Tissue-specific changes of type 1 angiotensin II receptor and angiotensin-converting enzyme mRNA in angiotensinogen gene-knockout mice

K Tamura¹, N Yokoyama¹, Y Sumida¹, T Fujita¹, E Chiba¹,
N Tamura², S Kobayashi¹, M Kihara¹, K Murakami³, M Horiuchi⁴
and S Umemura¹

¹Department of Internal Medicine II, Yokohama City University School of Medicine, Yokohama 236, Japan

²Department of Dermatology, Yokohama City University School of Medicine, Yokohama 236, Japan

³Institute of Applied Biochemistry, University of Tsukuba, Ibaraki 305, Japan

⁴Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

(Requests for offprints should be addressed to K Tamura, Department of Internal Medicine II, Yokohama City University School of Medicine, 3-9, Fukuura, Kanazawa-ku, Yokohama 236, Japan)

Abstract

This study examined whether type 1 angiotensin II receptor (AT₁) and angiotensin-converting enzyme (ACE) mRNAs are regulated during dietary salt loading in angiotensinogen gene-knockout (*Atg*^{-/-}) mice which are genetically deficient in endogenous production of angiotensin II. Wild-type (*Atg*^{+/+}) and *Atg*^{-/-} mice were fed a normal-salt (0.3% NaCl) or a high-salt (4% NaCl) diet for 2 weeks. The mRNA levels were measured by Northern blot analysis. In *Atg*^{+/+} mice, concentrations of plasma angiotensin peptides were decreased by salt loading, whereas the treatment increased the brainstem, cardiac, pulmonary, renal cortex, gastric and intestinal AT₁ mRNA levels. Salt loading also enhanced renal cortex ACE mRNA levels in *Atg*^{+/+} mice. Although plasma angiotensin peptides and urinary aldosterone excretion were not detected in *Atg*^{-/-} mice, salt loading increased blood pressure in *Atg*^{-/-} mice. In *Atg*^{-/-} mice, pulmonary, renal cortex, gastric and intestinal AT₁,

and renal cortex and intestinal ACE mRNA levels were higher than those in *Atg*^{+/+} mice. However, salt loading upregulated AT₁ mRNA expression only in the liver of *Atg*^{-/-} mice, and the treatment did not affect ACE mRNA levels in *Atg*^{-/-} mice. Furthermore, although the levels of ACE enzymatic activity showed the same trend with the ACE mRNA levels in the lung, renal cortex and intestine of both *Atg*^{-/-} and *Atg*^{+/+} mice, the results of radioligand binding assay showed that cardiac expression of AT₁ protein was regulated differently from AT₁ mRNA expression both in *Atg*^{-/-} and *Atg*^{+/+} mice. Thus, expression of AT₁ and ACE is regulated by salt loading in a tissue-specific manner that appears to be mediated, at least partly, by a mechanism other than changes in the circulating or tissue levels of angiotensin peptides.

Journal of Endocrinology (1999) **160**, 401–408

Introduction

The renin–angiotensin system (RAS) exerts a major influence on blood pressure as well as sodium and extracellular fluid balance through the generation of angiotensin II (Ang II), which has a variety of actions such as vasoconstrictor activity and stimulation of the production and release of aldosterone. The RAS has been strongly implicated in the development of several cardiovascular diseases including hypertension, and accumulating evidence from biochemical and molecular studies of angiotensin suggests that distinct local RASs with different regulatory mechanisms from the classical circulating RAS may exist (Griendling *et al.* 1993, Dzau & Re 1994).

Whether all components of local RASs are physiologically relevant is controversial, and the precise roles of these systems remain elusive, but it is interesting to speculate that a local RAS may augment the effects of Ang II on a particular tissue in specific physiological and pathophysiological processes. 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 (Griendling *et al.* 1993, Dzau & Re 1994, Tamura *et al.* 1995). 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 (Kohara *et al.* 1992, Lassegue *et al.* 1995, Sechi *et al.* 1996).

Recently, we and others generated angiotensinogen-deficient mice by gene targeting (Tanimoto *et al.* 1994, Kim *et al.* 1995, Niimura *et al.* 1995). 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.

