AT₁ Receptor Blockade Reduces Cardiac Calcineurin Activity in Hypertensive Rats

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Abstract—The possible role of calcineurin in the attenuation of cardiac hypertrophy and fibrosis by blockade of the angiotensin II type 1 (AT₁) receptor was investigated in Dahl salt-sensitive (DS) rats. The effect of the calcineurin inhibitor FK506 was also studied. DS rats progressively developed severe hypertension when fed a diet containing 8% NaCl from 7 weeks of age. In addition, marked cardiac hypertrophy and fibrosis were apparent and the activity of calcineurin and its mRNA expression in the myocardium was increased in these animals at 12 weeks in comparison with age-matched Dahl salt-resistant rats. The abundance of angiotensin-converting enzyme (ACE) and transforming growth factor (TGF)-β1 mRNAs was also increased in the hearts of DS rats at 12 weeks. Treatment of DS rats with a non-antihypertensive dose of the selective AT₁ receptor blocker candesartan (1 mg/kg per day) or FK506 (0.1 mg/kg per day) from 7 to 12 weeks attenuated both calcineurin activity and its mRNA expression in the heart, as well as the development of cardiac hypertrophy and fibrosis, without affecting cardiac function. Treatment with candesartan, but not FK506, prevented the upregulation of ACE and TGF-β1 gene expression. Both candesartan and FK506 prevented the load-induced induction of fetal-type cardiac genes. These results demonstrate that AT₁ receptor blockade attenuates the development of cardiac hypertrophy and fibrosis as well as the activation of calcineurin, without an antihypertensive effect, in rats with salt-sensitive hypertension. Calcineurin may be downstream from TGF-β1 in AT₁ receptor-mediated angiotensin II signaling in vivo. (Hypertension. 2002;40:168-174.)

Key Words: hypertension, sodium-dependent myocardium hypertrophy fibrosis angiotensin II

Evidence suggests that angiotensin II (Ang II) is a potent stimulator of cardiac hypertrophy.¹² There are at least 2 isoforms for Ang II receptors, which are designated as AT₁ and AT₂, and the AT₁ receptor is further subdivided into AT₁A and AT₁B. It is generally accepted that most of the traditional Ang II functions in the cardiovascular system are attributable to the AT₁ receptor.⁵ Angiotensin-converting enzyme (ACE) inhibitors or AT₁ receptor blockers induce the regression or prevent the development of left ventricular (LV) hypertrophy, both in animal models¹⁻⁶ and in hypertensive patients.⁷,⁸ However, it has proved difficult to determine whether the antagonistic effects of ACE inhibitors and AT₁ receptor blockers on Ang II-induced growth promotion or the concomitant systemic hemodynamic effects of these agents underlie their beneficial action with regard to this condition. A non-antihypertensive dose of an ACE inhibitor was shown to reverse LV hypertrophy in aortic banded rats⁹,¹⁰ and the antihypertrophic effect of an AT₁ receptor blocker was shown to be greater than that of hydralazine, despite the greater antihypertensive effect of hydralazine, in spontaneously hypertensive rats.⁶ These observations suggest that blood pressure reduction alone is not sufficient to prevent target organ damage and that the additional control of local or neurohumoral factors might also be required.

An intracellular signaling pathway that includes the Ca²⁺-dependent protein phosphatase calcineurin has been shown to underlie cardiac hypertrophy.¹¹ Calcineurin has also been shown to play a key role in the development of pressure overload–induced cardiac hypertrophy.¹²,¹³ A recent study suggested that calcineurin is involved in the development of cardiac hypertrophy induced by mineralocorticoid excess.¹⁴ Furthermore, treatment of cultured cardiac myocytes with Ang II or phenylephrine results in activation of calcineurin.¹⁵ However, the effect of the cardiac renin-angiotensin system (RAS) on calcineurin signaling in vivo has not been described.

We have, therefore, now investigated whether blockade of the cardiac RAS with a non-antihypertensive dose of candesartan, the AT₁ receptor blocker, attenuates the development of cardiac hypertrophy and fibrosis in rats with salt-sensitive hypertension, and, if so, whether this drug also inhibits calcineurin activation in vivo. To explore the mechanism by
which the AT1 receptor blocker suppresses calcineurin activation, we also studied the effects of FK506, the calcineurin inhibitor, on the expression of various cardiac genes. 

