Attenuation of Virus-Induced Myocardial Injury by Inhibition of the Angiotensin II Type 1 Receptor Signal and Decreased Nuclear Factor-Kappa B Activation in Knockout Mice

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OBJECTIVES
This study examined the role of angiotensin II (Ang-II) in a murine model of viral myocarditis.

BACKGROUND
Ang-II plays an important role in the pathophysiology of various cardiovascular disorders. However, the role of Ang-II in inflammatory heart diseases is not known.

METHODS
Four-week-old wild-type (WT) and Ang-II type 1 receptor (AT1R) knockout (KO) mice were inoculated with the encephalomyocarditis virus (EMCV). Survival, histopathology, expression of proinflammatory cytokines, and activity of nuclear factor-kappa B (NF-kB) in the heart were examined.

RESULTS
The 14-day survival was significantly increased in KO compared with WT mice. Histopathologic scores for myocardial necrosis (0.86 ± 0.69 vs. 2.44 ± 0.88, p < 0.01) and cellular infiltration (0.86 ± 0.38 vs. 2.33 ± 0.50, p < 0.01) were lower in KO than in WT mice. The expression of tumor necrosis factor-alpha (TNF-alpha) was increased 43.2-fold, that of interleukin-1-beta (IL-1-beta) 45.8-fold, and the activity of NF-kB 2.24-fold by EMCV inoculation in WT mice (each p < 0.01), but not in KO mice (5.9-fold, 6.3-fold, and 1.12-fold, respectively, each p = NS). The AT1R blocker also significantly attenuated the expression of proinflammatory cytokines and the activation of NF-kB in virus-inoculated WT mice. Intravenous Ang-II injection enhanced the activation of NF-kB (2.28-fold, p < 0.01) and increased the expression of TNF-alpha (2.31-fold, p < 0.01) and IL-1-beta (2.45-fold, p < 0.01) in heart tissue of WT but not KO mice.

CONCLUSIONS
These results indicate that the AT1R signal is obligatory for the development of virus-induced myocardial injury through the proinflammatory action of Ang-II via the NF-kB/cytokine pathway. (J Am Coll Cardiol 2003;42:2000–6) © 2003 by the American College of Cardiology Foundation

Angiotensin II (Ang-II), the main effector peptide of the renin-angiotensin system, plays a central role in the pathophysiology of various cardiovascular and renal disorders, including hypertension, atherosclerosis, myocardial infarction, cardiac hypertrophy, and congestive heart failure (1). Ang-II acts by binding to specific cellular receptors, AT1 and AT2 being the best characterized (2). The AT1 receptors (AT1R) mediate several important cardiovascular responses, such as vasoconstriction, vascular and cardiac remodeling, and cell survival/death. The AT1R blockers are effective in the treatment of various cardiovascular disorders. However, recent reports have found no difference between AT1R knockout (KO) mice and wild-type (WT) mice in the development of hypertrophy by acute pressure overload, as well as neointimal formation by vascular injury (3,4).

Viral myocarditis is one of the main causes of dilated cardiomyopathy and severe congestive heart failure. Angiotensin-converting enzyme inhibitors have been reported to attenuate virus-induced myocardial injury (5,6). In a classic murine model of acute viral myocarditis (7), we found that inoculation of the encephalomyocarditis virus (EMCV) increased the plasma concentrations of Ang-II, and that myocardial lesions were attenuated by angiotensin receptor blockade with candesartan cilexetil (8). However, downstream events responsible for the Ang-II–induced myocardial damage remain to be clarified.