Materials and Methods

Animals

Atg^{-/-} mice were generated by gene targeting as described previously (Tanimoto *et al.* 1994). Six-week-old male *Atg*^{-/-} (*n*=16) and *Atg*^{+/+} (*n*=16) mice were used in this study. The mice were housed under a 12/12-hour day/night cycle at a temperature of 25°C. All animals had free access to tap water and normal rat chow containing 0.3% NaCl. At 8 weeks of age, *Atg*^{-/-} and *Atg*^{+/+} mice were divided into two groups and placed on either a high-salt (4% NaCl) or a normal-salt (0.3% NaCl) diet. At 10 weeks of age, systolic blood pressure (SBP) was measured by tail-cuff plethysmography. Mice were killed by decapitation. Blood was centrifuged at 15 000 *g* for 10 min at 4 °C and plasma was stored at -80 °C. Fore-brain, heart, lung, liver, renal cortex, stomach and intestine were dissected out and immediately frozen in liquid nitrogen.

Radioimmunoassay for plasma renin activity, angiotensin peptides and aldosterone

Plasma renin activity (PRA) and plasma angiotensin I (Ang I) concentration were measured by a radioimmunoassay kit (RENIN RIABEAD Ang I kit, Dainabot Co., Ltd, Tokyo, Japan) (Tokita *et al.* 1994). Plasma Ang II concentration was determined by a specific direct radioimmunoassay, using an anti-Ang II antibody as described previously without an extraction procedure (Shimamoto *et al.* 1984). Before and after the experimental period, individual mice were placed in metabolic cages for 24-h urine collection. Urinary excretion of aldosterone was determined with a radioimmunoassay kit (SPAC-S aldosterone kit, Daiichi Radio-isotope Co., Tokyo, Japan).

Determination of angiotensin-converting enzyme activity

For measurement of angiotensin-converting enzyme (ACE) activity in the tissues, the tissues were homogenized in ACE homogenization buffer (50 mM HEPES,

pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 25 µM ZnCl₂, 1 mM phenylmethyl sulfonyl fluoride (PMSF)) and clarified by centrifugation for 15 min at 10 000 *g* (Esther *et al.* 1996). Tissue ACE activity was measured by a spectrophotometric assay kit using 3-(2-furylacryloyl)-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate (Sigma Co., St Louis, MO, USA) (Johansen *et al.* 1987). Total protein concentration in the tissue homogenates was calculated by the method of Lowry *et al.* (1951).

Determination of mRNA expression

Northern blotting analysis was performed essentially as described previously (Tamura *et al.* 1996, 1997, 1998). Total RNA from tissues was extracted using the guanidinium thiocyanate-cesium chloride centrifugation method (Chirgwin *et al.* 1979). Each RNA sample (20 µg) was denatured with 1 M glyoxal and 50% dimethyl sulfoxide, electrophoresed on 1.2% agarose gels, and transferred onto nylon membranes (GeneScreen Plus, DuPont-New England Nuclear, Boston, USA). Filters were prehybridized for 30 min at 60 °C in a solution consisting of 1% sodium dodecyl sulfate (SDS), 1 M NaCl, and 10% dextran sulfate. Hybridization proceeded for 18 h at 60 °C in the same solution containing 300 µg/ml denatured salmon sperm DNA and 1 × 10⁶ c.p.m./ml of the ³²P-labeled probes for angiotensinogen (Tanimoto *et al.* 1994), ACE (Soubrier *et al.* 1988) or type 1 Ang II receptor (AT₁) (Sugaya *et al.* 1995). Filters were washed twice with 2 × SSC (1 × SSC=0.15 M NaCl, 0.015 M sodium citrate) for 5 min at room temperature, twice with 2 × SSC and 1% SDS for 30 min at 60 °C, and twice with 0.1 × SSC for 15 min at room temperature. Filters were exposed to the imaging plate of a 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 ribosomal RNA gene (Raynal *et al.* 1984).

Determination of AT₁ binding activity

Radioligand binding assay was performed as described previously (Sumida *et al.* 1998). Briefly, the whole hearts were minced, homogenized, and centrifuged. The final pellet was resuspended in a buffer of 10 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, 8 mg/ml antipain, and 16 mg/ml leupeptin, and immediately used for radioligand receptor binding experiments. The incubation mixtures contained a suspension of membranes (approximately 140 to 300 µg protein), 70 pM [¹²⁵I]-[Sar¹,Ile⁸]-Ang II, incubation buffer and Ang II antagonist (Sar¹,Ile⁸-Ang II, CV-11974) at various concentrations (10 pM-10 mM), in a final volume of 200 µl. The samples were incubated for 120 min at 22 °C, followed by three washes with ice-cold buffer containing

10 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl₂, and 2 mg/ml bovine serum albumin. The radioactivity trapped on the filters was quantified with an automatic gamma counter (Beckman Gamma 5500). Nonspecific binding of ¹²⁵I-[Sar¹,Ile⁸]-Ang II was defined as the radioactivity that bound to membrane fractions and was not displaced by a high concentration of unlabeled Sar¹,Ile⁸-Ang II (1 mM). Specific ¹²⁵I-[Sar¹,Ile⁸]-Ang II binding displaced by CV-11974 was estimated as AT₁. Each binding assay was carried out in duplicate. Maximum binding capacity (B_{max}) and dissociation constant (K_d) were analyzed with the LIGAND computer program.

