Effects of Quinapril on Expression of eNOS, ACE, and AT1 Receptor in Deoxycorticosterone Acetate-Salt Hypertensive Rats

Kazuyoshi Hara, Naohiko Kobayashi, Shigeko Watanabe, Yusuke Tsubokou, and Hiroaki Matsuoka

Angiotensin II and nitric oxide (NO) may play a role in hypertensive cardiovascular remodeling. We evaluated the effects of long-term treatment with quinapril, an angiotensin converting enzyme (ACE) inhibitor, on expression of endothelial NO synthase (eNOS), ACE, and angiotensin II type 1 (AT1) receptor in the left ventricle and evaluated these relations to myocardial remodeling in deoxycorticosterone acetate (DOCA)-salt hypertensive rats. Deoxycorticosterone acetate-salt rats were induced with weekly injections of DOCA (30 mg/kg) and 1% saline in drinking water after right nephrectomy. Quinapril (DOCA-QUI, 10 mg/kg/day, subdepressor dose) or AT1 receptor antagonist TCV-116 (DOCA-TCV, 5 mg/kg/day, subdepressor dose) or vehicle (DOCA-V) were given after induction of DOCA-salt hypertension for 5 weeks, and age-matched sham-operated rats (ShC) served as a control group. The eNOS expression in the left ventricle were significantly decreased in DOCA-V compared with ShC, and were significantly increased in DOCA-QUI and DOCA-TCV compared with ShC and DOCA-V. The gene expression of ACE, AT1 receptor, and type I collagen mRNA were significantly increased in DOCA-V compared with ShC, and significantly suppressed in DOCA-QUI compared with DOCA-V. The DOCA-V rats demonstrated a significant increase of the wall-to-lumen ratio, perivascular fibrosis, and myocardial fibrosis, with all these parameters being significantly improved by quinapril. Myocardial remodeling in DOCA-salt hypertensive rats was significantly ameliorated by a subdepressor dose of quinapril, which may be due to an increase in eNOS mRNA and protein expression and a decrease in ACE and AT1 receptor mRNA expression in the left ventricle. Am J Hypertens 2001;14:321–330 © 2001 American Journal of Hypertension, Ltd.

Key Words: Angiotensin-converting enzyme inhibitor, angiotensin II, gene expression, hypertension, nitric oxide synthase, rats.

Left ventricular hypertrophy increases the incidence of ischemic heart disease, and is recognized as an important independent risk factor that determines the prognosis of hypertensive patients. Although vascular remodeling is usually an adaptive process that occurs in response to long-term changes in hemodynamic conditions, but it can contribute subsequently to the pathophysiology of vascular diseases and circulatory disorders. Hypertensive left ventricular hypertrophy is accompanied by structural remodeling of the myocardium that includes myocyte hypertrophy and interstitial and perivascular fibrosis of intramyocardial coronary arteries. Whereas the endothelium plays an important role in the regulation of vascular tone, and endothelial dysfunction has been shown clinically and experimentally to be a major component of the vascular disease in hypertension. In addition, the long-term blockade of nitric oxide synthase (NOS) produces not only hypertension but also pathologic structural changes of the coronary vasculature and fibrosis of the myocardium in animals. Furthermore, impaired endothelial-dependent vasodilation in hypertensive animal models could be improved by effective antihypertensive therapy. Indeed, we have reported that some antihypertensive drugs improve the impaired endothelial NOS (eNOS) expression or NOS activity in hypertensive rats.

