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Effects of Angiotensin Converting Enzyme Inhibition on Gene Expression of the Renin-Angiotensin System in Rats

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To investigate interaction of angiotensin converting enzyme (ACE) inhibitor with local tissue renin-angiotensin system (RAS), changes in gene expression of the RAS components in various tissues in response to chronic administration of an ACE inhibitor, enalapril, were examined in Sprague-Dawley male rats. Enalapril was administered in their drinking water (3～4 mg/day) over 8 wk. Plasma and renal ACE activity increased significantly after 4 and 8 wk of enalapril treatment. Renin levels of the plasma and kidney of the enalapril-treated rats markedly increased after 4 wk and decreased thereafter, but still remained significantly higher than those of control rats. Kidney mRNA levels of renin markedly increased after 4 and 8 wk of enalapril treatment, but those of angiotensinogen and ANG II-receptor subtypes, AT_{1A} and AT_{1B}, did not change significantly. The liver expressed genes for renin, angiotensinogen and AT_{1A} receptor subtype, but AT_{1B} receptor subtype mRNA was not detectable by RT-PCR. None of mRNA for these RAS components in the liver changed significantly by enalapril treatment. The hypothalamus showed mRNA expressions of renin, angiotensinogen, AT_{1A} and AT_{1B} receptor subtypes. AT_{1A} receptor subtype mRNA was more abundant than AT_{1B} receptor subtype in the hypothalamus as shown in the kidney. However, gene expression of the RAS components remained unchanged during 8-wk treatment of enalapril. In the present study, chronic ACE inhibition increased plasma and renal levels of ACE and renin, but did not affect mRNA levels of other RAS components such as angiotensinogen, ANG II receptor subtypes in the kidney. Gene levels of the RAS components in the liver and hypothalamus were not altered by chronic treatment of enalapril. These results suggest the differential expression of the RAS components in response to enalapril, and localized action and some degree of tissue specificity of enalapril.

Key Words: Enalapril, Northern blot, RT-PCR, Kidney, Liver, Brain

INTRODUCTION

An increasing number of evidences suggest angiotensins (ANG) are produced not only in the circulation, but also at tissue sites. Coexistance of all the components necessary for the local formation of ANG II has been demonstrated in a number of tissues, including the brain, kidney, adrenal, cardiac tissue, vessel wall and sex organs (Phillips et al, 1993; Danser 1996). ANG II generated by the local tissue renin-angiotensin system (RAS) may play a significant role through paracrine or autocrine effects. This theory is supported by the observations that tissue ANG II concentration was much greater than could be accounted for on the basis of circulating ANG II levels and can be altered independently of the plasma levels in response to various maneuvers (Campbell et al, 1991). Recently, we also demonstrated differential regulation of mRNA expression of the RAS components in the central and peripheral tissues under various physiological conditions such as sodium intake (Jo et al, 1996) and hemorrhage (Lee et al, 1997). From physiologic point of view, the tissue RAS implies that ANG-mediated functions of these target organs are locally regulated, independent

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of the circulating RAS.

ANG II has a wide range of cardiovascular effects, which are involved in pathophysiology of many cardiovascular diseases. Also its physiological roles in regulating blood pressure and body fluid homeostasis are well known (Lindpaintner & Ganten, 1991). The pathophysiologic significance of ANG converting enzyme (ACE) in the formation of ANG II was emphasized through clinical trials demonstrating that ACE inhibitors are useful in the treatment of cardiovascular diseases.

Administration of ACE inhibitors block the conversion of ANG I to ANG II and acutely reduces plasma levels of both ACE and ANG II, which correlate with the hypotensive effect (Johnston et al, 1988). However, prolonged antihypertensive effects of ACE inhibitors were not associated with the suppression of plasma levels of ACE and ANG II (Forslund et al, 1981; Unger et al, 1981; Keuneke et al, 1995). Thus, persistent ACE inhibition in tissues has been postulated after chronic administration of ACE inhibitors (Unger et al, 1985). In most studies, one or two components of the RAS were measured from various tissues (Forslund 1983; Tokita et al, 1995), but few studies have been done on the effects of ACE inhibitors on gene expression of RAS in different tissues (Keuneke et al, 1995; Sechi et al, 1996). Furthermore, changes in tissue levels of the RAS components showed some inconsistencies between the experiments.

