision March 16, 1998; revision accepted March 20, 1998.

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Selected Abbreviations and Acronyms

ACE	=	angiotensin-converting enzyme
Ang	=	angiotensin
AT _{1A}	=	angiotensin II type 1A receptor
AT ₂	=	angiotensin II type 2 receptor
BW	=	body weight
HW	=	wet tissue heart weight
RAS	=	renin-angiotensin system
SBP	=	systolic blood pressure

itation. Brain, heart, and kidney were dissected out and immediately frozen in liquid nitrogen.

Biochemical Assays

The concentrations of electrolytes were measured with an automated analyzer for routine laboratory tests (Hitachi-736). Plasma Ang I concentration was measured with a radioimmunoassay kit (Renin Riabead Ang I kit, Dainabot Co Ltd).¹¹ Plasma Ang II concentration was determined by a specific direct radioimmunoassay using an anti-Ang II antibody, as described previously, without an extraction procedure.¹² Urinary concentrations of aldosterone were determined with a radioimmunoassay kit (SPAC-S aldosterone kit, Daiichi Radio-isotope Co).

For measurement of ACE activity in the kidney, the kidneys were homogenized in ACE homogenization buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.5% Triton X-100, 25 μ mol/L ZnCl₂, 1 mmol/L PMSF) and clarified by centrifugation for 15 minutes at 10 000g.¹³ Renal ACE activity was measured by a spectrophotometric assay kit using 3-(2-furylacryloyl)-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate (Sigma Chemical Co).¹⁴ Total protein concentration in the kidney homogenates was calculated by the method of Lowry et al.¹⁵

RNA Isolation and Analysis

Total RNA from tissues was extracted with the guanidinium thiocyanate–cesium chloride centrifugation method.¹⁶ Each RNA sample (20 μ g) was denatured with 1 mol/L glyoxal and 50% DMSO, electrophoresed on 1.2% agarose gels, and transferred onto nylon membranes (GeneScreen Plus, DuPont-New England Nuclear). Fil-

ters were prehybridized for 30 minutes at 60°C in a solution consisting of 1% SDS, 1 mol/L NaCl, and 10% dextran sulfate. Hybridization proceeded for 18 hours at 60°C in the same solution containing 300 μ g/mL denatured salmon sperm DNA and 1 \times 10⁶ cpm/mL of the ³²P-labeled probes for angiotensinogen,⁸ renin,¹⁷ ACE,¹⁸ AT_{1A},¹⁹ or AT₂.²⁰ Filters were washed twice with 2 \times SSC (1 \times SSC=150 mmol/L NaCl, 15 mmol/L sodium citrate) for 5 minutes at room temperature, twice with 2 \times SSC and 1% SDS for 30 minutes at 60°C, and twice with 0.1 \times SSC for 15 minutes at room temperature. Dried filters were exposed to an imaging plate of Fujix Bio-Imaging Analyzer BAS2000 (Fuji Photo Film). Expression of mRNA was quantified with the BAS2000 computer analyzer and normalized to the signal generated by probing for the constitutively expressed 18S rRNA gene.²¹

Statistical Analysis

For statistical analysis of differences among groups, the unpaired Student's *t* test or ANOVA followed by Scheffé's *F* test was used. All quantitative data are expressed as mean \pm SE. A value of *P*<0.05 was considered to indicate statistical significance.

Results**SBP and Circulating Angiotensin Peptides in *Atg*^{+/+} and *Atg*^{-/-} Mice**

As shown in Table 1, BW of *Atg*^{-/-} mice at 10 weeks was lower than that of *Atg*^{+/+} mice. When fed the normal-salt diet, *Atg*^{-/-} mice had significantly lower SBP and the ratio of HW to BW (HW/BW ratio) than *Atg*^{+/+} mice. In *Atg*^{-/-} mice, SBP was significantly increased by the high-salt diet, whereas this diet had no effect on SBP in *Atg*^{+/+} mice and on HW/BW ratio in either *Atg*^{+/+} or *Atg*^{-/-} mice. Plasma Ang I concentration, plasma Ang II concentration, and urinary aldosterone levels were below the detection limit of the assay systems in *Atg*^{-/-} mice and were significantly decreased in *Atg*^{+/+} mice when they were fed the high-salt diet. Although *Atg*^{-/-} mice showed an increased urine output and a decreased urine osmolality compared with

