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

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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.


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, Niumura et al. 1995). Homozygous mutant ($\text{Atg}^{-/-}$) 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 $\text{Atg}^{-/-}$ mice.

**Materials and Methods**

**Animals**

$\text{Atg}^{-/-}$ mice were generated by gene targeting as described previously (Tanimoto et al. 1994). Six-week-old male $\text{Atg}^{-/-}$ ($n=16$) and $\text{Atg}^{+/+}$ ($n=16$) 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, $\text{Atg}^{-/-}$ and $\text{Atg}^{+/+}$ 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. Forebrain, 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 (RENNIN 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 mM ZnCl$_2$, 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-furylacyl)l]-phenylalanine-1-carboxylic acid (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 prehydrated 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 $1 \times 10^6$ c.p.m./ml of the $^{32}$P-labeled probes for angiotensinogen (Tanimoto et al. 1994), ACE (Soubrier et al. 1988) or type 1 Ang II receptor (AT$_1$) (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 AT$_1$ 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 MgCl$_2$, 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 $^{125}$I-[(Sar$^1$,Ile$^8$)]-Ang II, incubation buffer and Ang II antagonist (Sar$^1$,Ile$^8$)-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 MgCl\(_2\), 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 \( ^{125}\text{I}-[\text{Sar}^1,\text{Ile}^8]\)-Ang II was defined as the radioactivity that bound to membrane fractions and was not displaced by a high concentration of unlabeled \( ^{125}\text{I}-[\text{Sar}^1,\text{Ile}^8]\)-Ang II (1 mM). Specific \( ^{125}\text{I}-[\text{Sar}^1,\text{Ile}^8]\)-Ang II binding displaced by CV-11974 was estimated as AT1. Each binding assay was carried out in duplicate. Maximum binding capacity (B\(_{\text{max}}\)) 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 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 AT\(_1\) 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 AT\(_1\) mRNA levels, whereas in Atg\(^-\)/- mice the high-salt diet increased the hepatic AT\(_1\) mRNA levels.

As shown in Fig. 3, Atg\(^-\)/- mice had higher levels of pulmonary and renal cortex AT\(_1\) mRNA expression than Atg\(^+\)/+ mice when fed the normal-salt diet. The high-salt diet significantly increased the pulmonary and renal cortex AT\(_1\) mRNA levels in Atg\(^+\)/+ mice. In Atg\(^-\)/- mice, the treatment decreased the pulmonary AT\(_1\) mRNA levels but did not affect the renal cortex AT\(_1\) mRNA levels.
With respect to the regulation of ACE gene, the levels of pulmonary ACE mRNA expression were similar between Atg\(^{+/+}\) and Atg\(^{-/-}\) mice when fed a normal-salt (0.3% NaCl) or a high-salt (4% NaCl) diet for 2 weeks. 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 but not in Atg\(^{-/-}\) mice. 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 AT\(_1\) mRNA levels in Atg\(^{-/-}\) mice, we performed Northern blot analysis and radioligand binding assay of AT\(_1\) using hearts from Atg\(^{-/-}\) and Atg\(^{+/+}\) mice (Fig. 5). The levels of normal-salt diet, and the high-salt diet significantly increased the gastric and intestinal AT\(_1\) 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).
cardiac AT\textsubscript{1} mRNA expression were similar in \textit{Atg}\textsuperscript{+/+} and \textit{Atg}\textsuperscript{−/−} mice fed the normal-salt diet. The high-salt diet significantly increased AT\textsubscript{1} mRNA levels in \textit{Atg}\textsuperscript{+/+} mice, whereas the treatment did not affect cardiac AT\textsubscript{1} mRNA levels in \textit{Atg}\textsuperscript{−/−} mice. On the other hand, the results of radioligand binding assay showed that \textit{B}_{\text{max}}\textsubscript{max} of AT\textsubscript{1} was significantly increased in \textit{Atg}\textsuperscript{−/−} mice compared with \textit{Atg}\textsuperscript{+/+} mice both on normal- and high-salt diets, although \textit{K}_{d} was not different among the four groups.

\section*{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 \textit{Atg}\textsuperscript{−/−} mice do not produce angiotensin peptides at all and thus are chronically hypotensive, \textit{Atg}\textsuperscript{−/−} mice may be a genetically suitable hypotension model for analysis of the regulation of RAS gene expression \textit{in vivo}. The present study was performed to examine the effect of a high sodium intake on the expression of RAS (in particular AT\textsubscript{1}) genes in the tissues of \textit{Atg}\textsuperscript{−/−} mice which completely lack endogenous production of Ang II, and we showed that salt loading significantly increased SBP in \textit{Atg}\textsuperscript{−/−} mice and that expression of the RAS genes was regulated in a tissue-specific manner by salt loading in \textit{Atg}\textsuperscript{−/−} mice.

It is well known that conditions of increased RAS activity cause downregulation of AT\textsubscript{1}, whereas a decrease in the activity of the RAS upregulates the AT\textsubscript{1}. Previous studies showed that dietary salt loading enhanced the expression of AT\textsubscript{1} mRNA in the brainstem and kidney (Sandberg \textit{et al.} 1994, Jo \textit{et al.} 1996, Sechi \textit{et al.} 1996), and one of the possible mechanisms involved in salt-mediated regulation of AT\textsubscript{1} is changes of circulating Ang II levels; changes in sodium intake exert effects on the expression of AT\textsubscript{1} 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 \textit{et al.} 1984, Wang \& Du 1998). Dietary sodium loading is known to suppress

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Tissue} & \textbf{Atg\textsuperscript{+/+}} & \textbf{Atg\textsuperscript{−/−}} & \\
\hline
& \textbf{Normal-salt diet} & \textbf{High-salt diet} & \textbf{Normal-salt diet} & \textbf{High-salt diet} \\
\hline
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† \\
\hline
\end{tabular}
\caption{Tissue ACE activities of \textit{Atg}\textsuperscript{+/+} and \textit{Atg}\textsuperscript{−/−} mice fed a normal- or high-salt diet. ACE activities are expressed as units/mg tissue protein. Values are means \pm s.e.; \textit{n}=8 mice in each group.}
\end{table}
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.

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-[Sar3,Ile8]-Ang II and CV-11974).
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