Methods

Animals

Male inbred Dahl salt-sensitive (DS) and salt-resistant (DR) rats were obtained from Eisai (Tokyo, Japan). Rats were handled in accordance with the guidelines of Nagoya University Graduate School of Medicine. Weaning rats were fed laboratory chow containing 0.3% NaCl until 7 weeks of age, when the diet was switched to laboratory chow containing 8% NaCl. Systolic blood pressure was measured weekly by the indirect tail-cuff method. Rats were divided into 4 groups; (1) DR rats (n=5), (2) DS rats (n=6), (3) DS rats (n=6) administered a non-antihypertensive dose of candesartan (1 mg/kg body weight daily; Takeda Chemical Industries), and (4) DS rats (n=6) administered FK506 (0.1 mg/kg body weight daily; Fujisawa Industries). Candesartan was given orally by a gastric tube from 7 to 12 weeks of age. The dose of candesartan was determined from the results of a preliminary study and from those of a previous study. FK506 was given intramuscularly from 7 to 12 weeks of age. This dose of FK506 has been shown to prevent a load-induced elevation of calcineurin activity in this model. At 12 weeks of age, all rats were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and killed, and the hearts were removed and subjected to further analysis.

Echocardiography

At 12 weeks of age, rats were subjected to transthoracic echocardiography as previously described. In brief, M-mode echocardiography was performed with an Acuson system with a 13-MHz transducer (Sequoia Ultrasound System). LV end-diastolic (LVDd) and end-systolic (LVd) dimensions and the thickness of the interventricular septum (IVS) and posterior wall (PW) were measured, and fractional shortening (FS) was calculated as follows: FS=[(LVDd–LVDd)/LVDd]×100%.

Histology

LV was fixed with ice-cold 4% paraformaldehyde for 16 to 24 hours and embedded in paraffin. Transverse sections (3 μm thickness) were prepared and stained either with hematoxylin-eosin for routine histological examination or with Azan Mallory solution to evaluate the extent of fibrosis. The myocyte cross-sectional area was measured from myocytes that were cut transversely and exhibited both a histological examination or with Azan Mallory solution to evaluate the extent of fibrosis. The myocyte cross-sectional area was measured from myocytes that were cut transversely and exhibited both a

Real-Time Quantitative RT-PCR

Total RNA was extracted from LV tissue and treated with DNase with the use of a spin-vacuum (SV) total RNA isolation kit (Promega). Complementary DNA was then synthesized from 2 μg of total RNA with an oligo(dT)18 primer and SuperScript II reverse transcriptase (Gibco BRL). Quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis was performed with a Prism 7700 Sequence Detector (Perkin-Elmer), as previously described, with primers and TaqMan probes specific for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), ACE, the AT1 receptor, and transforming growth factor (TGF)-β1 and calcineurin Aβ mRNAs (Table 1). TaqMan rodent GAPDH control reagents (Perkin-Elmer) were used for detection of GAPDH mRNA as an internal standard. The PCR products of each target gene were subcloned by TA cloning (pGEM-T Easy, Promega) and verified by sequencing. Serial dilutions of cloned plasmid DNA were analyzed for each target gene to determine standard curves for quantitative analysis.

Assay of Calcineurin Activity

The activity of calcineurin was determined as described elsewhere. LV tissue was homogenized in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 2 mmol/L EDTA, 2 mmol/L EGTA, 0.1% Triton X-100, 0.5 mmol/L dithiothreitol, and protease inhibitors. A calcineurin substrate, casein, was first phosphorylated by protein kinase A in the presence of [γ-32P]ATP. A 25-μL reaction mixture, containing 50 mmol/L HEPES (pH 7.5), 1 mmol/L dithiothreitol, 0.1 mmol/L MnCl2, 1 mmol/L CaCl2, 1.5 μmol/L calmodulin, 0.2 μmol/L calyculin A, and 100 μg/mL 32P-labeled casein, was incubated with 3 μL of tissue extract for 10 minutes at 30°C. The amount of 32P-labeled inorganic phosphate in the supernatant was measured with a liquid scintillation counter. The activity was corrected for the percentage of the mean value for age-matched control DR rats.