TABLE 1. Characteristics of *Atg*^{+/+} and *Atg*^{-/-} Mice Fed a Normal- or High-Salt Diet

Characteristic	<i>Atg</i> ^{+/+}		<i>Atg</i> ^{-/-}	
	Normal Salt (0.3% NaCl)	High Salt (4.0% NaCl)	Normal Salt (0.3% NaCl)	High Salt (4.0% NaCl)
SBP, mm Hg	95 \pm 6.3	108 \pm 4.5	70 \pm 2.8†	95 \pm 5.3*
BW, g	36.4 \pm 1.1	37.9 \pm 0.9	31.2 \pm 0.8†	32.6 \pm 0.9†
HW, g	0.22 \pm 0.01	0.20 \pm 0.01	0.14 \pm 0.01†	0.13 \pm 0.01†
HW/BW ratio, $\times 10^{-3}$	6.02 \pm 0.46	5.29 \pm 0.19	4.49 \pm 0.23†	4.09 \pm 0.18†
KW, g	0.30 \pm 0.02	0.34 \pm 0.02	0.29 \pm 0.03	0.34 \pm 0.03
KW/BW ratio, $\times 10^{-3}$	8.12 \pm 0.25	8.95 \pm 0.39	9.34 \pm 0.81	10.49 \pm 0.83
p-Ang I, pmol/mL	1.85 \pm 0.19	1.20 \pm 0.12*	ND	ND
p-Ang II, fmol/mL	51.9 \pm 13.7	15.0 \pm 1.4*	ND	ND
U _{Na} V, μ mol/d	175.9 \pm 30.2	1168.4 \pm 48.5*	124.2 \pm 32.0	1052.9 \pm 93.6*
U _K V, μ mol/d	318.9 \pm 40.5	458.6 \pm 22.1*	227.0 \pm 26.4	422.7 \pm 35.5*
U _{ald} V, pmol/d	6.08 \pm 1.39	1.78 \pm 0.39*	ND	ND

KW indicates wet tissue weight of left kidney; p-Ang I, plasma Ang I concentration; p-Ang II, plasma Ang II concentration; ND, not detected; U_{Na}V, urinary sodium excretion; U_KV, urinary potassium excretion; and U_{ald}V, urinary aldosterone excretion. Values are mean \pm SE; n=6 mice in each group.