Systemic angiotensin converting enzyme (ACE) is...
probably most important in regulating vasomotor tone, whereas angiotensin (Ang) II produced in the tissues excerts local trophic effects that modulate gene expression. Moreover, two main subtypes of Ang II receptor, type 1 (AT1) and type 2 (AT2) receptor, have been identified to date.\(^5\) Most of the known effects of Ang II are mediated through the AT1 receptor, and stimulation of the AT1 receptor produces vasoconstriction, proliferation, and extracellular matrix formation. Whereas, ACE inhibitors may have particular efficacy in the treatment of cardiovascular disease, and their beneficial effects including prevention of mortality have been established in large clinical trials.\(^6,7\) These effects may not only be due to the ACE inhibitor action of decreasing the synthesis of Ang II, but also to the ability of these drugs to attenuate the degradation of endogenous bradykinin by inhibition of kininase II, an enzyme that is functionally indistinguishable from ACE.\(^8\) Although in deoxycorticosterone acetate (DOCA)-salt hypertensive rats the development and maintenance of hypertension have been believed to be independent of the circulating renin-angiotensin system, because the plasma renin level in this model is lower than that in normotensive control rats, and ACE inhibitors and Ang II receptor antagonists have no effect on blood pressure (BP).\(^9\) It is well recognized that there are two kinds of the renin-angiotensin system, circulating and local, and particular interest to evaluate the local renin-angiotensin system under the condition of low renin hypertensive animal models. Recently, some investigators have evaluated the gene expression of local tissue, kidney\(^20\) and adrenal gland,\(^21\) renin-angiotensin system in DOCA-salt hypertensive rats. However, very few studies have evaluated the gene expression of eNOS, ACE, and AT1 receptor mRNA in the left ventricle (LV) and whether the beneficial effects of ACE inhibitor on myocardial remodeling is associated with direct local gene expression of eNOS, ACE, and AT1 receptor mRNA in DOCA-salt hypertensive rats. The purpose of the present study was to evaluate the effects of long-term treatment with a subdepressor dose of quinapril, an ACE inhibitor, on expression of eNOS, ACE, and AT1 receptor mRNA in the LV, and evaluate these relations to myocardial remodeling including type I collagen mRNA in DOCA-salt hypertensive rats.

**Methods**

**Animal Models and Experimental Designs**

All procedures were in accordance with institutional guidelines for animal research. Twenty-four male normotensive Wistar rats (Oriental Bioservice Kanto Inc., Ibaragi, Japan) aged 6 weeks were used, and DOCA-salt hypertension was induced in 16 rats as described previously.\(^2,22\) Rats received weekly subcutaneous injections of DOCA (30 mg/kg) after right nephrectomy and were given 1% saline for drinking water (DOCA-V, n = 8). The remaining 8 DOCA-salt rats were treated with quinapril (Welfide Co., Osaka, Japan) in drinking water with 1% saline for 5 weeks, fresh drug solution being prepared daily (DOCA-QUI, n = 8). The drug was given at an average dose of 10 mg/kg/day by being weekly adjusted to the drinking habits of the animals. The 10 mg/kg/day of quinapril was not expected to influence BP in rats, according to our preliminary data (data not shown). Age-matched sham-operated rats (ShC, n = 8) served as a control group. Systolic blood pressure (SBP) was measured by the tail-cuff method (model MK-1100, Muromachi Kikai, Tokyo, Japan) before the operation and at 1-week intervals thereafter (protocol 1). Numbers of animal model and experimental designs of protocols 2, 3, and 4 were treated in the same manner as described above for protocol 1. Rats were housed at a constant temperature (25°C ± 1°C), and fed a standard laboratory rat chow (0.4% sodium content).