Statistical Analysis

Data are expressed as mean±SEM. Differences among groups were assessed by 1-way factorial analysis of variance (ANOVA). Within-group comparisons were performed by 2-way repeated-measures ANOVA. When a significant difference was detected, intergroup
Figure 1. Time course of changes in blood pressure in DR rats, untreated DS rats, and candesartan- and FK506-treated DS rats. Data are mean±SEM. *P<0.05 versus age-matched DR rats.

comparisons were carried out by Fisher’s multiple comparisons test. P<0.05 was considered statistically significant.

Results

Attenuation of the Development of Load-Induced Cardiac Hypertrophy and Fibrosis by a Non-Antihypertensive Dose of Candesartan

Blood pressure from 7 to 12 weeks of age was similar among DS rats treated with candesartan or FK506 and untreated DS rats (Figure 1). None of the DS rats died during candesartan or FK506 treatment. The LV weight (LVW) of 12-week-old untreated DS rats was increased by 24% compared with that of age-matched DR rats, and this increase was attenuated by 55% by treatment with the non-antihypertensive dose of candesartan and completely prevented by the FK506 treatment (Table 2). The LVW/tibial length (TL) ratio was 27% greater in untreated DS rats than in DR rats, and this overload-induced increase in LVW/TL was reduced by 53% by candesartan and completely prevented by FK506.

Echocardiography revealed that the thickness of both the IVS and PW was greater and that the LVDd was smaller in untreated DS rats, and candesartan- and FK506-treated DS rats. Treatment with candesartan attenuated the increases in IVS and PW thickness, as well as the decrease in LVDd in DS rats, whereas FS was not affected by this drug. These results indicate that long-term treatment with candesartan inhibited LV remodeling and preserved cardiac function without an antihypertensive effect. Treatment with FK506 prevented the development of cardiac hypertrophy without any impairment of cardiac function.

Microscopic analysis revealed that hemodynamic overload for 5 weeks increased the cross-sectional area of cardiac myocytes in DS rats by 65%, compared with that apparent in DR rats (Figure 2). Treatment of DS rats with candesartan reduced this effect by 48%, and the FK506 treatment completely prevented the load-induced increase in cardiomyocyte hypertrophy. Moreover, marked perivascular and interstitial fibrosis was detected in untreated DS rats at 12 weeks of age (Figure 3). This increase in cardiac fibrosis was significantly reduced by treatment with candesartan and completely suppressed by the FK506 treatment.

Inhibition of Load-Induced Activation of Calcineurin by a Non-Antihypertensive Dose of Candesartan

Calcineurin activity in the hearts of untreated DS rats at 12 weeks of age was increased by 43% compared with that of age-matched DR rats, indicating that hemodynamic overload, not the high-salt diet, induces calcineurin activation in the hearts of DS rats (Figure 4). The load-induced increase in calcineurin activity was reduced by 70% by treatment with a non-antihypertensive dose of candesartan and completely prevented by the FK506 treatment. The abundance of calcineurin Aβ mRNA was increased in the hearts of untreated DS rats compared with that apparent in DR rats, and this effect of hemodynamic overload was inhibited by treatment with candesartan or FK506.

Role of Endogenous Ang II in Load-Induced Reprogramming of Cardiac Gene Expression

Hemodynamic overload for 5 weeks resulted in upregulation of the expression of fetal-type genes, such as those for ANP, BNP, and β-MHC, in untreated DS rats (Figure 5). Treatment with candesartan or FK506 during the same period inhibited the increase in the expression level of these genes. Expression of the ACE gene was also upregulated in the hearts of untreated DS rats, whereas that of the AT1A receptor gene was increased in untreated DS rats relative to that in DR rats.
not affected (Figure 6). Treatment of DS rats with candesartan prevented the load-induced upregulation of ACE gene expression and downregulated the expression of the AT_{1A} receptor gene. Treatment of DS rats with FK506 did not significantly affect the expression of ACE or AT_{1A} receptor genes. Finally, the abundance of TGF-β1 mRNA was increased in the hearts of untreated DS rats compared with that apparent in DR rats, and candesartan, but not FK506, inhibited this effect of hemodynamic overload (Figure 6).