**P*<0.05 vs normal-salt diet, †*P*<0.05 vs *Atg*^{+/+} mice within the same salt group.

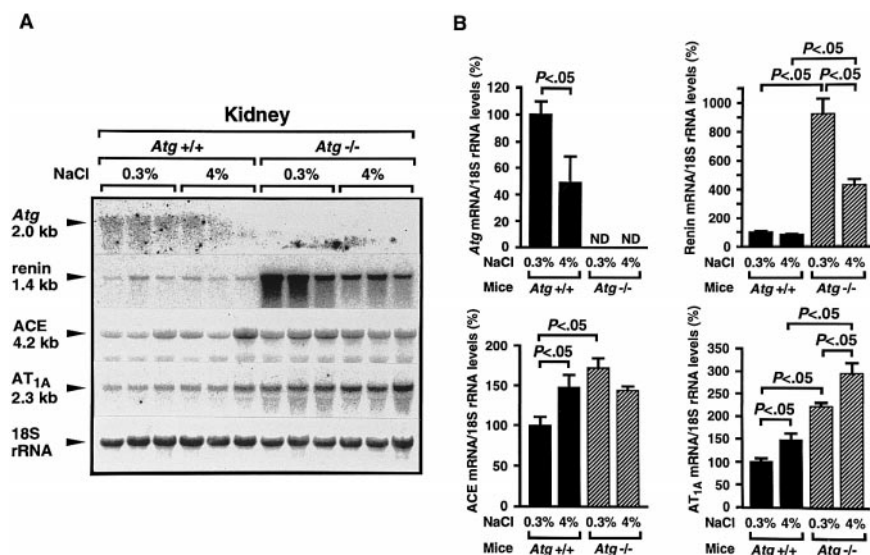


Figure 1. A, Representative Northern blot analysis showing the expression of angiotensinogen (*Atg*), renin, ACE, and AT_{1A} mRNAs in the kidney of *Atg*^{+/+} and *Atg*^{-/-} mice. Total RNA (20 μg) was isolated from the kidney of *Atg*^{+/+} and *Atg*^{-/-} mice, electrophoresed, and hybridized with probes for *Atg*, renin, ACE, AT_{1A} mRNAs, and 18S rRNA. B, Relative *Atg*, renin, ACE, and AT_{1A} mRNA levels (n=6 in each group). The levels of mRNA expression were measured as radioactivities using a BAS2000 Imaging Analyzer, normalized relative to the radioactivity generated by probing for 18S rRNA expression, and expressed relative to those achieved with RNA from the kidney of *Atg*^{+/+} mice fed a normal-salt (0.3% NaCl) diet. Data are mean±SE from the 6 separate experiments.

Atg^{+/+} mice,²² urinary excretions of sodium and potassium were similar in these mice (Table 1).

Effects of Salt Loading on mRNA Expression of RAS in *Atg*^{+/+} and *Atg*^{-/-} Mice

Because the level of renal renin expression in *Atg*^{-/-} mice is reported to be much higher than in *Atg*^{+/+} mice^{8,23} and the kidney plays a critical role in the maintenance of cardiovascular homeostasis, we examined expression of the RAS components in the kidney of *Atg*^{+/+} and *Atg*^{-/-} mice and analyzed the effect of salt loading on expression of RAS as determined by Northern blot analysis (Figure 1). In the kidney, angiotensinogen mRNA is expressed in *Atg*^{+/+} mice but not in *Atg*^{-/-} mice, and the high-salt diet decreased angiotensinogen mRNA levels in *Atg*^{+/+} mice. *Atg*^{-/-} mice had higher levels of renin, ACE, and AT_{1A} mRNA expression than *Atg*^{+/+} mice when fed the normal-salt diet; the high-salt diet significantly increased AT_{1A} mRNA levels in both *Atg*^{-/-} and *Atg*^{+/+} mice and ACE mRNA levels in *Atg*^{+/+} mice but decreased renin mRNA levels in *Atg*^{-/-} mice. The ACE mRNA levels in *Atg*^{+/+} mice were comparable to those in *Atg*^{-/-} mice fed the high-salt diet, and *Atg*^{-/-} mice still had 5.3- and 2.0-fold higher levels of renin and AT_{1A} mRNA expression than *Atg*^{+/+} mice, respectively, when fed the high-salt diet. The levels of ACE enzymatic activity showed the same trend with the ACE mRNA levels in the kidney of both *Atg*^{-/-} and *Atg*^{+/+} mice (Table 2).

TABLE 2. Renal ACE Activities of *Atg*^{+/+} and *Atg*^{-/-} Mice Fed a Normal- or High-Salt Diet

<i>Atg</i> ^{+/+}		<i>Atg</i> ^{-/-}	
Normal Salt (0.3% NaCl)	High Salt (4.0% NaCl)	Normal Salt (0.3% NaCl)	High Salt (4.0% NaCl)
53.3±3.9	67.7±3.7*	83.0±4.8†	72.8±4.5

Renal ACE activities are expressed as units/mg tissue protein. Values are mean±SE; n=6 mice in each group.

*P<0.05 vs normal-salt diet, †P<0.05 vs *Atg*^{+/+} mice within the same salt group.