**Protocol 1: Reverse Transcription-Polymerase Chain Reaction**

After 5 weeks of treatment, the rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and decapitated, and the heart was immediately excised. The LV was carefully separated from the atria and right ventricle, weighted, immediately frozen in liquid nitrogen, and stored at −80°C until extraction of total RNA. Total RNA was prepared as previously described.\(^2,4\) Reverse transcription-polymerase chain reaction was performed by standard methods with 1 μg of total RNA. First-strand cDNA was synthesized with random primers and Molony murine leukemia virus reverse transcriptase (Promega, Madison, WI). Polymerase chain reaction amplification was then performed with synthetic gene-specific primers for eNOS (upstream primer, 5′-ATCCAGTAAACAGA-CATGCCA-3′; downstream primer, 5′-CAGGAAAGTA-AGTGAAGC-3′; product length, 693 bp),\(^2,5\) ACE (forward primer, 5′-ATCACGTTCATCATCCATGT-3′; reverse primer, 5′-AGGAAGAGCAAGCAGCCTG-3′; product length, 403 bp),\(^2,6\) AT1 receptor (forward primer, 5′-GCCAAGTCACCTGCTAC-3′; reverse primer, 5′-AATTTTTTCCCAGAAAACC-3′; product length, 494 bp),\(^2,7\) and type I collagen (upstream primer, 5′-TTGTTCGGTTCTCAGGGAAGTTAG-3′; downstream primer, 5′-TTGTTCACAGGTTCTTTCT-3′; product length, 254 bp),\(^2,8\) using a DNA polymerase chain reaction kit (Perkin Elmer, Norwalk, CT) for 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min. Parallel amplification of rat glyceraldehyde-3-phosphate-dehydrogenase was performed for reference with primers as described.\(^2,9\) Reaction conditions were optimized to obtain reproducible and reliable amplification within the logarithmic phase of the reaction, as determined by preliminary experiments. The reaction was linear to 35 cycles with use of the ethidium bromide detection method. Polymerase chain reaction products were separated by electrophoresis on a 2% agarose gel...
containing ethidium bromide and were visualized by ultraviolet-induced fluorescence. The intensity of each band was quantified using a densitometer. The resulting densities of the eNOS, ACE, AT1 receptor, and type I collagen bands were expressed relative to the corresponding densities of the glyceraldehyde-3-phosphate-dehydrogenase bands from the same RNA sample.9–13,30

Protocol 2: Western Blot Analysis

The LV was homogenized (25% w/v) in 10 mmol/L HEPES buffer, pH 7.4, containing 320 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L DTT, 10 μg/mL leupeptin, and 2 μg/mL aprotinin at 0° to 4°C with a polytron homogenizer. Homogenate was centrifuged at 1000 g for 5 min at 4°C and the resulting supernatant was used as a postnuclear fraction. Protein concentrations were determined with bovine serum albumin as a standard protein.31 The postnuclear fraction (eNOS: 50 μg of protein) of sample was subjected to SDS-PAGE using 10% gels.32 The proteins in the gels were transferred electrophoretically to polyvinylidene difluoride sheets for 1 h at 2 mA/cm² as described.33 The sheets were immunoblotted with an anti-eNOS antibody (Transduction Laboratories, Lexington, KY) in a buffer containing 10 mmol/L Tris/HCl, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20, and 5% skim milk followed by peroxidase-conjugated goat anti-mouse IgG (Amersham Life Science Inc., Arlington Heights, IL).33 The eNOS proteins transferred to the sheets were detected using the enhanced chemiluminescence immunoblotting detection system (Amersham Life Science Inc.). The amount of protein was quantified using a densitometer in a linear range and expressed as percent relative to that in non-treated rat.11,13

Protocol 3: Nitrite Production in Myocardium Slices

The LV was used for the assay of nitrite production within 24 h. Three 50-μm sections of each myocardium were cut on a vibratome and incubated in a buffer (pH 7.2) containing 25 mmol/L HEPES (Sigma, St. Louis, MO), 140 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl₂, 1 mmol/L MgCl₂, and 5 mmol/L glucose for 48 h at 37°C. The supernatant was used for the assay of NO₂⁻ production, and the amount of NO₂⁻ was corrected by the protein amount measured by the Bradford method (Bio-Rad, Richmond, CA). Nitrite was measured with an autoanalyzer (TCI-NOX 1000m; Tokyo Kasei Kogyo, Tokyo, Japan) using the Griess method.9,10,13,14,23