Statistics

For statistical analysis of differences among groups, the unpaired Student's *t*-test or analysis of variance followed by Scheffe's *F*-test were used. All quantitative data are expressed as means ± s.e. A value of *P* < 0.05 was considered to be statistically significant.

Results

SBP, PRA, plasma angiotensin peptides and urinary aldosterone in Atg+/+ and Atg-/- mice

As shown in Fig. 1, body weight 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. In *Atg* -/- mice, SBP was significantly increased by the high-salt diet, whereas this diet had no effect on SBP in *Atg* +/+ mice. PRA, plasma Ang I and Ang II concentrations and urinary aldosterone excretion were below the detection limit of the assay systems in *Atg* -/- mice, and were significantly decreased in *Atg* +/+ mice when fed the high-salt diet.

Tissue RAS gene expression in Atg+/+ and Atg-/- mice

Since angiotensinogen, which is the unique substrate of RAS and has been strongly suggested to be involved in human hypertension, is abundantly expressed in the liver and brainstem, we first examined the expression of angiotensinogen mRNA in these tissues of *Atg* +/+ and *Atg* -/- mice and analyzed the effect of salt-loading on expression by Northern blot analysis (Fig. 2). The BAS2000 analyzer system used in this study is able to count radioactivity directly from the imaging plate which is exposed to radioactive material (Amemiya & Miyahara 1988). The imaging plate of BAS2000 is more sensitive (about 100-fold) than the conventional X-ray film in the detection of radioactivity, and has a wide dynamic range with good linear relationship with radioactivity, thus allowing us to calculate accurately the mRNA value. We

Figure 1 (A) Body weight, (B) systolic blood pressure, (C) plasma (p) Ang I concentration, (D) PRA, (E) plasma (p) Ang II concentration and (F) urinary (u) aldosterone excretion in *Atg* +/+ and *Atg* -/- mice fed a normal-salt (0.3% NaCl) or a high-salt (4% NaCl) diet for 2 weeks. Values are means ± s.e. (*n*=8). ND, not detected.

repeated the quantitation of the mRNA value and used the data from two independent experiments. Angiotensinogen mRNA is expressed in *Atg* +/+ mice but not in *Atg* -/- mice and the high-salt diet had no significant effect on the levels of hepatic and brainstem angiotensinogen mRNA. Both hepatic and brainstem AT₁ 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 the brainstem AT₁ mRNA levels, whereas in *Atg* -/- mice the high-salt diet increased the hepatic AT₁ mRNA levels.

As shown in Fig. 3, *Atg* -/- mice had higher levels of pulmonary and renal cortex AT₁ mRNA expression than *Atg* +/+ mice when fed the normal-salt diet. The high-salt diet significantly increased the pulmonary and renal cortex AT₁ mRNA levels in *Atg* +/+ mice. In *Atg* -/- mice, the treatment decreased the pulmonary AT₁ mRNA levels but did not affect the renal cortex AT₁ mRNA levels.

Figure 2 Expression of AT₁ and angiotensinogen (Atg) mRNA in the liver and brainstem of *Atg*^{+/+} and *Atg*^{-/-} mice fed a normal-salt (0.3% NaCl) or a high-salt (4% NaCl) diet for 2 weeks. (A) Representative results of Northern blot analysis of total RNA (20 µg) for AT₁ and Atg mRNA and 18S ribosomal RNA (18S). (B) Bar graphs show relative AT₁ and Atg mRNA levels. The mRNA levels were measured using a BAS2000 Imaging Analyzer, normalized to the signal generated by probing for 18S, and expressed relative to those achieved with RNA from tissues of *Atg*^{+/+} mice fed a normal-salt (0.3% NaCl) diet. Values are means ± S.E. (n=8). ND, not detected.

With respect to the regulation of ACE gene, the levels of pulmonary ACE mRNA expression were similar between *Atg*^{+/+} and *Atg*^{-/-} mice when fed the normal-salt diet, and the high-salt diet had no significant effect on such levels. In the renal cortex, *Atg*^{-/-} mice had higher levels of ACE mRNA expression than *Atg*^{+/+} mice when fed the normal-salt diet, and the high-salt diet significantly increased ACE mRNA levels in *Atg*^{+/+} mice. The renal cortex ACE mRNA levels in *Atg*^{+/+} mice were comparable to those in *Atg*^{-/-} mice fed the high-salt diet.

