Contribution of Endothelin-1 to Myocardial Injury in a Murine Model of Myocarditis

Acute Effects of Bosentan, an Endothelin Receptor Antagonist

Koh Ono, MD, PhD; Akira Matsumori, MD, PhD; Tetsuo Shioi, MD, PhD; Yutaka Furukawa, MD, PhD; Shigetake Sasayama, MD, PhD

Background—Endothelin (ET) is one of the most important contributing factors in the pathophysiology of cardiovascular diseases. However, little is known about its role in myocarditis.

Methods and Results—Four-week-old DBA/2 mice were inoculated with the encephalomyocarditis virus. Expression levels of ET-converting enzyme-1 (ECE-1) and prepro-ET-1 mRNA were significantly increased at 7 and 14 days after virus inoculation. Plasma and myocardial ET-1 levels were significantly higher in infected than noninfected mice between 5 and 14 days after virus inoculation. Immunohistological analyses revealed that not only endothelial cells and myocytes but also infiltrating mononuclear cells produced ET-1 protein at 7 days. Oral bosentan, a mixed ET-1 receptor antagonist, was administered after virus inoculation in doses of 0 (control group), 10, or 100 mg · kg⁻¹ · d⁻¹, and the animals were killed on day 14. Mean heart weight/body weight ratios were 8.3 ± 1.8 versus 11.2 ± 2.4 versus 10.8 ± 2.4 in the bosentan 100 mg · kg⁻¹ · d⁻¹ versus 10 mg · kg⁻¹ · d⁻¹ versus control groups, respectively (P < 0.05). Corresponding histological scores for myocardial necrosis were 2.0 ± 0.2 versus 2.9 ± 0.3 versus 3.0 ± 0.4 (P < 0.05), and cellular infiltration scores were 2.3 ± 0.3 versus 2.9 ± 0.4 versus 3.3 ± 0.4 (P < 0.05). Animals killed on day 5 had significantly smaller necrotic areas after treatment with bosentan 100 mg · kg⁻¹ · d⁻¹ than the group treated with a lower dose or the control group, despite the absence of differences in virus titers.

Conclusions—This study suggests that ET-1 plays an important pathophysiological role in viral myocarditis. Treatment with bosentan had a cardioprotective effect without modifying viral replication. (Circulation. 1999;100:1823-1829.)

Key Words: myocarditis ■ cardiomyopathy ■ endothelin ■ viruses

The concentration of endothelin (ET)-1, a potent vasoconstrictor, is increased in various pathophysiological conditions, such as hypertension, atherosclerosis, myocardial ischemia, and heart failure. Several ET antagonists have been discovered in recent years, which will help elucidate the mechanisms by which ET mediates its effects.

Dilated cardiomyopathy (DCM) is a disease of the heart muscle of unknown cause that progresses to severe heart failure and, in its end stage, can only be remedied by heart transplantation. Several etiological factors have been proposed, viral myocarditis being the most important one. Several ET antagonists have been discovered in recent years, which will help elucidate the mechanisms by which ET mediates its effects.

ET has recently been reported to be produced by macrophages in response to bacterial lipopolysaccharide and human immunodeficiency virus-1 glycoprotein 120. These observations suggest that ET plays a role in inflammatory disorders such as myocarditis.

This study was designed to examine the pathophysiological role of ET-1 and the effects of bosentan, a mixed ETα and ETβ receptor antagonist, in a murine model of myocarditis.

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.

RNA Preparation and cDNA Synthesis

For the RNA preparation, hearts of infected and control mice were removed on days 0, 3, 7, and 14 (n = 4 at each time point). Total RNA was prepared from tissues by the guanidinium isothiocyanate/phenol/chloroform isoamyl alcohol isolation method. One microgram of total RNA template was subjected to first-strand cDNA synthesis in a 40-μL reaction containing 5 OD random hexamer, 1 U ribonuclease inhibitor, 10 mmol/L dNTP, 200 U Moloney murine leukemia virus reverse transcriptase, and first-strand buffer (Gibco/BRL). The reaction mixture was incubated at 37°C for 60 minutes.
heated to 70°C for 5 minutes to denature the reverse transcriptase, then cooled on ice for 3 minutes. Forty milliliters of water was then added to each sample, and the synthesized cDNA was stored at −20°C until use.