This study was performed to examine the chronic effects of enalapril, an ACE inhibitor, on gene expression of the RAS in various tissues. To minimize the interexperimental variations, changes in mRNA expression for renin, angiotensinogen, AT_1 receptor subtypes (AT_{1A} and AT_{1B}) from a single set of animals were examined by using Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR). The kidney and liver were selected because they are the sources of plasma renin and angiotensinogen, respectively. In addition, the hypothalamus was also selected because the brain RAS is important for rapid regulation of blood pressure (Lee et al, 1995), and development and maintenance of hypertension (Iyer et al, 1996; Phillips et al, 1997).

METHODS

Animals

Ten-week old male Wistar rats weighing 250~280 g drank either tap water only or water mixed with enalapril (3~4 mg/day), an ACE inhibitor for 8 weeks. On 4 or 8 wk, rats were decapitated, and the trunk blood samples were collected for measurements of plasma ACE activity and renin concentration. The kidney, liver and hypothalamus were removed from each rat and frozen in liquid nitrogen, and then kept in deep freezer (−70°C) until total RNA isolation.

Measurement of plasma and renal ACE activity

For the measurement of plasma ACE activity, blood sample was centrifuged at 2,500 rpm for 20 min at 4°C. Renal cortical slices were weighed and homogenized in ice-cold 20 mM Tris buffer, pH 8.3. The homogenate was centrifuged at 3,000 rpm for 30 min at 4°C. The supernatant and plasma samples were stored at −20°C until assay.

ACE activity was measured by the rate of generation Gly-Gly from Hip-Gly-Gly substrate using colorimetric assay described by Neels et al (1983). The plasma and renal samples were then incubated with 30 mM Hip-Gly-Gly for 30 min at 37°C. Tungstate (100 g/L) and 0.33 M sulfuric acid were added and centrifuged at 6,000 rpm for 10 min at 4°C. The supernatant was mixed with 100 mM borate, pH 9.6 and 60 mM picrylsulfonic acid and incubated for 10 min at 37°C. Optical density was measured at a wavelength of 420 nm with a spectrophotometer (Ultra spec 3,000, Pharmacia Biotech).

Measurement of plasma and renal renin levels

The plasma and supernatant of the renal cortical preparation samples were incubated with excess exogenous angiotensinogen at 37°C, pH 7.4, in the presence of protease inhibitors [3.4 mM 8-hydroxyquinolone sulfate, 0.25 mM EDTA, 0.1 mM phenylmethyl-sulfonyl fluoride, 1.6 mM dimercaprol, and 5 mM sodium tetra-thionate] for one hour. The source of angiotensinogen was renin-free plasma from rats nephrectomized 48 hours prior to experiment. The ANG I generated was measured by radioimmunoassay described earlier (Lee et al, 1995).

Table 1. Primer sequences for angiotensinogen, renin, AT_{1A} and AT_{1B} receptor subtypes, and GAPDH used in this study

Gene	Primer sequence	Priduct size	References
Angiotensinogen			
(s)	CTGACCCAGTTCTTGCTGCC	723bp	Yoshio et al.(1993)
(as)	TGGGGGTTATCCACTCTGCC		
Renin			
(s)	AGGCAGTGACCCTCAACATTACCAG	362bp	burnham et al.(1987)
(as)	CCAGTATGCACAGGTCATCGTTCCT		
AT_{1A}			
(s)	GCACACTGGCAATGTAATGC	385bp	Kitami et al.(1992)
(as)	GTTGAACAGAACAAGTGACC(s)		
AT_{1B}			
(s)	GCCTGCAAGTGAAGTGATTT	204bp	Kitami et al.(1992)
(as)	TTTAACAGTGGCTTTGCTCC		
GAPDH			
(s)	ATCAAATGGGGTGATGCTGGTGCTG	505bp	Tso et al.(1985)
(as)	CAGGTCTCCAGGCGGCATGTCAGTT		