Growing attention is being paid to the regulation and function of transcription factors, such as nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1) during tissue injury (9–11). Ang-II activates various nuclear transcription factors, including the signal transducer and activator transcription factor, AP-1, NF-kB, and cyclic adenosine monophosphate (cAMP) response element–binding (CREB) protein (9–13). Among these, NF-kB is of special interest, as it plays a pivotal role in the control of several genes, including cytokines, chemokines, adhesion molecules, cyclooxygenase-2, and inducible nitric oxide synthase.
Several reports have shown that its inhibition prevents inflammatory responses and attenuates the expression of proinflammatory cytokines (15,16). We have previously reported that pimobendan, a phosphodiesterase inhibitor, protects against viral myocarditis through its inhibition of NF-κB activation (17,18). We have also recently found that myocarditis and inhibits the expression of proinflammatory cytokines (14). In vitro and ex vivo studies have shown that Ang-II induces the expression of cytokines (21–26). However, the influence of Ang-II on the expression of cytokines in the heart has not been described in vivo.

Recent reports have emphasized the important role played by cytokines in the pathophysiology of viral myocarditis. Ang-II is capable of producing inflammatory changes, such as vascular inflammation and chemotaxis of immune cells by signals through its AT1R (10,20). However, the influence of Ang-II on the expression of cytokines in the heart has not been described in vivo.

This study was performed to clarify the role and mechanisms of Ang-II signaling in murine viral myocarditis by inoculation of EMCV to AT1R KO mice.

**METHODS**

**Animal model.** To obtain Ang-II type 1a (AT1a)–deficient heterozygous (AT1a+/−) mice that have a C57BL/6 background, a germ-line chimera derived from TT2 embryonic stem cells with a previously described targeted mutation of the AT1a gene was back-crossed for five generations with C57BL/6 mice. The resulting AT1a+/−F5 mice were intercrossed to generate the homozygous (AT1a−/−) mice. The AT1a−/− mice were then inbred to obtain an appropriate number of animals for the present study. The C57BL/6J strain (obtained from Clea Japan Inc., Tokyo, Japan) was used as WT (AT1a+/+) mice (3,4,27–30). Male mice, 28 days or 7 weeks of age, were housed in a special pathogen-free animal facility at the Kyoto University Hospital before the experiments.

**Experimental viral myocarditis.** Twenty-eight-day-old male KO mice and WT mice were used. The experiments were performed as described previously (5). Briefly, 10 plaque-forming units (pfu) of the myocardiotrophic variant of EMCV were inoculated intraperitoneally on day 0. Survival was measured daily up to 14 days, and the amount of cytokine messenger ribonucleic acid (mRNA) and NF-κB activities in ventricular tissue, harvested on day 5, was measured in surviving mice (n = 10 each).

All experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University.

**Intravenous injection of Ang-II.** Seven-week-old male AT1R KO mice and WT mice were anesthetized with 0.5 mg/g vertin (2,2,2-tribromoethanol). Ang-II, 4 ng/g in 100 μl phosphate-buffered saline (PBS), or 100 μl PBS was injected as a bolus into the jugular vein (31). The mice were euthanized for measurements of intracardiac NF-κB activity and proinflammatory cytokine mRNA at prespecified times after the injection.

**Administration of AT1R blocker.** Candesartan cilexetil (Takeda Chemical Industries, Osaka, Japan), dissolved in 5% arabic gum, in a dose of 10 mg/kg per mouse daily, was administered orally to WT mice, starting on day 0 after EMCV inoculation (8). Control-infected WT mice received the vehicle only. Mice were sacrificed on day 5 for later analyses (n = 9 each).

**Histopathologic examination.** For histopathologic studies, hearts from surviving mice (n = 8 each) were harvested on day 6, fixed in 3.7% formalin, and embedded in paraffin. The ventricle was sliced transversely, stained with hematoxylin-eosin, and examined by light microscopy. The extent of cellular infiltration and myocardial necrosis was graded as follows: 0 = no lesion; 1+ = lesions involving <25% of myocardium; 2+ = lesions involving 25% to 50% of myocardium; 3+ = lesions involving 50% to 75% of myocardium; 4+ = lesions involving >75% of myocardium. The extent of infiltration and necrosis was scored blindly by two independent trained observers, and the scores were averaged. Details of the method have been described previously (17).

**Measurement of cytokine mRNA in heart tissue.** Total RNA was isolated from the ventricles by signals through its AT1R (10,20). In vitro and ex vivo measurements of intracardiac NF-κB activity were performed as described previously (17).