**Discussion**

We have shown that the AT_{1} receptor blocker candesartan attenuated the development of cardiac hypertrophy and fibrosis, without an antihypertensive effect, in rats with salt-sensitive hypertension. Furthermore, AT_{1} receptor blockade also prevented the increase in calcineurin activity and its mRNA expression normally observed in the hearts of these animals. These results suggest that calcineurin may contribute to AT_{1} receptor-mediated Ang II signaling in vivo.

**Figure 2.** Cross-sectional area of cardiac myocytes in the hearts of 12-week-old DR rats (A, B), untreated DS rats (C, D), and DS rats treated with candesartan (E, F) or FK506 (G, H). Data are mean±SEM. *P<0.05 versus DR rats; †P<0.05 versus untreated DS rats.

**Figure 3.** Relative area of fibrosis in the hearts of 12-week-old DR rats (A, B), untreated DS rats (C, D), and DS rats treated with candesartan (E, F) or FK506 (G, H). Data are mean±SEM. *P<0.05 versus DR rats; †P<0.05 versus untreated DS rats.
Ang II has been implicated in the development of cardiac hypertrophy and fibrosis associated with hemodynamic overload. In vivo studies have shown that the abundance of angiotensinogen, ACE, and AT1A receptor mRNAs in the heart is increased in response to pressure overload in various species. In the present study, the amount of ACE mRNA was increased in the hearts of 12-week-old DS rats compared with that in the hearts of age-matched DR rats, suggesting that the cardiac RAS was activated in the former animals. The expression of the AT1A receptor gene, however, did not differ between the hearts of these 2 groups of rats. Although the reason for this finding is not clear, a recent study observed an increase in expression of ACE gene, but no change in AT1A receptor gene expression in hypertensive cardiac hypertrophy of DS rats. Mechanical stretch of cultured rat cardiomyocytes induced upregulation of the expression of both ACE and AT1A receptor genes, whereas Ang II increased the expression of the ACE gene but downregulated that of the AT1A receptor gene. It is thus possible that upregulation of AT1A receptor gene expression, induced by pressure overload, was counteracted by the opposite effect, induced by activation of the local RAS in the hearts of 12-week-old DS rats. In addition, treatment of cardiac fibroblasts with Ang II was shown not to affect the expression of renin, angiotensinogen, and ACE genes but to inhibit the expression of the AT1A receptor gene. It is, therefore, also possible that the level of AT1A receptor gene expression apparent in the hearts of 12-week-old DS rats represents the net effect of pressure overload on cardiac myocytes and fibroblasts.

A non-antihypertensive dose of candesartan suppressed the LV (and cardiomyocyte) hypertrophy and prevented the upregulation of ANP, BNP, and β-MHC genes normally apparent in 12-week-old DS rats, consistent with the results of previous studies with other animal models of hypertrophy. Furthermore, candesartan induced downregulation of ACE and AT1A receptor genes in DS rats, consistent with the previous observation that losartan, another AT1 receptor blocker, inhibited Ang II–induced upregulation of RAS genes, as well as the stretch-induced increase in AT1A receptor gene expression in cardiac myocytes in vitro. Candesartan also induced downregulation of TGF-β1 gene in DS rats, which is supported by a recent study with TGF-β1–deficient mice demonstrating that TGF-β1 is an important mediator of the hypertrophic growth response of the heart to Ang II. TGF-β1 has been shown to induce the expression of fetal-type genes in cultured cardiomyocytes. Together, these results indicate that AT1 receptor blockers, in a manner independent of their antihypertensive effects, are able to attenuate the development of hypertensive LV hypertrophy through antagonism of cardiac AT1 receptors.