We also examined the expression of RAS component mRNAs in the gastrointestinal tract (Fig. 4). *Atg*^{-/-} mice had higher levels of gastric and intestinal AT₁ mRNA expression than *Atg*^{+/+} mice when fed the

Figure 3 Expression of AT₁ and ACE mRNA in the lung and renal cortex of *Atg*^{+/+} and *Atg*^{-/-} mice fed a normal-salt (0.3% NaCl) or a high-salt (4% NaCl) diet for 2 weeks. (A) Representative results of Northern blot analysis of total RNA (20 µg) for AT₁ and ACE mRNA and 18S ribosomal RNA (18S). (B) Bar graphs show relative AT₁ and ACE mRNA levels. The mRNA levels were measured as described in the legend of Fig. 1. Values are means ± S.E. (n=8).

normal-salt diet, and the high-salt diet significantly increased the gastric and intestinal AT₁ mRNA levels in *Atg*^{+/+} mice but not in *Atg*^{-/-} mice. The gastric angiotensinogen mRNA is expressed in *Atg*^{+/+} mice but not in *Atg*^{-/-} mice and the high-salt diet increased gastric angiotensinogen mRNA levels in *Atg*^{+/+} mice. *Atg*^{-/-} mice had higher levels of ACE mRNA expression than *Atg*^{+/+} mice both on normal- and high-salt diets. The levels of ACE enzymatic activity showed the same trend with the ACE mRNA levels in the lung, renal cortex and intestine of both *Atg*^{-/-} and *Atg*^{+/+} mice (Table 1).

Finally, to examine whether angiotensin binding to receptor is altered in relation to differences in AT₁ mRNA levels in *Atg*^{-/-} mice, we performed Northern blot analysis and radioligand binding assay of AT₁ using hearts from *Atg*^{-/-} and *Atg*^{+/+} mice (Fig. 5). The levels of

Figure 4 Expression of AT₁ and angiotensinogen (*Atg*) in the stomach, and of AT₁ and ACE mRNA in the intestine of *Atg*^{+/+} and *Atg*^{-/-} mice fed a normal-salt (0.3% NaCl) or a high-salt (4% NaCl) diet for 2 weeks. (A) Representative results of Northern blot analysis of total RNA (20 µg) for AT₁, *Atg* and ACE mRNA and 18S ribosomal RNA (18S). (B) Bar graphs show relative AT₁, *Atg* and ACE mRNA levels. The mRNA levels were measured as described in the legend of Fig. 1. Values are means $\pm$ S.E. ($n=8$). ND, not detected.

cardiac AT₁ mRNA expression were similar in *Atg*^{+/+} and *Atg*^{-/-} mice fed the normal-salt diet. The high-salt diet significantly increased AT₁ mRNA levels in *Atg*^{+/+}

mice, whereas the treatment did not affect cardiac AT₁ mRNA levels in *Atg*^{-/-} mice. On the other hand, the results of radioligand binding assay showed that $B_{\max}$ of AT₁ was significantly increased in *Atg*^{-/-} mice compared with *Atg*^{+/+} mice both on normal- and high-salt diets, although K_d was not different among the four groups.

Discussion

Previous studies of regulation of the RAS components in genetically or experimentally hypertensive animals showed widespread abnormalities of RAS gene expression which were modulated in some tissues by the development of hypertension. However, little is known about regulation of the RAS genes in hypotensive animals. Since *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*. The present study was performed to examine the effect of a high sodium intake on the expression of RAS (in particular AT₁) genes in the tissues of *Atg*^{-/-} mice which completely lack endogenous production of Ang II, and 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.

It is well known that conditions of increased RAS activity cause downregulation of AT₁, whereas a decrease in the activity of the RAS upregulates the AT₁. Previous studies showed that dietary salt loading enhanced the expression of AT₁ mRNA in the brainstem and kidney (Sandberg *et al.* 1994, Jo *et al.* 1996, Sechi *et al.* 1996), and one of the possible mechanisms involved in salt-mediated regulation of AT₁ is changes of circulating Ang II levels; changes in sodium intake exert effects on the expression of AT₁ genes, and the elevation or suppression of circulating Ang II accompanying restriction of sodium intake or sodium loading respectively may be involved in these effects (Aguilera & Catt 1981, Catt *et al.* 1984, Wang & Du 1998). Dietary sodium loading is known to suppress