In the brain, angiotensinogen mRNA is expressed in *Atg*^{+/+} mice but not in *Atg*^{-/-} mice, and the high-salt diet had no significant effect on levels of angiotensinogen mRNA (Figure 2). Both AT_{1A} and AT₂ mRNA levels in *Atg*^{+/+} mice were comparable to those in *Atg*^{-/-} mice fed the normal-salt diet. In *Atg*^{+/+} mice, the high-salt diet significantly increased AT_{1A} and AT₂ mRNA levels, whereas in *Atg*^{-/-} mice the high-salt diet had no effect on such levels. The levels of cardiac ACE, AT_{1A}, and AT₂ mRNA expression were similar in *Atg*^{+/+} and *Atg*^{-/-} mice fed the normal-salt diet (Figure 3).²⁴ The high-salt diet significantly increased ACE, AT_{1A}, and AT₂ mRNA levels in *Atg*^{+/+} mice. In *Atg*^{-/-} mice, the high-salt diet did not affect cardiac ACE or AT_{1A} mRNA levels, but it significantly increased cardiac AT₂ mRNA expression to 1.4-fold higher than that in *Atg*^{+/+} mice fed the high-salt diet.

Discussion

Previous studies of regulation of the RAS components in genetically or experimentally hypertensive animals showed widespread abnormalities of RAS gene expression that were modulated in some tissues by the development of hypertension.¹⁻³ However, little is known about regulation of the RAS genes in hypotensive animals. Because *Atg*^{-/-} mice do not produce angiotensin peptides at all and thus are chronically hypotensive, *Atg*^{-/-} mice may be a genetically suitable hypotension model for analysis of the regulation of RAS gene expression in vivo. In the present study, we showed that salt loading significantly increased SBP in *Atg*^{-/-} mice and that expression of the RAS genes was regulated in a tissue-specific manner by salt loading in *Atg*^{-/-} mice.

Consistent with the results of previous studies,^{8,23} we showed an increase in the levels of renin mRNA in the kidney of *Atg*^{-/-} mice compared with *Atg*^{+/+} mice. The mRNA levels of ACE and AT_{1A} were also increased in the kidney of *Atg*^{-/-} mice. Upregulation of ACE and AT_{1A} mRNA levels in *Atg*^{-/-} mice was observed in the kidney but not in either the brain or heart when these mice were fed the normal-salt diet. These results suggest that expression of the renal ACE and AT_{1A} genes is specifically activated in *Atg*^{-/-} mice. Previous studies