Total RNA isolation and Northern blot analysis

Total RNA was isolated according to the method described by Chomczynski & Sacchi (1987) with slight modifications in the final step. The RNA pellet was dissolved in formamide and kept at −20°C until assay. Aliquots of total RNA (20~40 μg) were separated on a 1% agarose gel containing 0.66 M of formaldehyde in 1×MOPS buffer (0.02 M MOPS, pH 7.0; 5 mM sodium acetate; 1 mM EDTA, pH 8.0). RNA was transferred to a nitrocellulose membrane by capillary action in 20×SSC (1×SSC; 0.15 M sodium chloride, 0.015 M sodium citrate). The membrane was baked in a vacuum oven at 80°C for 2 hr and prehybridized for 4 hr at 42°C in a solution containing 50% formamide, 6×SSC, 5 Denhardt's solution (0.1% each of Ficoll 400, polyvinyl-pyrrolidine and nuclease-free bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS) and 0.1 mg/ml salmon sperm DNA. [^{32}P] dCTP-labeled cDNA was added to the solution and the membrane was incubated for 18~20 hrs at 42°C. After hybridization, the membrane was sequentially washed in 2×SSC, 0.2% SDS for 5 min at room temperature and for 30 min at 42°C. The membrane was washed thereafter in 0.1×SSC, 0.1% SDS at 60°C until the signal and background were distinguishable. The resulting membrane was exposed to X-ray film for 1~4 days at −70°C. After autoradiography, the membrane was deprobed in 0.5% SDS by boiling, and was rehybridized with 18S cDNA probe for loading control.

Reverse transcriptase-polymerase chain reaction (RT-PCR): The nucleotide sequence of the primers and references are presented in Table 1. Total RNA (2~4 g) was primed with oligo (dT) primers, and the first strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, U.S.A.) in a 30 μl reaction volume for 90 min at 37°C. With 1~2 μl (330~660 ng of total RNA) of this reaction mixture, PCR was carried out in a 25 μl reaction mixture containing 0.1% formamide. PCR was conducted in a DNA Thermal Cycler (Perkin-Elmer, Cetus) and cyclic amplification profiles were as follows: 94°C for 1 min, 56°C for 1 min, 72°C for 1.5 min. In a preliminary experiment, we conducted PCR with serial dilutions of reverse-transcribed RNA from different tissues. PCR cycles and the amount of template were carefully selected according to the relative abundance of mRNAs in different tissues.

Densitometric analysis: The autoradiogram and polaroid film was scanned using an Epson (ES-600) scanner with a resolution of 70 DPI and the maximum gray scale unit of 256. The resulting image was analyzed using NIH-Image analysis program (NIH, Betheda, MD). The scale of each band was expressed by multiplying the mean density and the total area of the band.

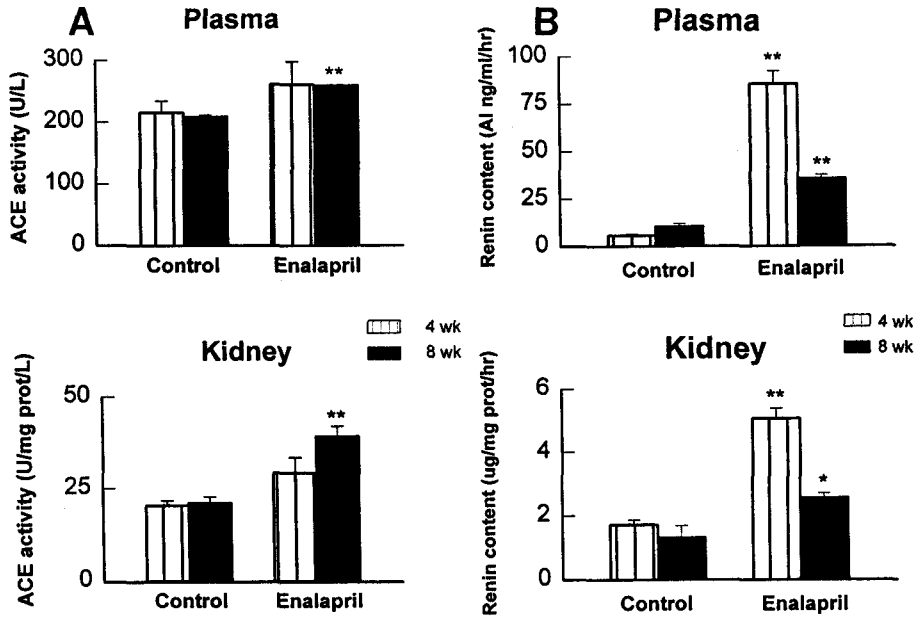

Fig. 1. (A) Angiotensin converting enzyme (ACE) activity and (B) renin levels in the plasma and kidney cortical tissue of rats after 4 or 8 wk of enalapril treatment. $*p<0.05$, $**p<0.01$, vs. corresponding control rats (each group, n=4). $^{\#}p<0.01$, vs. 4 wk of enalapril treatment

Statistical analysis: All data are presented in mean ±SE. Unpaired t-test was used to compare the means of different groups. Differences were considered statistically significant if $P<0.05$.