Protocol 4: Histologic Examination and Evaluation of Myocardial Remodeling

Histologic examination was studied as described in detail previously.9–13,30,34 To assess any thickening of the coronary arterial wall and perivascular fibrosis, the transsec-
Administration of quinapril (380 ± 15 beats/min). Body weight was also similar among the three groups. The LV mass of the DOCA-V was significantly increased compared to that of ShC in body weight-corrected values (2.78 ± 0.07 mg/g vs. 1.91 ± 0.04 mg/g, *P < .01), and significantly decreased in DOCA-QUI (2.41 ± 0.06 mg/g, **P < .01) compared with the DOCA-V after 5 weeks of treatment with quinapril.

Reverse Transcription-Polymerase Chain Reaction for eNOS, ACE, AT1 Receptor, Type I Collagen mRNA Expression in the Left Ventricle

The level of eNOS mRNA in the LV was significantly decreased in DOCA-V compared with ShC, and significantly increased in DOCA-QUI compared with ShC and DOCA-V (Figs. 2, 3A). The ACE mRNA levels were significantly increased in DOCA-V compared with ShC and significantly decreased in DOCA-QUI compared with DOCA-V (Figs. 2, 3B). The AT1 receptor and type I collagen mRNA levels were significantly greater in DOCA-V than in ShC and was significantly less in DOCA-QUI than in DOCA-V (Figs. 2, 4A,B).

Western Blot Analysis of eNOS Protein

The eNOS protein mass in the LV was significantly decreased in DOCA-V compared with ShC, and significantly increased in DOCA-QUI compared with ShC and DOCA-V (Fig. 5A,B).
Nitrite Production in the Myocardium Slices

The myocardium slices of DOCA-V produced significantly less NO$_2^-$ than those in ShC. The NO$_2^-$ production was significantly increased in DOCA-QUI compared with ShC and DOCA-V (Fig. 6).

Myocardial Remodeling

Micrographs of small coronary arteries with Masson’s trichrome stain among three groups are shown in Fig. 7. The wall-to-lumen ratio was significantly increased in DOCA-V compared with ShC, and was significantly decreased by quinapril treatment (Fig. 8A). The degree of perivascular fibrosis was significantly greater in DOCA-V than in ShC, and was also significantly decreased by quinapril treatment (Fig. 8B). Compared with ShC, myocardial fibrosis was significantly greater in DOCA-V, and was significantly less in DOCA-QUI than in DOCA-V (Fig. 8C).

**FIG. 4.** Effect of chronic quinapril treatment on AT1 receptor (A), and type I collagen (B) mRNA expression. Total RNA was assayed by reverse transcription-polymerase chain reaction with gene-specific primers for AT1 receptor, type I collagen, and and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each panel represents the mean densities of the AT1 receptor (A) and type I collagen (B) bands in relation to the bands obtained for GAPDH. Values are expressed as means ± SEM. n = 8 per group. *P < .05 v sham-operated control group (ShC). †P < .05 v deoxycorticosterone acetate (DOCA)-salt hypertensive rats (DOCA-V). DOCA-QUI = DOCA-salt hypertensive rats treated with quinapril.

**FIG. 5.** Effect of chronic quinapril treatment on endothelial nitric oxide synthase (eNOS) protein expression level. A) Typical Western blot of eNOS in the left ventricle of rat treated with quinapril. Western blot analysis using anti-eNOS antibodies were performed. Lane 1 represents sham-operated control group (ShC), lane 2 deoxycorticosterone acetate (DOCA)-salt hypertensive rats (DOCA-V), and lane 3 DOCA-salt hypertensive rats treated with quinapril (DOCA-QUI). B) Group data depicting relative optical densities of left ventricular eNOS protein bands in the study groups. Values are expressed as means ± SEM. n = 8 per group. *P < .05 v ShC. †P < .01 v DOCA-V.