Table 1 Tissue ACE activities of *Atg*^{+/+} and *Atg*^{-/-} mice fed a normal- or high-salt diet. ACE activities are expressed as units/mg tissue protein. Values are means $\pm$ S.E.; $n=8$ mice in each group

	<i>Atg</i> ^{+/+}		<i>Atg</i> ^{-/-}	
	Normal-salt diet	High-salt diet	Normal-salt diet	High-salt diet
Tissue				
Lung	226.3 $\pm$ 21.3	232.1 $\pm$ 23.5	227.7 $\pm$ 24.8	236.9 $\pm$ 19.5
Renal cortex	52.6 $\pm$ 4.1	67.1 $\pm$ 3.9*	82.3 $\pm$ 5.7†	75.1 $\pm$ 7.6
Intestine	21.3 $\pm$ 2.8	23.7 $\pm$ 3.1	36.1 $\pm$ 4.3†	40.7 $\pm$ 4.8†

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

Figure 5 Expression of AT₁ mRNA and receptor protein in the heart of *Atg*^{+/+} and *Atg*^{-/-} mice fed a normal-salt (0.3% NaCl) or a high-salt (4% NaCl) diet for 2 weeks. (A) Bar graphs show relative AT₁ mRNA levels of Northern blot analysis of total RNA (20 µg). The mRNA levels were measured as described in the legend of Fig. 1. Values are means ± S.E. (n=4). (B) Bar graphs show cardiac radioligand binding activity of AT₁. Membrane solution prepared from mouse heart was incubated with [¹²⁵I]-[Sar¹,Ile⁸]-Ang II, and AT₁ binding activity was determined by competition binding experiments using Ang II antagonists ([¹²⁵I]-[Sar¹,Ile⁸]-Ang II and CV-11974).

the circulating RAS and to decrease circulating levels of Ang II. In this study, salt loading increased AT₁ mRNA levels in the brainstem, lung, renal cortex, stomach, intestine and heart of *Atg*^{+/+} mice, and *Atg*^{-/-} mice had higher levels of pulmonary, renal cortex, gastric and intestinal AT₁ mRNA than *Atg*^{+/+} mice when fed the normal-salt diet. Because Ang II downregulates expression of the AT₁ gene (Lassegue *et al.* 1995, Nickenig & Murphy 1996), the decrease in circulating and tissue Ang II levels by salt loading in *Atg*^{+/+} mice and the genetic deficiency of endogenous production of Ang II in *Atg*^{-/-} mice may be involved in the upregulation of AT₁ gene expression in the lung, renal cortex, stomach and intestine.

On the other hand, there was no difference in AT₁ mRNA expression in the liver, brainstem and heart between *Atg*^{+/+} and *Atg*^{-/-} mice when fed the normal-salt diet. In these tissues, salt loading increased the brainstem and cardiac AT₁ mRNA levels in *Atg*^{+/+} mice but not in *Atg*^{-/-} mice, and the treatment enhanced the hepatic AT₁ mRNA levels in *Atg*^{-/-} mice but not in *Atg*^{+/+} mice. Thus, complete lack of angiotensin peptides may affect the salt-mediated regulation of AT₁ gene in these tissues while the basal expression of AT₁ gene is similar in *Atg*^{+/+} and *Atg*^{-/-} mice. Ang II is well known to be a potent inhibitor of renin production in the kidney. However, in our recent study using *Atg*^{-/-} mice, the neuronal-type nitric oxide synthase (N-NOS) expression in the macula densa is inversely regulated by salt intake and the enzyme activity is functionally linked to renal renin production, thereby suggesting that salt-modulated renal N-NOS and renin expressions are independent of angiotensin formation in *Atg*^{-/-} mice (Kihara *et al.* 1998). Based on our results, there are obviously additional factors other than Ang II that may influence the salt-mediated expression of AT₁, as with the renal renin expression. Although the mechanisms responsible for these different responses to salt loading between *Atg*^{+/+} and *Atg*^{-/-} mice are not known, several recent studies indicated sodium chloride itself was capable of upregulating the vascular AT₁ gene expression independently of Ang II *in vitro* and *in vivo* (Nickenig *et al.* 1998, Wang & Du 1998). In addition, it is known that changes in osmolarity in the sense of hypotonic or hypertonic stress induce second messenger pathways such as calcium, cAMP, inositol phosphate and the mitogen-activated protein (MAP) kinase cascade (Maeda *et al.* 1994, Sadoshima *et al.* 1996), although the intracellular pathways mediating salt-induced modulation of AT₁ expression are unknown. Previous studies showed that aldosterone and glucocorticoid increased expression of the AT₁ gene whereas the expression was downregulated by phorbol esters, estrogens, and Ang II (Ullian *et al.* 1992, Sato *et al.* 1994). In the present study, the urinary excretion of aldosterone is not detectable in *Atg*^{-/-} mice, although others have found an angiotensin-independent mechanism