The RNA concentration was measured spectrophotometrically. First-strand complementary deoxyribonucleic acid (cDNA) was synthesized using the Superscript Preamplification System for First-Strand cDNA Synthesis (GIBCO BRL, Gaithersburg, Maryland). From our previous observations in this animal model, a peak in the expression of proinflammatory cytokine mRNA in the heart occurs five to seven days after EMCV inoculation (32). Therefore, we measured proinflammatory cytokine mRNAs in the heart using a real-time quantitative polymerase chain reaction (PCR) method (TaqMan PCR) with the ABI PRISM 7700 Sequence Detection System and TaqMan PCR Core Reagent Kit (Perkin-Elmer Corp., Foster City, California), performed according to the manufacturer’s protocol. One μl of first-strand cDNA was used in the following assay. The following forward (F) and reverse (R) oligonucleotides and
probes (P) were used for the quantification of cytokines and glyceraldehyde dehydrogenase (GAPDH) mRNA. The conditions for TaqMan PCR were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min: primer sequence for mouse interleukin-1-beta (IL-1-beta); F, 5’-CAACCAACAAGTGATATTTCCTCATG-3’; primer sequence for mouse IL-1-beta; R, 5’-GATCCACCTCTCACGCTGA-3’; primer sequence for mouse IL-1-beta; P, 5’-CTGTGATATTGAAAGACGGGACAACCACCAC-3’; primer sequence for mouse tumor necrosis factor-alpha (TNF-alpha); F, 5’-CATCTCTCAGAAAATTCGAGTACAA-3’; primer sequence for mouse TNF-alpha; R, 5’-TGGGAGTAGAAGTTGCAACAAGTGTA-CAACC-3’; primer sequence for mouse TNF-alpha; P, 5’-CACCCTGTAGCAACACCAGTGGGA-3’; GAPDH: F, 5’-TACCGTCTAGCACAACCAAGTGGA-3’; GAPDH: R, 5’-GGCATGGACTGTGGTCATGA-3’; GAPDH: P, 5’-TGCATCCTGACACCACACCGTCTTAG-3’.

The cytokine mRNA was corrected by the amount of GAPDH mRNA.

**Measurements of intramyocardial virus concentrations.** Measurements of virus concentration in heart tissue harvested on day 5 were made as previously described (8). Each value represents the average of two experiments. Virus concentrations are expressed as pfu/g heart tissue.

**Electrophoretic mobility shift assay.** Nuclear factor-kappa B plays an important role in the development of inflammatory disorders. Recently, it has been reported that Ang-II activates NF-kB in vitro and in vivo (10,20). To investigate the mechanism of action of AT1R signaling on inflammatory cytokines, and NF-kB activities were made by electrophoretic mobility shift assay (EMSA).

Nuclear extracts from ventricular tissue samples were prepared using a previously described method (14). A double-stranded 22-base-pair oligonucleotide (5’-AGTTGAGGGGACTTTCCCAGGC-3’) (Promega, Madison, Wisconsin) containing the consensus sequence for NF-kB binding was end-labeled using (gamma-32P)dATP and T4 polynucleotide kinase (Promega) and purified using a G-25 Sephadex column (Pharmacia Biotech, Piscataway, New Jersey). The binding reactions were performed in a final volume of 10 μl containing 8 μg of nuclear protein, 10 mmol/l Tris-HCl (pH 7.5), 50 mmol/l NaCl, 1 mmol/l MgCl2, 0.5 mmol/l EDTA, 0.5 mmol/l dithiothreitol, 4% glycerol (vol./vol.), and 1 μg poly(dI-dC)·poly(dI-dC). After 10-min of pre-incubation at 4°C, the labeled probe (~100,000 cpm/reaction) was added to each reaction mixture and incubated for 20 min at 22°C. The DNA–protein complexes were then separated on 4% non-denaturing polyacrylamide gels in one-half diluted (one-half concentration) tris-EDTA buffer. Gels were vacuum-dried, exposed to Fuji imaging plates (Fuji Photo Film Co., Tokyo, Japan), and analyzed with a Fujix bioimaging analyzer BAS 2000 (Fujix, Tokyo, Japan). For supershift assays, 0.3 μg anti-p65 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California) was added to the reaction mixture immediately after the addition of the radiolabeled probe. For competition assays, 100-fold molar excess of unlabeled NF-kB consensus oligonucleotide was added to the reaction mixture.