Semiquantitative Analysis of Endothelin-Converting Enzyme-1 and prepro-ET-1 mRNA Expression
cDNA stock (2 μL) was amplified by polymerase chain reaction (PCR) for each sample. Each PCR reaction mixture contained 100 μmol/L dNTP, 0.5 μmol/L specific primer, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% gelatin, and 0.25 U Taq polymerase (Cetus) in a 20-μL volume. Oligonucleotide primers for endothelin-converting enzyme-1 (ECE-1), prepro-ET-1, and β-actin gene were purchased from Oligos Etc, Inc. A sense primer and an antisense primer for each were designed to cross introns to avoid confusion between mRNA and genomic DNA. They were synthesized by use of the published cDNA sequences for ECE-1, prepro-ET-1, and β-actin. The actual sequences of the oligonucleotides were as follows: ECE-1, sense: 5'-GCATTGGCACAGCTTGATTGGGG-3' and antisense: 5'-CTTCACCTCCAAGGAGTCTCAG-3'; prepro-ET-1, sense: 5'-CGGTTGCTCGTCTCTTGATG-3' and antisense: 5'-GATGTCAATTGTGGCTTGGCG-3'; β-actin, sense: 5'-ATGGAGTACATGATCTCG-3' and antisense: 5'-ATGGATGATTGTCGTACAGT-3'.

[α-32P]dCTP was included in the reaction to quantify the PCR products. These mRNAs were analyzed by 25 cycles of amplification in a thermal cycler (Cetus) for semiquantification. Each cycle consisted of denaturation at 94°C for 1 minute, annealing at 60°C for 2 minutes, and extension at 72°C for 1 minute. Thirty percent portions of the PCR reaction products were then resolved by electrophoresis on a 4% polyacrylamide gel and examined with a Fujix (Japan) bioimaging analyzer BAS 2000. Representative data showing the amplification of prepro-ET-1 are illustrated in Figure 1. In these experimental conditions, a linear correlation between the amount of cDNA and the yield of PCR products was found.

Plasma and Heart ET-1 Concentration
In this animal model of myocardial infarction, the heart ET-1 concentration tends to remain higher than the plasma level, and tissue ET-1 seems to play an important pathophysiological role. Therefore, we examined the time course of plasma and heart ET-1 concentrations after virus inoculation. Blood was obtained before and 5, 7, and 14 days after virus inoculation, transferred to chilled tubes containing aprotinin (1000 KIU/mL) and Na₂EDTA (1 mg/mL), and immediately centrifuged at 4°C. Plasma samples were stored at −80°C until ET-1 assay. The plasma concentration of ET-1 was measured by use of 100 μL of mouse plasma and a specific sandwich-enzyme immunoassay system. Heart tissues were homogenized with PBS containing 0.02% sodium azide with an ultrasound processor (Astrason, Misonix Inc). The homogenates were centrifuged at 15,000 rpm for 15 minutes, and the supernatants were collected and stored at −80°C until assay.

Immunohistochemical Analysis
Immunohistochemical studies of heart specimens were performed as previously described. The hearts of infected mice were removed on day 7, fixed in 10% neutral buffered formalin, and embedded in paraffin. Sections 2 μm thick were cut, and the slices were deparaffinized with xylene and rehydrated by passage through gradually more diluted ethanol solutions, finishing with water. Endogenous peroxidase was suppressed by treatment with 3% hydrogen peroxide in methanol for 10 minutes. Nonspecific background staining was limited by preincubation with 10% normal goat serum for 30 minutes. The sections were incubated at 37°C for 1 hour with the primary antibody, rabbit anti–ET-1 (IHC6901, Peninsula Laboratories, Inc), diluted to 1:50. Biotinylated goat anti-rabbit IgG (DAKO) diluted to 1:300 was used as the secondary antibody. Incubation with the secondary antibody was carried out at room temperature for 30 minutes. After incubation in the avidin–horseradish peroxidase complex (Vector Laboratories), peroxidase was visualized with 3',3'-diaminobenzidine. Counterstaining was performed with Mayer’s hematoxylin (Wako Pure Chemical Industries). The primary antibody and the absorption test were omitted to prepare the control.