**Effect of AT1 Receptor Antagonist on Systemic Hemodynamics, Left Ventricular Weight, eNOS, and AT1 Receptor mRNA Expression**

The SBP in DOCA-V and DOCA-TCV was similar and in DOCA-TCV it was significantly higher than in ShC.
Heart rate was similar among three groups, and was not changed by the administration of TCV-116 (385 ± 17 beats/min). Body weight was also similar among the three groups. The LV mass of the DOCA-TCV was significantly decreased in DOCA-TCV (2.49 ± 0.07 mg/g, P < .01) compared with the DOCA-V in body weight-corrected values. The level of eNOS mRNA in the LV was significantly increased in DOCA-TCV compared with ShC and DOCA-V (Figs. 9, 10A). AT1 receptor mRNA levels were significantly decreased in DOCA-TCV compared with DOCA-V (Figs. 9, 10B).

**Discussion**

The present study demonstrated that the production of eNOS mRNA and protein expression and NOS activity is downregulated and ACE and AT1 receptor mRNA expression is upregulated in the LV of DOCA-salt hypertensive rats. The chronic administration of an ACE inhibitor quinapril increased eNOS expression and decreased ACE and AT1 receptor mRNA expression. In addition, long-term treatment with AT1 receptor antagonist TCV-116 also increased eNOS mRNA expression and decreased AT1 receptor mRNA expression. Moreover, after 5 weeks of treatment, quinapril effectively improved left ventricular hypertrophy and myocardial remodeling, and decreased type I collagen mRNA expression. These results suggested that chronic renin-angiotensin system blockade improves the myocardial remodeling and ameliorates left ventricular fibrosis factor. Therefore, it is strongly suggested that increased eNOS and decreased ACE and AT1 receptor expression play an important role in the amelioration of myocardial remodeling in DOCA-salt hypertensive rats.

The ACE inhibitors have been widely used in the treatment of hypertension and heart failure. Although the primary action of these agents is the inhibition of systemic and local formation of Ang II, a number of experimental and clinical data suggest that other dilator mechanisms may be involved in the hypotensive effect of ACE inhibitors. Because ACE is identical to the kinase II of the kallikrein-kinin system that inactivates bradykinin by liberating the carboxy-terminal dipeptide phenylalanyl-arginine, it has been suspected that a significant part of the BP lowering effect of ACE inhibitors in vivo is mediated by the accumulation of kinins. Kinins are vasodilators through the release of different autacoids, mainly generated by the endothelium. Moreover, it has been demonstrated that decrease in Ang II production as well as inhibition of kinase II lead to activation of bradykinin B2 receptors, and the bradykinin causes intracellular calcium increase by binding with B2 receptors in the endothelial cells, and enhancing NO production through eNOS activation.

In the present study, chronic administration of an ACE inhibitor quinapril increased eNOS mRNA and protein expression and improved left ventricular hypertrophy and myocardial remodeling. Several in vivo experimental studies have been reported. Linz et al demonstrated that late treatment of spontaneously hypertensive rats with the ACE inhibitor ramipril extended lifespan from 21 to 30 months and showed the regression of left ventricular hypertrophy and the improvement of cardiac and vascular dysfunction, which is comparable to the lifespan of untreated normotensive Wistar-Kyoto rats. In addition, these pivotal beneficial effects of ACE inhibition were BP lowering agents.
reduction and upregulation of eNOS expression. Moreover, they discussed that the ramipril-induced inhibition of the endogenous breakdown of kinins mediated the increase in survival, because cotreatment with the bradykinin receptor antagonist icatibant suppressed the enhanced aortic cGMP content after high- and low-dose treatment with ramipril.\textsuperscript{41} Takemoto et al\textsuperscript{7} suggested that the long-term blockade of NO synthesis with chronically administered $N^\omega$-nitro-L-arginine methyl ester (L-NAME) produced vascular structural changes and hypertrophy and fibrosis of the myocardium, and that the ACE inhibitor temocapril prevented the coronary vascular and myocardial remodeling in L-NAME-induced hypertensive rats. We also evaluated the effects of long-term treatment with the ACE inhibitor imidapril on gene expression of eNOS mRNA in the LV and examined these relations to coronary vascular and myocardial remodeling in L-NAME-induced hypertensive rats. Downregulation of eNOS mRNA expression in the LV of this models was significantly increased, and myocardial remodeling was significantly ameliorated by subdepressor dose of imidapril.\textsuperscript{12} Furthermore, in the failing heart of Dahl salt-sensitive hypertensive rats, subdepressor dose of imidapril may ameliorate the endothelial damage not only by the inhibiting production of Ang II but also by promoting eNOS and inhibiting inducible NOS mRNA and protein expression in the LV, and this increased eNOS mRNA and protein level may have a role in the improvement of congestive heart failure and myocardial remodeling.\textsuperscript{11} These results indicate that eNOS expression may have an important role in beneficial cardioprotective effect of myocardial remodeling.