RESULTS

Effects of enalapril on plasma and renal ACE activity and renin levels

Plasma and renal ACE activity increased significantly after 4 and 8 wk of treatment with enalapril, an ACE inhibitor (Fig 1A). At 4 and 8 wk, plasma ACE activity was 215±19 and 208±3 U/L, respectively, in the controls, and increased to 261±37 and 260±1 U/L ($P<0.01$) in the enalapril treated rats. A significant increase in the kidney ACE activity was also found in the rats receiving enalapril.

Renin levels of the plasma and kidney of the enalapril-treated rats markedly increased at 4 wk and decreased thereafter, but still remained significantly higher than those of the control rats (Fig 1B). Plasma renin concentration at 4 and 8 wk was 5.7±0.9 and 10.6±1.4 ng ANG I/ml/h in the controls and 85.5±6.7 and 35.7±1.9 ng ANG I/ml/h in the enalapril treated rats. Increase in plasma renin concentration was in parallel with kidney renin levels (1.7±0.1 and 1.3±0.4 in the controls vs. 5.1±0.3 and 2.6±0.1 μg ANG I/mg prot/h in the enalapril treated rats).

Effects of enalapril on gene expression of the RAS components in the central and peripheral tissues

Kidney mRNA levels of renin and angiotensinogen in the control and enalapril-treated rats were determined by Northern blot (Fig. 2) and RT-PCR analysis (Fig. 3), and those levels were compared. Densitometric analysis indicated that ratios of renal renin to 18S mRNA in Northern blot (Fig. 2B) as well as those of renin mRNA in RT-PCR markedly increased after 4 and 8 wk of treatment of enalapril, but those of renal angiotensinogen mRNA were not significantly altered by enalapril treatment (Fig. 3B). These results demonstrate the validity of these techniques for detecting changes in gene expression. Renal mRNA levels of ANG II-receptor subtypes, AT_{1A} and AT_{1B}, too, were not altered by enalapril treatment (Fig. 3B).

The liver expressed genes for renin, angiotensinogen, and AT_{1A} receptor subtype (Fig. 4). AT_{1B} receptor subtype mRNA was not detectable by

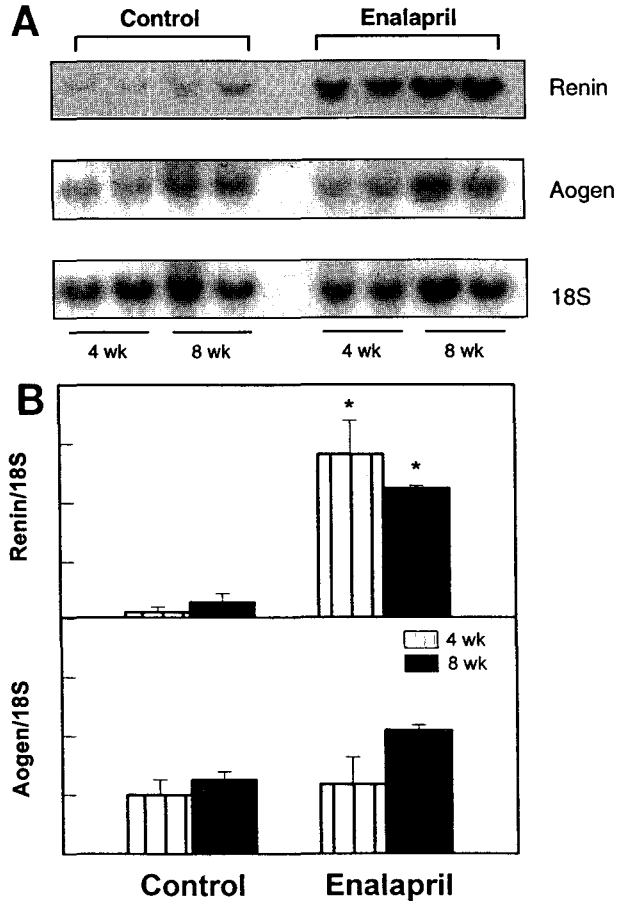

Fig. 2. (A) Northern blot analysis of mRNA for renin and angiotensinogen (Aogen) in the kideny cortex of rats after 4 or 8 wk of enalapril treatment. Each lane represents one rat and the amount of RNA used was 20 μg. (B) Densitometric analyses of Northern blot corrected for 18S. The values are expressed in arbitrary densitometric unit. $*p<0.05$, vs. corresponding control rats (each group, n=4).

