An Angiotensin II Receptor Antagonist Reduces Myocardial Damage in an Animal Model of Myocarditis

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Background  Recently, an angiotensin-converting enzyme inhibitor was shown to have a beneficial effect on virus-induced myocardial injury. We investigated the effect of a new angiotensin II type 1 receptor antagonist, (±)-1-(cyclohexylcarbonyloxy)ethyl 2-ethoxy-1-[2"-(1H-tetrazol-5-yl)biphenyl-4-yl][methyl]-1H-benzimidazole-7-carboxylate (TCV-116), in an animal model of viral myocarditis induced by encephalomyocarditis virus.

Methods and Results  Four-week-old DBA/2 mice were inoculated with the encephalomyocarditis virus. TCV-116 (3 mg/kg) was given 1 day before (1 or 10 mg/kg) or 2 days after virus inoculation (0.3 mg/kg). Control mice received the vehicle only. All drugs were administered orally on a daily basis, and the animals were killed on day 14. When treatment started 1 day before inoculation, the survival of mice receiving 10 mg/kg of TCV-116 improved (17 of 20 [85%] versus 14 of 22 [64%] control mice), but the difference was not significant. Heart weight (106±24 mg versus 133±33 mg; P<.05), histological scores for myocardial necrosis (1.1±0.3 versus 2.3±1.2; P<.01), cellular infiltration (1.4±0.7 versus 2.6±1.3; P<.05), and calcification (1.1±0.3 versus 2.1±1.1; P<.01) were significantly decreased in mice given TCV-116 at 3 mg/kg compared with the vehicle control mice. The plasma angiotensin II level was significantly higher in infected mice than in noninfected mice (71.8±30.2 versus 31.8±22.5 pg/mL; P<.05). TCV-116 did not inhibit viral replication in the heart.

Conclusions  This study suggests that angiotensin II plays an important pathophysiological role in viral myocarditis. Treatment with TCV-116, an angiotensin II receptor antagonist, had a cardioprotective effect.

Methods

Experimental Infection  Four-week-old inbred male DBA/2 mice were inoculated intraperitoneally with 0.1 mL of the M variant of EMC virus diluted in Eagle’s minimal essential medium (EMEM) to a concentration of 100 plaque-forming units (pfu)/mL.

Drug Preparation  TCV-116 (Fig 1) was synthesized by Takeda Chemical Industries, Ltd. Since TCV-116 is insoluble in water, drug solutions were prepared by suspending the compound in 5% gum arabic.

Experimental Design  

Experiment 1  Since most mice die of congestive heart failure within 14 days after virus inoculation in our model, we observed survival for 14 days in the present study. To investigate the effect of TCV-116 on survival, the drug was given 1 day before virus inoculation at a dose of 1 mg/kg per day (n=20) or 10 mg/kg per day (n=20), while control mice received vehicle only (n=22). Other mice were given the drug 2 days after virus inoculation at a dose of 0.3 mg/kg per day (n=21) or 3 mg/kg per day (n=21), while control mice received vehicle only. Animals were killed on day 14. For the histopathological study, hearts of mice treated 2 days after virus inoculation were used.

Experiment 2  Our previous study showed that viral replication was maximal at 4 to 5 days after inoculation, so we studied the effect of TCV-116 on viral replication at day 5. Mice were inoculated with EMC virus and were given oral TCV-116 (3 mg/kg) or vehicle on day 2 after virus inoculation. Four mice from the TCV-116 group and six from the control group were killed on

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The renin-angiotensin system is one of the most important factors in the pathophysiology of cardiovascular diseases such as hypertension and congestive heart failure. Many drugs that modify the activity of this system have been developed, and angiotensin-converting enzyme (ACE) inhibitors such as captopril have been reported to be beneficial in patients with congestive heart failure.1-4 Dilated cardiomyopathy (DCM) is a heart muscle disease of unknown origin that progresses to severe heart failure and can only be treated with heart transplantation in the terminal stage. Many etiological factors have been proposed, although viral myocarditis is thought to be the most important cause. We developed an animal model of DCM induced by encephalomyocarditis virus (EMC) virus that features myocardial lesions similar to those seen in human DCM.5,6 We recently demonstrated that an ACE inhibitor, captopril, is effective in treating EMC virus myocarditis.7 In the present study, we investigated the pathophysiological role of angiotensin II and the effect of a new nonpeptide angiotensin II type 1 receptor antagonist, (±)-1-(cyclohexylcarbonyloxy)ethyl 2-ethoxy-1-[2"-(1H-tetrazol-5-yl)biphenyl-4-yl][methyl]-1H-benzimidazole-7-carboxylate (TCV-116),8 in our animal model of EMC virus myocarditis.
day 5, and their hearts were used for a plaque assay to assess the myocardial viral titer.