for aldosterone synthesis, in particular, during chronic depletion of extracellular fluid volume in *Atg*^{-/-} mice (Okubo *et al.* 1997). Furthermore, the vasopressin and catecholaminergic systems may be altered by lack of angiotensin peptides in *Atg*^{-/-} mice (Umemura *et al.* 1998). Therefore, it is possible that alterations of hormonal levels and signaling pathways in *Atg*^{-/-} mice may change the ultimate response of regulation of AT₁ gene by Ang II in a tissue-specific manner.

In the present study we found a discrepancy between AT₁ mRNA and protein levels in the heart of *Atg*^{+/+} and *Atg*^{-/-} mice, although AT₁ protein contents were not analyzed in other tissues. Examples of dissociation between mRNA levels and protein contents are known to occur, and a previous study showed that Ang II infusion had no effect on AT₁ mRNA levels but that it downregulated AT₁ density through a posttranscriptional mechanism (Sechi *et al.* 1996). Further studies are clearly needed to elucidate a mechanism responsible for posttranscriptional modulation induced by genetic deficiency of angiotensin peptides.

Although tissue ACE may play an important role in the local production of Ang II (Johnston 1994), little is known regarding the regulation of the tissue ACE gene by altered sodium intake. Previous studies showed a significant increase in ACE mRNA in response to a high-sodium diet and an Ang II-mediated downregulation of ACE gene expression in rats (Schunkert *et al.* 1993, Kreutz *et al.* 1995). In the present study, *Atg*^{-/-} mice had higher levels of renal cortex and intestinal ACE mRNA than *Atg*^{+/+} mice when fed the normal-salt diet. However, salt loading increased the renal cortex ACE mRNA levels in *Atg*^{+/+} mice, but the treatment had no effect on renal cortex ACE mRNA in *Atg*^{-/-} mice. Thus, complete lack of angiotensin peptides may make the ACE gene unable to respond to salt loading in the tissues of *Atg*^{-/-} mice. Similar to the case of AT₁, ACE gene expression is regulated by glucocorticoid (Dasarathy *et al.* 1992, Fishel *et al.* 1995), and it is also possible that alterations of hormonal levels and signaling pathways in *Atg*^{-/-} mice may change the ultimate response of regulation of ACE gene by Ang II in a tissue-specific manner. Furthermore, ACE plays an important role in both the RAS and the kinin-kallekrein system. Therefore, enhanced ACE activities in the kidney and intestine of *Atg*^{-/-} mice may have several influences on the physiological functions of these tissues through the possible effect of ACE on the kinin-kallekrein system, although further studies are obviously needed to clarify this issue.

In conclusion, the findings obtained in the present study suggest that dietary salt loading exerts its 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.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan, the Uehara Memorial Foundation, Ichiro Kanehara Foundation, and Yokohama Foundation for Advancement of Medical Science. Dr Kouichi Tamura is supported by a Postdoctoral Fellowship for Research Abroad of the Japan Society for the Promotion of Science.