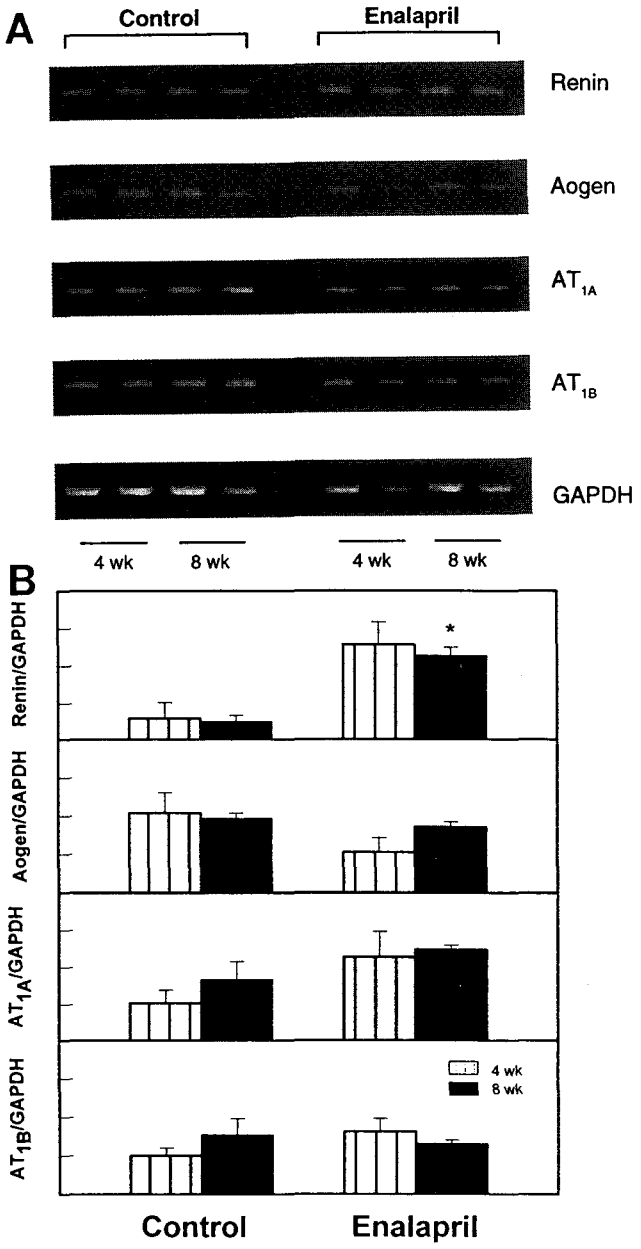

Fig. 3. (A) Ethidium bromide-stained gel of the PCR products for renin, angiotensinogen (Aogen), AT_{1A} and AT_{1B} receptor mRNAs in the kidney of rats after 4 or 8 wk of enalapril treatment. (B) Densitometric analyses of PCR products corrected for GAPDH in each rat. The values are expressed in arbitrary densitometric unit. $*p<0.05$, vs. corresponding control rats (each group, n=4).

RT-PCR in the liver. None of mRNA for these RAS components was changed significantly by enalapril treatment (Fig. 4B). The hypothalamus showed mRNA expressions of renin, angiotensinogen, AT_{1A} and AT_{1B} receptor subtypes (Fig. 5). AT_{1A} receptor subtype mRNA was more abundant than AT_{1B} receptor subtype in the brain as in the kidney. However, gene expression of the RAS components remained unchanged during 8-wk treatment of enalapril.