The circulating renin-angiotensin system has long been known to regulate vasoconstriction and aldosterone secretion, thus participating in BP control and fluid homeostasis. In addition, it has become apparent that local tissue renin-angiotensin systems play a major role in the regulation of growth processes.\textsuperscript{21} Moreover, ACE inhibitors appear to have a greater potential, experimentally and clinically, for inducing both normalization of cardiovascular structural hypertrophy and the prerequisite lowering of arterial pressure.\textsuperscript{16,17,35–37} These effects may not only be due to the ACE inhibitor action of decreasing the synthesis of Ang II, but also to the ability of these drugs to attenuate the degradation of endogenous bradykinin by inhibition of kinase II, an enzyme that is functionally indistinguishable from ACE.\textsuperscript{18} Whereas to evaluate the role of local tissue renin-angiotensin system and humoral factors in vascular remodeling we performed our experi-

**FIG. 9.** Typical gel electrophoresis of reverse transcription-polymerase chain reaction of left ventricular endothelial nitric oxide synthase (eNOS) mRNA, AT1 receptor mRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Lane 1 represents sham-operated control group, lane 2 deoxycorticosterone acetate (DOCA)-salt hypertensive rats, and lane 3 DOCA-salt hypertensive rats treated with TCV-116.
FIG. 10. Effect of chronic TCV-116 treatment on ventricular endothelial nitric oxide synthase (eNOS) (A) and AT1 receptor (B) mRNA expression. Total RNA was assayed by reverse transcription-polymerase chain reaction with gene-specific primers for eNOS, AT1 receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each panel represents the mean densities of the eNOS (A) and AT1 receptor (B) in relation to the bands obtained for GAPDH. Values are expressed as means ± SEM. n = 6 per group. *P < .05 v ShC; †P < .05, ‡P < .01 v DOCA-V. ShC = sham-operated control group; DOCA-V = deoxycorticosterone acetate (DOCA)-salt hypertensive rats; DOCA-TCV = DOCA-salt hypertensive rats treated with TCV-116.

In conclusion, we evaluated the effects of long-term treatment with the ACE inhibitor quinapril on expression of eNOS, ACE, AT1 receptor, and type I collagen in the LV and evaluated these relations to myocardial remodeling in DOCA-salt hypertensive rats. The present results show that downregulation of eNOS mRNA and protein expression and NOS activity was significantly increased and upregulation of ACE and AT1 receptor mRNA expression was significantly decreased in DOCA-V by quinapril treatment. Myocardial remodeling of DOCA-salt hypertensive rats was significantly ameliorated by a subpressor dose of quinapril, which may be due to an increase in eNOS mRNA expression and a decrease in ACE and AT1 receptor mRNA expression in the LV.

Acknowledgment

We thank Kazumi Akimoto, PhD, for technical assistance with RT-PCR, Noriko Suzuki for preparing and staining...
tissue sections for histologic investigation, and Yasuko Mamada for technical assistance. We thank WelFide Co. for supplying quinapril.

References