**Statistical analysis.** Survival data were analyzed by Kaplan-Meier plots, and survival differences between groups were tested by the Mantel-Cox log-rank test. Statistical comparisons of histologic scores, concentrations of proinflammatory cytokines, and NF-kB activities were made by the unpaired two-tailed Student t test. The standard volume lines were analyzed by simple linear regression. A p value <0.05 was considered statistically significant. Data are expressed as the mean value ± SEM. Each value represents the average of three experiments.

**RESULTS**

**Survival of animals.** The 14-day survival rate in the KO group (35%, n = 20) was significantly higher than that in the WT group (15%, n = 20; p < 0.05) (Fig. 1).

**Histopathologic examinations and heart weight to body weight ratio (HW/BW).** Neither myocardial necrosis nor cellular infiltration was observed in either group with PBS inoculation. In the KO group infected with EMCV, cellular infiltration (0.86 ± 0.38 vs. 2.33 ± 0.50) and myocardial necrosis (0.86 ± 0.69 vs. 2.44 ± 0.88) scores were significantly lower than in the WT group (p < 0.01 each) (Fig. 2). After EMCV inoculation, the HW/BW ratio increased significantly as a result of myocardial inflammation (7,8,18,20,32). In the WT group infected with EMCV, HW/BW increased significantly compared with PBS inoculation (3.582 ± 0.453 vs. 3.240 ± 0.115, p < 0.05), whereas no significant increase was observed in the KO group (3.179 ± 0.356 vs. 2.960 ± 0.238, p = NS).

**Measurement of cytokines in heart tissue.** The inoculation of EMCV increased the expression of TNF-alpha and IL-1-beta significantly in WT (43.2-fold and 45.8-fold, respectively; both p < 0.01), but not in KO mice (5.9-fold and 6.3-fold, respectively; both p = NS) (Fig. 3).
Myocardial virus concentration. On day 5, the mean myocardial virus concentration in the heart of KO mice was significantly higher (0.128 ± 0.034 log [pfu]/g) than that in WT mice (0.092 ± 0.031 log [pfu]/g, p < 0.05), as a result of a weaker inflammatory response in KO than in WT mice after EMCV infection.

Binding activity of NF-kB. Figure 4 shows the activity of NF-kB in heart tissue from KO and WT mice inoculated with EMCV versus saline. The reaction was confirmed to be specific by competition and supershift assay (Fig. 4A). There was no difference in NF-kB activity between the two groups in noninoculated mice. In contrast, EMCV inoculation significantly activated NF-kB in the WT group (2.24-fold, p < 0.01) (Figs. 4B and 4C), but not in KO mice (1.12-fold, p = NS).

Effects of AT1 receptor blocker on viral myocarditis. We then examined whether selective pharmacologic blockade of AT1R with candesartan cilexetil in WT mice mimicked the altered inflammatory response observed in AT1R KO mice. On day 5, the virus-induced activation of NF-kB (0.57-fold, p < 0.05) and the expression of TNF-alpha (0.37-fold, p < 0.01) and IL-1-beta (0.20-fold, p < 0.05) in heart tissue were significantly attenuated by AT1R blocker (ARB) (Fig. 5).