DISCUSSION

In an attempt to investigate chronic effects of ACE inhibition on tissue RAS, changes in gene expression of the RAS components in response to chronic administration of enalapril were examined in the kidney, liver, hypothalamus and brainstem. The present study demonstrated the differential expression

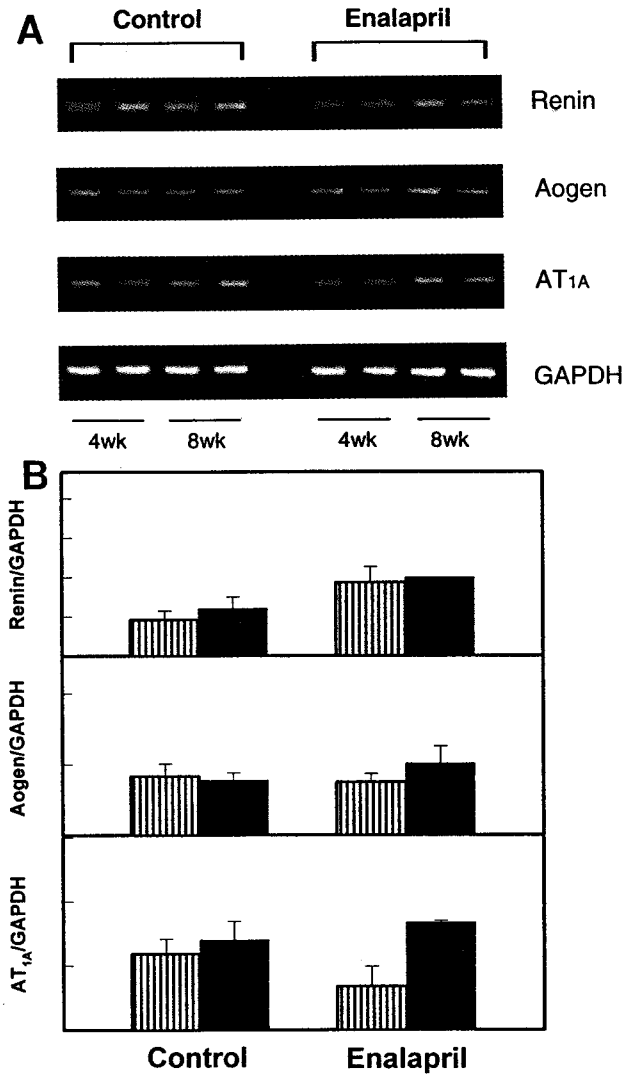

Fig. 4. (A) Ethidium bromide-stained gel of the PCR products for renin, angiotensinogen (Aogen), AT_{1A} and AT_{1B} receptor mRNAs in the liver of rats after 4 or 8 wk of enalapril treatment. (B) Densitometric analyses of PCR products corrected for GAPDH in each rat. The values are expressed in arbitrary densitometric unit (each group, n=4).