**Experiment 3**

To determine the plasma angiotensin II level in infected and noninfected mice, 15 four-week-old mice were inoculated with the virus, and whole-body blood was obtained from the orbital venous plexus on day 5 (n=5), day 7 (n=4), or day 14 (n=6). Blood was also obtained from noninfected mice (n=5). Blood was mixed with Trasirl/EDTA and centrifuged to separate the plasma, which was stored at -70°C. The angiotensin II level was measured by ELISA using a mouse anti-human angiotensin II antibody. Molecular cloning and sequence analysis of the mouse angiotensinogen gene showed a strong homology to human angiotensinogen, and the deduced amino acid sequence revealed that angiotensin I and angiotensin II have the same amino acid sequence in humans and mice. Therefore, the antibody should also cross-react with mouse angiotensin II and show a high level of specificity. Each experiment was done only once, because the susceptibility of mice to the virus varied slightly from litter to litter.

**Histological Examination**

The hearts were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The extent of myocardial calcification, necrosis, and cellular infiltration was blindly graded by two observers and was scored as follows: 0, no lesions; 1+, lesions involving <25% of the myocardium; 2+, lesions involving 25% to 50% of the myocardium; 3+, lesions involving 50% to 75% of the myocardium; and 4+, lesions involving >75% of the myocardium. The scores assigned by the two observers were averaged.

**Myocardial Viral Titer**

For the assay of viral replication, hearts were ground with autoclaved sea sand in EMEM. After centrifugation, 0.1 mL of the supernatant was inoculated onto human amniotic cell monolayers for the plaque-forming assay. The viral titer was expressed as log_{10} pfu/mg heart.

**Statistical Analysis**

Survival was analyzed by the Kaplan-Meier method. Statistical analysis of data on body weight, heart weight, histological score, myocardial virus concentration, and plasma angiotensin II concentration was performed by one-way ANOVA.

**Results**

**Experiment 1—Effect of TCV-116 on Survival, Body and Heart Weight, and Myocardial Histology**

**Survival**

When TCV-116 treatment was started 1 day before virus inoculation, 13 of 20 mice (65%) given 1 mg/kg survived, as did 17 of 20 mice (85%) given 10 mg/kg. Survival was better than in the vehicle control group (14 of 22 mice, 64%), but the difference was not significant (Table). The survival rate of the control mice was different in each experiment, because the susceptibility of the animals to the virus was variable. When treatment was started 2 days after virus inoculation, 10 of 21 mice (48%) survived at 0.3 mg/kg, 12 of 21 mice (57%) survived at 3 mg/kg, and 10 of 21 mice (48%) survived in the vehicle control group. The differences between these groups were not significant.

**Body and Heart Weight**

Although body weight was similar in each group, heart weight was significantly lower in the mice given TCV-116 at 3 mg/kg per day (106±24 mg, P<.05) than in the control group (133±33 mg) (Fig 2).

**Myocardial Histology**

The histological score for myocardial necrosis was 2.5±1.2 in the control group, 1.9±0.7 in mice given TCV-116 at 0.3 mg/kg, and 1.1±0.3 in mice treated at 3 mg/kg. The respective scores for cellular infiltration were 2.6±1.3, 2.1±0.5, and 1.4±0.7, and the respective scores for calcification were 2.1±1.1, 1.9±0.7, and 1.1±0.3. In mice given TCV-116 at 3 mg/kg per day, all scores were significantly lower than in the control group (Fig 3). Representative histological findings are shown in Fig 4.

**Experiment 2—Effect of TCV-116 on Viral Replication**

On day 5 after viral inoculation, the viral titer was 3.1±0.8 log_{10} pfu/mg in the control group and 2.8±0.6 log_{10} pfu/mg in the TCV-116–treated group. There was no significant difference between the two groups.

**Experiment 3—Plasma Angiotensin II Levels**

The plasma angiotensin II level in infected mice was 43.0±12.4 pg/mL on day 5, 51.3±13.4 pg/mL on day 7, and 71.2±18.5 pg/mL on day 14.
and 71.8±30.2 pg/mL on day 14. The angiotensin II level on day 14 was significantly higher in infected mice than in noninfected mice on day 0 (22.2±17.8 pg/mL, P<.05) and on day 14 (31.8±22.4 pg/mL, P<.05) (Fig 5).

**Discussion**

Various types of heart disease cause congestive heart failure. Among them, viral myocarditis is one of the most important causes of DCM.9-15 The goals of therapy in patients with DCM are to improve cardiac function and to inhibit the progression of myocardial necrosis.