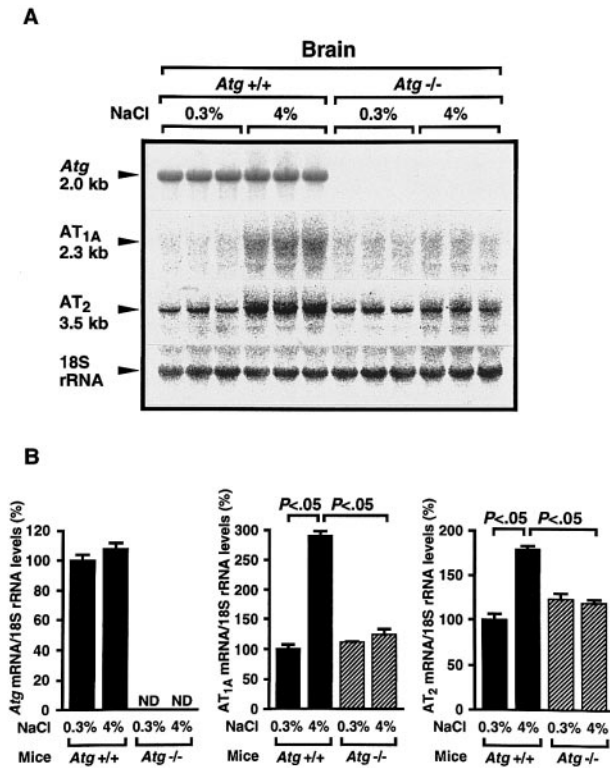


Figure 2. A, Representative Northern blot analysis showing the expression of angiotensinogen (*Atg*), *AT*_{1A}, and *AT*₂ mRNAs in the brain of *Atg*^{+/+} and *Atg*^{-/-} mice. Total RNA (20 μg) was isolated from the brain of *Atg*^{+/+} and *Atg*^{-/-} mice, electrophoresed, and hybridized with probes for *Atg*, *AT*_{1A}, *AT*₂ mRNAs, and 18S rRNA. B, Relative *Atg*, *AT*_{1A}, and *AT*₂ mRNA levels (n=6 in each group). The levels of mRNA expression were measured as radioactivities using a BAS2000 Imaging Analyzer, normalized relative to the radioactivity generated by probing for 18S rRNA expression, and expressed relative to those achieved with RNA from the brain of *Atg*^{+/+} mice fed a normal-salt (0.3% NaCl) diet. Data are mean±SE from the 6 separate experiments.

revealed that the kidney in *Atg*^{-/-} mice was undergoing numerous pathological changes, including marked vascular hypertrophy, interstitial inflammation, atrophic changes in the tubules and papilla, and increased expression of growth factors and neuronal nitric oxide synthase genes,^{9,10,23} as well as increased urine output and decreased urine osmolality compared with *Atg*^{+/+} mice.²² In contrast to these remarkable abnormalities in the kidney, no appreciable abnormality was found in the brain or heart of *Atg*^{-/-} mice.⁹ Thus, these pathological changes may be involved in increases in the mRNA levels of ACE and *AT*_{1A} in the kidney in *Atg*^{-/-} mice fed the normal-salt diet. In particular, *Atg*^{-/-} mice have reduced renal medullas; thus, their renal RNA is enriched in RNA from the renal cortex where ACE is produced. This could explain the elevations of ACE mRNA levels observed in *Atg*^{-/-} mice. Another possibility is that the higher mRNA levels of ACE and *AT*_{1A} in the kidney of *Atg*^{-/-} mice than of *Atg*^{+/+} mice may be due to complete disruption of the negative feedback of Ang II on ACE- and *AT*_{1A}-expressing cells in the kidney of *Atg*^{-/-} mice.^{4,5} Further study is needed to clarify the mechanisms responsible for these increases in the kidney of *Atg*^{-/-} mice.

Tissue ACE in the heart may play a role in the pathogenesis of cardiac hypertrophy and remodeling.²⁵ However, little is