References

- Aguilera G & Catt K 1981 Regulation of vascular angiotensin II receptors in the rat during altered sodium intake. *Circulation Research* **49** 751–758.
- Anemiyi Y & Miyahara J 1988 Imaging plate illuminates many fields. *Nature* **336** 89–90.
- Catt KJ, Mendelsohn FAC, Millan MA & Aguilera G 1984 The role of angiotensin II receptors in vascular regulation. *Journal of Cardiovascular Pharmacology* **6** S575–S586.
- Chirgwin JM, Pryzbyla AE, MacDonald RJ & Rutter WJ 1979 Isolation of biologically active ribonucleic acid from sources enriched in nuclease. *Biochemistry* **18** 5294–5299.
- Dasarathy Y, Lanzillo JJ & Fanburg BI 1992 Stimulation of bovine pulmonary artery endothelial cell ACE by dexamethasone: involvement of steroid receptors. *American Journal of Physiology* **263** L645–L649.
- Dzau VJ & Re R 1994 Tissue angiotensin system in cardiovascular medicine: a paradigm shift? *Circulation* **89** 493–498.
- Esther CR Jr, Howard TE, Marino EM, Goddard JM, Capecchi MR & Bernstein KE 1996 Mice lacking angiotensin-converting enzyme have low blood pressure, renal pathology, and reduced male fertility. *Laboratory Investigation* **74** 953–965.
- Fishel RS, Eisenberg S, Shai SY, Redden RA, Bernstein KE & Berk BC 1995 Glucocorticoids induce angiotensin-converting enzyme expression in vascular smooth muscle. *Hypertension* **25** 343–349.
- Griendling KK, Murphy TJ & Alexander RW 1993 Molecular biology of the renin-angiotensin system. *Circulation* **87** 1816–1828.
- Jo H, Yang E-K, Lee W-J, Park K-Y, Kim H-J & Park J-S 1996 Gene expression of central and peripheral renin-angiotensin system components upon dietary sodium intake in rats. *Regulatory Peptides* **67** 115–121.
- Johansen KB, Marstein S & Aas P 1987 Automated method for the determination of angiotensin-converting enzyme in serum. *Scandinavian Journal of Clinical and Laboratory Investigation* **47** 411–414.
- Johnston CI 1994 Tissue angiotensin converting enzyme in cardiac and vascular hypertrophy, repair, and remodeling. *Hypertension* **23** 258–268.
- Kihara M, Umemura S, Yabana M, Sumida Y, Nyui N, Tamura K, Kadota T, Kishida R, Murakami K, Fukamizu A & Ishii M 1998 Dietary salt loading decreases the expression of neuronal-type nitric oxide synthase and renin in the juxtaglomerular apparatus of angiotensinogen gene-knockout mice. *Journal of American Society of Nephrology* **9** 355–362.
- Kim H-S, Kregel JH, Kluckman KD, Hagaman JR, Hodgins JB, Best CF, Jennette JC, Coffman TM, Maeda N & Smithies O 1995 Genetic control of blood pressure and the angiotensinogen locus. *Proceedings of the National Academy of Sciences of the USA* **92** 2735–2739.
- Kohara K, Brosnihan KB, Ferrario CM & Milsted A 1992 Peripheral and central angiotensin II regulates expression of genes of the renin-angiotensin system. *American Journal of Physiology* **262** E651–E657.