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Cardiac fibrosis is a pathological feature associated with hypertension and cardiac hypertrophy. Untreated DS rats at 12 weeks of age exhibited an increase in the extent of interstitial and perivascular fibrosis in the LV myocardium, consistent with previous observations with this animal model. Furthermore, the amount of TGF-β1 mRNA was also increased in the hearts of 12-week-old DS rats compared with that in hearts of age-matched DR rats. TGF-β1 is a potent stimulator of extracellular matrix production by cardiac fibroblasts. In addition, Ang II directly stimulates the proliferation, as well as the production, of extracellular matrix proteins by cardiac fibroblasts, and TGF-β1 participates in the Ang II–induced synthesis of collagens by these cells. In the present study, candesartan reduced both the extent of cardiac fibrosis and the amount of TGF-β1 mRNA in the hearts of DS rats, suggesting that AT1 receptor signaling through TGF-β1 contributes to the development of fibrosis apparent in these animals. These observations are also consistent with the results of experimental studies suggesting that Ang II induces cardiac fibrosis, not as a result of its hypertensive effect, but by a direct action on the heart. Together, these results thus indicate that AT1 receptor blockers, in a manner independent of their antihypertensive effects, are able to attenuate cardiac fibrosis associated with hypertension and that inhibition of TGF-β1 gene expression is important for the attenuation of fibrosis by AT1 receptor blockade.

We have shown that the activity of calcineurin and its mRNA expression in the LV myocardium were increased in 12-week-old DS rats compared with that in age-matched DR rats, consistent with previous observations with this animal model. The increase in calcineurin activity in the DS rat heart observed in the present study was inhibited by treatment with the non-antihypertensive dose of candesartan. The dose of FK506 used here also prevented the increase in calcineurin activity in the hearts of DS rats, which is consistent with previous results. These observations thus suggest that inhibition of endogenous Ang II–induced activation of calcineurin is important for the attenuation of cardiac hypertrophy by the AT1 receptor blocker apparent in 12-week-old DS rats. In the present study, the candesartan treatment was associated with reduced perivascular and interstitial fibrosis in the DS rat heart. FK506 suppressed the development of cardiac fibrosis, consistent with a previous study showing that the load-induced increase in the extent of cardiac fibrosis in this model was inhibited by FK506 in a dose-dependent manner. The FK506 treatment prevented the upregulation of fetal-type genes but did not affect ACE, AT1A receptor, or TGF-β1 gene expression in DS rats, suggesting that calcineurin may be downstream from TGF-β1 in AT1 receptor–mediated Ang II signaling. Furthermore, TGF-β was shown to stimulate Ca2+ influx into a fibroblast cell line, resulting in an increase in the cytosolic Ca2+ concentration, which might, in turn, activate the downstream Ca2+ effector calcineurin. Together, inhibition of TGF-β1 gene expression and a consequent reduction in calcineurin activity in cardiac myocytes and fibroblasts may contribute to the AT1 receptor blocker–induced attenuation of cardiac hypertrophy and fibrosis observed in 12-week-old DS rats.

Although several studies demonstrated that the AT1 receptor plays an important role in the regulation of blood pressure and apoptosis, most of the cardiovascular effects of Ang II were believed to be mediated by the AT1 receptor. The signaling mechanisms and physiological functions of the AT2 receptor still remain uncertain, particularly in the heart. A number of studies suggest that AT1 and AT2 receptors are functionally antagonistic. However, Ichihara et al and Mifune et al showed that lack of AT2 receptors prevented the development of cardiac hypertrophy and fibrosis. The regulation of AT1 and AT2 receptors in cardiac hypertrophy and fibrosis would be targeted for future investigation because the effects of the AT1 receptor should be taken into account in animals receiving chronic treatment of selective AT1 receptor blockade.

In conclusion, we have shown that the AT1 receptor blocker candesartan, in a manner independent of its antihypertensive effect, attenuates the development of cardiac hypertrophy and fibrosis and also reduces cardiac calcineurin activity in rats with salt-sensitive hypertension. These results suggest that calcineurin contributes to AT1 receptor–mediated Ang II signaling in vivo. Given the multifactorial nature of hypertrophic signaling, many regulatory pathways likely coordinate participate in the overall hypertrophic response. Further investigations are required to elucidate the connections among AT1 and AT2 receptors, calcineurin, and other intracellular signaling molecules and thereby provide a better understanding of the regulation of cardiac hypertrophy and fibrosis.

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