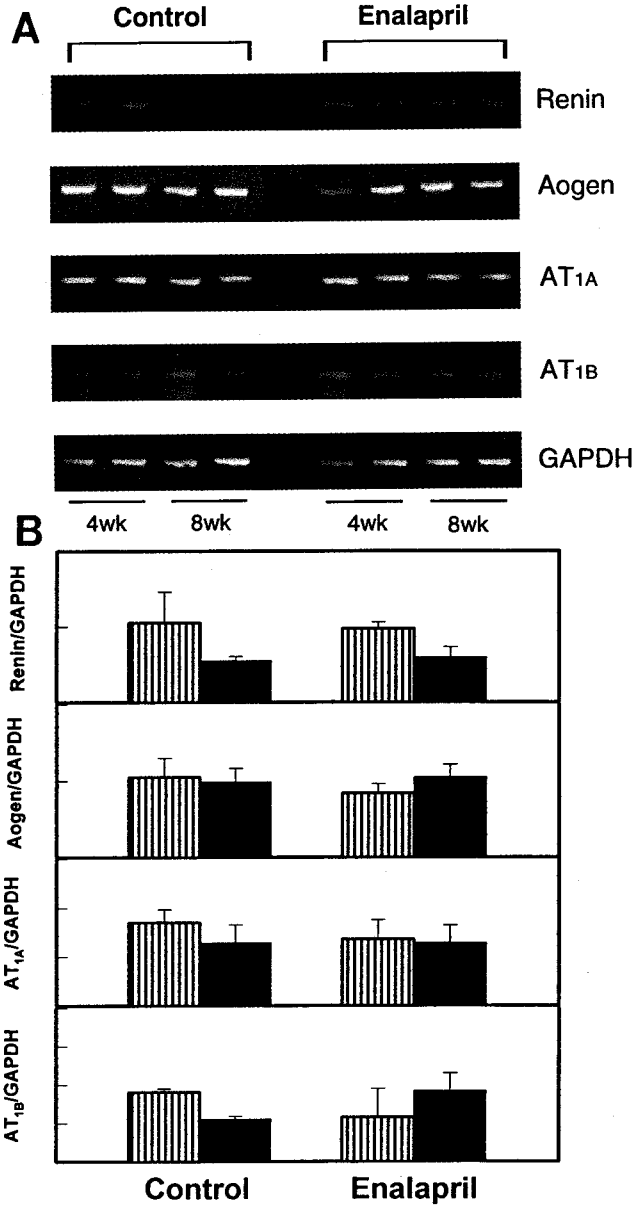

Fig. 5. (A) Ethidium bromide-stained gel of the PCR products for renin, angiotensinogen (Aogen), AT_{1A} and AT_{1B} receptor mRNAs in the hypothalamus of rats after 4 or 8 wk of enalapril treatment. (B) Densitometric analyses of PCR products corrected for GAPDH in each rat. The values are expressed in arbitrary densitometric unit (each group, n=4).

of the RAS components in various tissues in response to enalapril.

Acute effect of ACE inhibitors is a decreased blood pressure by inhibiting plasma ACE and lowering plasma ANG II (Holck et al, 1986). However, after chronic administration of ACE inhibitors, a dissociation between the hypotensive effect and suppression of plasma RAS has been observed by many investigators. As shown in the present study along with others (Forslund et al, 1981 & 1983; Johnston et al, 1988; Tokita et al, 1995; Nishida et al, 1997), chronic ACE inhibition increased the plasma levels of both ACE and renin. Chronic treatment of ACE inhibitors in rats increased serum ACE activity in a dose-dependent manner (Forslund et al, 1981; Forslund 1983) and plasma ANG II levels significantly above

baseline (Wilkes, 1984; Mento & Wilkes 1987). Different ACE inhibitors produce different effects on the plasma levels of ACE and ANG II. Infusion of perindopril into rats for 6 days significantly inhibited plasma ACE activity and ANG II, whereas that of captopril increased plasma ACE activity without altering plasma ANG II concentration (Tokita et al, 1995). Both perindopril and captopril markedly increased plasma renin concentration. Enalapril and captopril produced similar effects on the plasma ACE and ANG II levels.

It was suggested that the plasma ACE activity may not be the only factor that determines the long-term effect of ACE inhibitors on blood pressure. Prolonged antihypertensive action of ACE inhibitors may be related to persistent ACE inhibition in tissues (Unger et al, 1985). However, renal ACE activity and renin level also increased after chronic treatment of ACE inhibitors as shown in the present and other studies (Keuneke et al, 1995; Tokita et al, 1995). On the other hand, Campbell et al (1991) reported that kidney ANG II was reduced in rats treated with oral perindopril for 7 days, despite a failure to reduce plasma ANG II level. Thus, further studies are required to verify reduced ANG II formation by ACE inhibitors in various tissues.

In the present study, chronic treatment with enalapril increased plasma renin concentration and renal levels of renin mRNA and activity without altering renal mRNA levels of angiotensinogen or ANG II AT_1 receptor subtypes. On the other hand, in liver and hypothalamus, gene expressions of all the RAS components examined were not altered by enalapril treatment. Limited data are available on effects of ACE inhibition on the gene expression of the RAS components in the various tissues. It has been reported that chronic administration of ACE inhibitors increased plasma renin activity in parallel with kidney renin mRNA levels without altering renal or hepatic angiotensinogen mRNA (Keuneke et al, 1995; Sechi et al, 1996), or renal AT_1 receptor mRNA levels (Sechi et al, 1996). These results are consistent with the present study. However, Keuneke et al (1995) reported that the hypothalamic angiotensinogen mRNA level was suppressed by some of ACE inhibitors such as lisinopril and cilazapril, but not by captopril. They suggested that different lipophilicity of drugs determined accumulation of the drugs in specific tissues and produced different results. In the brain, ACE was inhibited by ACE inhibitors mainly in structures with a deficient blood brain barrier (Chai et al, 1992; Sakaguchi et al, 1987). Although most ACE inhibitors are unable to penetrate the blood brain barrier, high doses of more lipophilic drugs seem to penetrate the barrier especially after chronic administration (Chai et al, 1992).

Taken together, when determining the mRNA levels of the RAS components in various tissues, the present results suggest a differential expression of the RAS components in response to ACE inhibition, and localized action and some degree of tissue specificity of ACE inhibition, although the reasons for the effects are not yet determined.

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