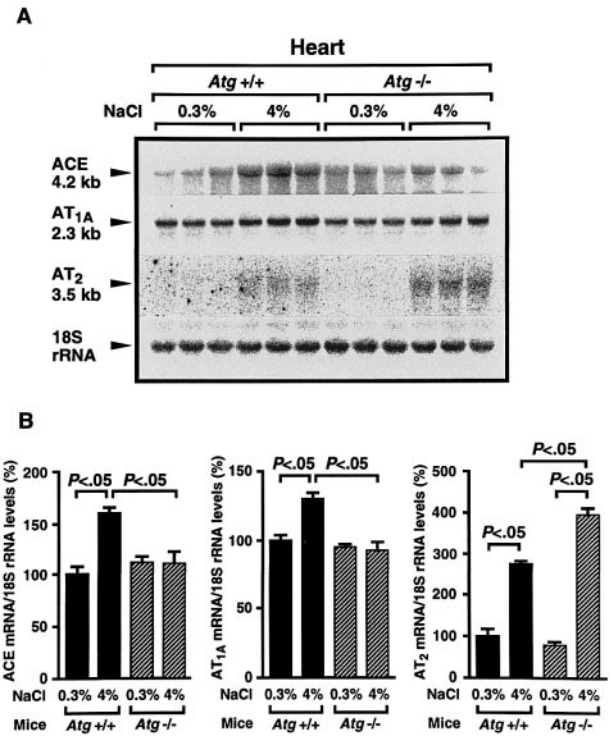


Figure 3. A, Representative Northern blot analysis showing the expression of ACE, *AT*_{1A}, and *AT*₂ mRNAs in the heart of *Atg*^{+/+} and *Atg*^{-/-} mice. Total RNA (20 μg) was isolated from the heart of *Atg*^{+/+} and *Atg*^{-/-} mice, electrophoresed, and hybridized with probes for ACE, *AT*_{1A}, *AT*₂ mRNAs, and 18S rRNA. B, Relative ACE, *AT*_{1A}, and *AT*₂ mRNA levels (n=6 in each group). The levels of mRNA expression were measured as radioactivities using a BAS2000 Imaging Analyzer, normalized relative to the radioactivity generated by probing for 18S rRNA expression, and expressed relative to those achieved with RNA from the heart of *Atg*^{+/+} mice fed a normal-salt (0.3% NaCl) diet. Data are mean±SE from the 6 separate experiments.

known regarding the regulation of the cardiac ACE gene by altered sodium intake. In this study, salt loading increased the cardiac ACE mRNA levels in *Atg*^{+/+} mice but not in *Atg*^{-/-} mice. Because a previous study showed a significant increase in cardiac ACE mRNA in response to a high-sodium diet in both Wistar-Kyoto and stroke-prone spontaneously hypertensive rats,²⁶ the findings in the present study suggest that angiotensin peptides are necessary for a salt-mediated increase in the cardiac ACE mRNA levels.

Similar to the regulation of cardiac ACE gene, salt loading increased the brain and cardiac *AT*_{1A} mRNA levels in *Atg*^{+/+} mice but not in *Atg*^{-/-} mice. Previous studies showed that high sodium intake increased expression of the *AT*_{1A} mRNA in the brain and kidney.^{7,27,28} Dietary sodium loading is known to suppress the circulating RAS and to decrease circulating levels of Ang II. Because Ang II downregulates expression of the *AT*_{1A} gene,⁵ the decrease in circulating Ang II levels by salt loading may upregulate *AT*_{1A} gene expression. In the present study, there was no significant difference in *AT*_{1A} mRNA expression in the brain and heart in *Atg*^{+/+} and *Atg*^{-/-} mice when fed the normal salt diet, and salt loading increased the *AT*_{1A} mRNA levels in *Atg*^{+/+} mice but not in *Atg*^{-/-} mice. Thus, complete lack of angiotensin peptides may make the *AT*_{1A} gene unable to respond to salt loading in the brain and heart.

In contrast to the AT_{1A} gene, much less is known about the regulation of the AT₂ gene. Abundant expression of AT₂ has been found in the mesenchymal tissues of a developing rat fetus, indicating an important role of AT₂ in growth, development, and apoptosis.^{29,30} Previous studies reported an increase in AT₂ expression in the hypertrophic myocardium of experimental hypertensive rats and showed that the process of cardiac remodeling after myocardial infarction induced not only AT_{1A} but also AT₂ expression.^{31,32} In the present study, salt loading increased the brain AT₂ mRNA levels in *Atg*^{+/+} mice but not in *Atg*^{-/-} mice. On the other hand, the treatment upregulated the cardiac AT₂ mRNA levels in both *Atg*^{+/+} and *Atg*^{-/-} mice, and the activated levels of cardiac AT₂ mRNA were higher in *Atg*^{-/-} mice than in *Atg*^{+/+} mice. Therefore, these results suggest that the complete lack of angiotensin peptide activates the salt-mediated expression of cardiac AT₂ mRNA but inhibits the salt-mediated expression of brain AT₂ mRNA.

In conclusion, the findings obtained in the present study suggest that dietary salt loading exerts a systemic influence on the RAS component genes differently in *Atg*^{+/+} and *Atg*^{-/-} mice. Future research must elucidate the systemic and cellular mechanisms by which salt intake modulates the expression of RAS genes and the possible roles of angiotensin peptides in these mechanisms.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan; the Ichiro Kanehara Foundation; the Uehara Memorial Foundation; the Yokohama Foundation for Advancement of Medical Science; and the Naito Memorial Foundation.

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