Activation of NF-kB and induction of proinflammatory cytokines by Ang-II in intact heart tissue. Ang-II is capable of producing inflammatory changes by signals through its AT1R. To further clarify the role of AT1R signaling in viral myocarditis, we injected Ang-II intravenously and measured NF-kB activity and mRNA expressions of TNF-alpha and IL-1-beta of the hearts of WT and KO mice. Thirty minutes after the injection of Ang-II, at the peak of NF-kB activity in this model, a significant increase in NF-kB activity was confirmed in WT mice (2.28-fold, p < 0.01) (Figs. 6A and 6B). In contrast, no significant increase was observed in KO mice (1.01-fold, p = NS) after the injection of Ang-II. In addition, at 90 min, significant increases in the mRNA expression of TNF-alpha (2.31-fold, p < 0.01) and IL-1-beta (2.45-fold, p < 0.01) were measured in WT but not KO mice (Figs. 6C and 6D). These results indicate that Ang-II is able to induce a proinflammatory response in intact heart tissue.

DISCUSSION

After the inoculation of EMCV to AT1R KO mice, the inhibition of AT1R signaling attenuated the virus-induced myocardial injury associated with the suppression of NF-kB activation and with the decreased expression of proinflammatory cytokines. We also observed that selective pharmacologic blockade of AT1R by ARB in WT mice mimicked the altered inflammatory response observed in AT1R KO mice. Furthermore, the intravenous injection of Ang-II in WT mice activated NF-kB and induced the expression of proinflammatory cytokines. These observations point to a
deleterious role played by Ang-II in EMCV-induced myocarditis and suggest that the stimulation of AT1R alone is sufficient to activate NF-kB and induce the expression of cytokines in intact cardiac tissue.

Ang-II is capable of producing inflammatory changes mediated by AT1R (20). Overexpression of both angiotensinogen and renin genes, or of the AT1R gene, causes focal necrosis and cellular infiltration in the heart and kidney of transgenic mice, associated with the activation of NF-kB (33,34). The production of reactive oxygen species and the expression of adhesion molecules, chemokine, and other mediators seem to be involved in these processes (33,34). Two of the transcription factors activating the responsible genes are NF-kB and AP-1 (33,34). In heart tissue, Ang-II induces NF-kB in cardiac fibroblasts (35), cardiomyocytes (24,36), and vascular smooth muscle cells (37). Although this study did not precisely identify the effector cells, cardiac fibroblasts seem to be the most likely targets, as our recent in vitro study showed that EMCV activates NF-kB in noncardiomyocytes, but not in cardiomyocytes (19).

The effects of Ang-II stimulation on cytokine expression are not well characterized. In vitro, Ang-II induces the expression of TNF-alpha (21) and IL-6 (22) in cardiac fibroblasts and of TNF-alpha and transforming growth factor-beta in cardiomyocytes (23,25). A decrease in the lipopolysaccharide-induced expression of serum TNF-alpha by angiotensin-converting enzyme inhibition in mice has also been reported (26). Kalra et al. (25) have recently reported that ex vivo Ang-II stimulation activates NF-kB and AP-1 (33,34). In heart tissue, Ang-II induces NF-kB in cardiac fibroblasts (35), cardiomyocytes (24,36), and vascular smooth muscle cells (37). Although this study did not precisely identify the effector cells, cardiac fibroblasts seem to be the most likely targets, as our recent in vitro study showed that EMCV activates NF-kB in noncardiomyocytes, but not in cardiomyocytes (19).

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cytokines in the pathophysiology of viral myocarditis (32,38) and the apparent correlation between the severity of disease evolution and amounts of intracardiac proinflammatory cytokine production (32). Exogenous cytokines or neutralizing antibodies influence many effectors of immune responses in vivo, thereby modulating the course of viral myocarditis (17,39). In our experiments, blockade of AT1R signaling attenuated the virus-induced myocardial injury associated with the suppression of NF-κB activation and with the decreased expression of proinflammatory cytokines. We have also recently found that NF-κB inhibitor prevents the development of EMCV myocarditis and inhibits the expression of proinflammatory cytokines in cardiac tissues (19). These results strongly suggest that blockade of the AT1R/NF-κB/cytokine pathway is therapeutic in viral myocarditis.

Conclusions. Blockade of Ang-II signaling was beneficial in this murine model of EMCV-induced myocarditis. This may be explained by the suppression of the proinflammatory effects of Ang-II via the NF-κB/cytokine pathway.

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References