- Kreutz R, Fernandez Alfonso MS, Liu Y, Ganten D & Paul M 1995 Induction of cardiac angiotensin I-converting enzyme with dietary NaCl-loading in genetically hypertensive and normotensive rats. *Journal of Molecular Medicine* **73** 243–248.
- Lassegue B, Alexander RW, Nickenig G, Clark M, Murphy TJ & Griendling KK 1995 Angiotensin II down-regulates the vascular smooth muscle AT₁ receptor by transcriptional and post-transcriptional mechanisms: evidence for homologous and heterologous regulation. *Molecular Pharmacology* **48** 601–609.
- Lowry OH, Rosebrough NJ, Farr AL & Randall RJ 1951 Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275.
- Maeda T, Wurgler-Murphy SH & Saito H 1994 A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369** 242–245.
- Nickenig G & Murphy TJ 1996 Enhanced AT₁ receptor mRNA degradation and induction of polyribosomal mRNA binding proteins by angiotensin II in vascular smooth muscle cells. *Molecular Pharmacology* **50** 743–751.
- Nickenig G, Strehlow K, Roeling J, Zolk O, Knorr A & Böhm M 1998 Salt induces vascular AT₁ receptor overexpression *in vitro* and *in vivo*. *Hypertension* **31** 1272–1277.
- Niimura F, Labosky PA, Kakuchi J, Okubo S, Yoshida H, Oikawa T, Ichiki T, Naftilan AJ, Fogo A, Inagami T, Hogan BLM & Ichikawa I 1995 Gene targeting in mice reveals a requirement for angiotensin in the development and maintenance of kidney morphology and growth factor regulation. *Journal of Clinical Investigation* **96** 2947–2954.
- Okubo S, Niimura F, Nishimura H, Takemoto F, Fogo A, Matsusaka T & Ichikawa I 1997 Angiotensin-independent mechanism for aldosterone synthesis during chronic extracellular fluid volume depletion. *Journal of Clinical Investigation* **99** 855–860.
- Raynal F, Michot B & Bachelier J-P 1984 Complete nucleotide sequence of mouse 18S rRNA gene: comparison with other available homologs. *FEBS Letters* **167** 263–268.
- Sadoshima J, Qiu Z, Morgan JP & Izumo S 1996 Tyrosine kinase activation is an immediate and essential step in hypotonic cell swelling-induced ERK activation and c-fos gene expression in cardiac myocytes. *EMBO Journal* **15** 5535–5546.
- Sandberg K, Ji H & Catt KJ 1994 Regulation of angiotensin II receptors in rat brain during dietary sodium changes. *Hypertension* **23** 1137–1141.
- Sato A, Suzuki H, Murakami M, Nakazato Y, Iwaita Y & Saruta T 1994 Glucocorticoid increases angiotensin II type 1 receptor and its gene expression. *Hypertension* **23** 25–30.
- Schunkert H, Ingelfinger JR, Hirsch AT, Pinto Y, Remme WJ, Jacob H & Dzau VJ 1993 Feedback regulation of angiotensin converting enzyme activity and mRNA levels by angiotensin II. *Circulation Research* **72** 312–318.
- Sechi LA, Griffin CA, Giacchetti G, Valentin JP, Llorens-Cortes C, Corvol P & Schambelan M 1996 Tissue-specific regulation of type 1 angiotensin II receptor mRNA levels in the rat. *Hypertension* **28** 403–408.
- Shimamoto K, Ishida H, Nakahashi Y, Nishitani T, Hosoda S, Yokoyama T, Tanaka S & Iimura O 1984 A very sensitive direct radioimmunoassay system for plasma angiotensin II and its clinical application in various hypertensive diseases. *Japanese Circulation Journal* **48** 1228–1235.
- Soubrier F, Alhenc-Gelas F, Hubert C, Allegrini J, John M, Tregear G & Corvol P 1988 Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proceedings of the National Academy of Sciences of the USA* **85** 9386–9390.
- Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, Goto S, Imaizumi K, Hisada Y, Otsuka A, Uchida H, Sugiyama M, Fukuta K, Fukamizu A & Murakami K 1995 Angiotensin type 1a receptor-deficient mice with hypotension and hyperreninemia. *Journal of Biological Chemistry* **270** 18719–18722.
- Sumida Y, Umemura S, Tamura K, Kihara M, Kobayashi S, Ishigami T, Yabana M, Nyui N, Ochiai H, Fukamizu A, Miyazaki H, Murakami K & Ishii M 1998 Increased cardiac angiotensin II receptors in angiotensinogen-deficient mice. *Hypertension* **31** 45–49.
- Tamura K, Umemura S, Fukamizu A, Ishii M & Murakami K 1995 Recent advances in the study of renin and angiotensinogen genes: from molecules to the whole body. *Hypertension Research* **18** 7–18.
- Tamura K, Umemura S, Nyui N, Yamakawa T, Yamaguchi S, Ishigami T, Tanaka S, Tanimoto K, Takagi N, Sekihara H, Murakami K & Ishii M 1996 Tissue specific regulation of angiotensinogen gene expression in spontaneously hypertensive rat. *Hypertension* **27** 1216–1223.
- Tamura K, Umemura S, Yamakawa T, Nyui N, Hibi K, Watanabe Y, Ishigami T, Yabana M, Tanaka S, Sekihara H, Murakami K & Ishii M 1997 Modulation of tissue angiotensinogen gene expression in genetically obese hypertensive rats. *American Journal of Physiology* **272** R1704–R1711.
- Tamura K, Umemura S, Sumida Y, Nyui N, Kobayashi S, Ishigami T, Kihara M, Sugaya T, Fukamizu A, Miyazaki H, Murakami K & Ishii M 1998 Effect of genetic deficiency of angiotensinogen on renin-angiotensin system. *Hypertension* **32** 223–227.
- Tanimoto K, Sugiyama F, Goto Y, Ishida J, Takimoto E, Yagami K, Fukamizu A & Murakami K 1994 Angiotensinogen-deficient mice with hypotension. *Journal of Biological Chemistry* **269** 31334–31337.
- Tokita Y, Franco-Saenz R, Reimann EM & Mulrow PJ 1994 Hypertension in the transgenic rat TGR(mRen-2)27 may be due to enhanced kinetics of the reaction between mouse renin and rat angiotensinogen. *Hypertension* **23** 422–427.
- Ullian ME, Schelling JR & Linas SL 1992 Aldosterone enhances angiotensin II receptor binding and inositol phosphate responses. *Hypertension* **20** 67–73.
- Umemura S, Kihara M, Sumida Y, Yabana M, Ishigami T, Tamura K, Nyui N, Hibi K, Murakami K, Fukamizu A & Ishii M 1998 Endocrinological abnormalities in angiotensinogen-gene knockout mice: studies of hormonal responses to dietary salt loading. *Journal of Hypertension* **16** 285–289.
- Wang DH & Du Y 1998 Regulation of vascular type 1 angiotensin II receptor in hypertension and sodium loading: role of angiotensin II. *Journal of Hypertension* **16** 467–475.

Received 5 June 1998

Revised manuscript received 17 August 1998

Accepted 20 October 1998