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Fig 3. Effect of TCV-116 on myocardial histological changes in DBA/2 mice with encephalomyocarditis virus myocarditis at 14 days after virus inoculation. The histological scores for necrosis, cellular infiltration, and calcification were significantly lower in mice given TCV-116 at 3 mg/kg per day. Data are expressed as mean±SD.

Fig 4. Tissue sections showing histological changes at 14 days after the inoculation of encephalomyocarditis virus. A, Multiple and extensive lesions of the heart in a mouse from the vehicle control group. B, Cardiac lesions are less severe in a mouse given TCV-116 at 3 mg/kg per day. C, Myocardial necrosis, cellular infiltration, and calcification are prominent in a heart from the vehicle control group. D, Only slight myocardial necrosis, cellular infiltration, and calcification are seen in the heart from a mouse given TCV-116 at 3 mg/kg per day. Hematoxylin and eosin stain; original magnification: panels A and B, ×16; panels C and D, ×180.

Fig 5. Plasma angiotensin II levels in mice infected with the encephalomyocarditis virus and noninfected mice. Angiotensin II began to increase on day 5 in the infected mice and rose further on days 7 to 14. Data are expressed as mean±SD.
and fibrosis. ACE inhibitors are known to reduce cardiac preload and afterload and have a beneficial effect on congestive heart failure.16–19 Accordingly, the pathophysiological role of the renin-angiotensin system in congestive heart failure and DCM has attracted considerable attention.20–24

Evidence that there is an endogenous renin-angiotensin system in the heart includes the demonstration of mRNA for angiotensinogen and renin,25 angiotensin I–converting enzyme,26 and angiotensin II receptors,27,28 as well as the detection of radioimmunoactivity for angiotensin I and II.29 Uprogulation of left ventricular angiotensinogen30 and angiotensin I–converting enzyme mRNA26 has been described in association with pressure-overload cardiac hypertrophy, suggesting that the cardiac renin-angiotensin system may be activated in cardiac hypertrophy.

In the present study, we investigated the effect of a newly developed angiotensin II receptor antagonist, TCV-116, on murine myocarditis. TCV-116 is a nonpeptide, orally active, noncompetitive antagonist of the AT1 receptor.8 In our model, TCV-116 decreased myocardial cell necrosis and cellular infiltration when the treatment was initiated during the acute stage of myocarditis. The drug also showed a tendency to reduce mortality when treatment was started early after infection.

TCV-116 shows a protective effect at a dose of 3 mg/kg. It is difficult to compare doses directly in different animal species, but on the basis of body surface area, a given dose in mice is similar to a dose that is 12 times lower in humans.31 Thus, a dose of 3 mg/kg in mice is equivalent to a dose of 0.25 mg/kg in humans, which is the dose used for clinical trials of TCV-116 in the treatment of hypertension.

In addition to its hemodynamic effects, Tan et al32 showed that exogenous and endogenous angiotensin II has a cardiotoxic effect without causing systemic hypertension and that its harmful effect is blocked by captopril. A recent study showed that TCV-116 was as effective as an ACE inhibitor for improving albuminuria, renal function, glomerulosclerosis, and survival in spontaneously hypertensive rats with reduced renal mass.23 Angiotensin II is considered to be a major factor in maintaining homeostasis of the circulation as well as water and electrolytes. However, recent studies have shown that angiotensin II has additional functions. Molecular cloning and sequence analysis of the angiotensinogen gene have shown that angiotensinogen is closely related to α-1-antitrypsin, antithrombin III, and α-1-antichymotrypsin, which belong to the same superfamily of serine protease inhibitors.34–36 Production of angiotensinogen in rat liver was reported to increase in response to the induction of acute inflammation.37 Another study showed that interleukin-1 or tumor necrosis factor enhanced angiotensin production by cultured HepG2 cells though transcriptional upregulation of NFκB–like protein, a common transcriptional factor in the response to inflammation and tissue injury.38 These findings suggest that angiotensinogen has an additional role in inflammatory reactions. Thus, elevated plasma angiotensin II levels in infected mice in the present study may have been caused by a combination of the development of heart failure and virus-induced inflammation.

In this model, TCV-116 was considered to act by blocking the cardiotoxicity of the high levels of circulating angiotensin II in the infected mice. Other studies have indicated that angiotensin II has a stimulatory effect on the growth of vascular smooth muscle cells or fibroblasts.39–41 suggesting that it may be involved in tissue remodeling after inflammatory injury.42 Therefore, angiotensin II receptor antagonist therapy may also modify cardiac remodeling after viral inflammation. Further investigation is needed to clarify how TCV-116 influences viral cardiac damage and contributes to cardioprotection.

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