Drug Preparation
Because bosentan is insoluble in water, drug solutions were prepared by suspending the compound in 5% gum arabic.

Experimental Design
Because, in our model, most mice die of congestive heart failure within 14 days after virus inoculation, survival in the present study was measured up to 14 days. The effects of oral bosentan 10 mg · kg⁻¹ · d⁻¹ (n=21) or 100 mg · kg⁻¹ · d⁻¹ (n=21) on survival and histological changes were examined after virus inoculation;
control mice received the vehicle only. The animals were killed on
day 14 with an overdose of pentobarbital sodium (500 mg/kg, IP).

**Histological Examination**

The hearts were fixed in 10% formalin, embedded in paraffin,
sectioned, and stained with hematoxylin and eosin. The extent of
myocardial necrosis and cellular infiltration was graded 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% to 100% of the myocardium. The scores assigned by 2
observers were averaged.

**Virus Titers of Murine Hearts**

To study the effects of bosentan on virus replication, hearts of
infected mice were removed aseptically on day 5, cut in halves along
the short axis, weighed, and homogenized in 2 mL of EMEM. After
centrifugation at 15 000g for 15 minutes at 4°C, 0.1 mL of super-
natant was inoculated into FL cell monolayers for 60 minutes at
37°C in 5% CO₂. Cells were then overlaid with 3 mL of medium
containing 4% FCS and 1% methylcellulose. After 2 days of
incubation at 37°C in a humidified atmosphere containing 5% CO₂,
cells were fixed with acetic acid and methanol (in a ratio of 1:3) and
stained with crystal violet (1%), and plaques were counted with an
inverted microscope. The myocardial virus titer was expressed as
\[ \log_{10} \text{pfu/mg}. \]

**Measurement of the Necrotic Area**

The other halves of the hearts were fixed in 10% formalin, embedded
in paraffin, sectioned, and stained with Masson’s trichrome to study
the histological effects of bosentan on day 5. On a 0.1-mm-square
microgrid, the necrotic area was measured by a blinded observer as
the ratio of the number of microgrid cross points in the necrotic area
to the overall number of cross points on the left ventricle.¹²

**Statistical Analysis**

Values are expressed as mean±SEM. Data were analyzed by 1-way
ANOVA, with multiple comparisons by Fisher’s protected least
significant difference. In all analyses, statistical significance was
declared at a 95% confidence level.

**Results**

**Heart ECE-1 and prepro-ET-1 mRNA Expressions**

To determine whether the ET system is activated in myocar-
ditis, the temporal profile of mRNA expression encoding for
ECE-1 and ET-1 was examined first. Figure 2 shows that both
levels of mRNA expression rose significantly after viral
inoculation. At 7 and 14 days after virus inoculation, ECE-1
mRNA levels had increased 4.6-fold (\( P<0.01 \)) and 4.7-fold
(\( P<0.01 \)), respectively. A significant 3.3-fold increase in
prepro-ET-1 mRNA was already apparent at 3 days
(\( P<0.01 \)). The prepro-ET-1 mRNA peaked at 7 days (6.6-
fold increase, \( P<0.01 \)) and remained increased through day
14 (3.9-fold, \( P<0.01 \)) after virus inoculation.

**Plasma and Heart ET-1 Concentration**

Plasma ET-1 concentration peaked at day 5 (6.3±0.7 pg/mL,
\( P<0.01 \)) and remained elevated until day 14 (4.4±0.5 pg/mL,
\( P<0.05 \)) after virus inoculation (Figure 3, left). Myocardial ET-1
concentration peaked at day 7 (214±22 pg/g tissue, P<0.01) and remained increased until day 14 (266±36 pg/g tissue, P<0.05) after virus inoculation (Figure 3, right). The plasma and myocardial ET-1 concentrations in the noninfected control mice remained unchanged.

**Immunohistochemical Study**

Immunohistochemical studies showed that not only endothelial cells and myocytes but also infiltrating mononuclear cells were positive for ET-1 at 7 days after virus inoculation (Figure 4, top). The study of a negative control is shown in Figure 4, bottom.

**Effects of Treatment With Bosentan**

**Survival**

Twelve of 21 mice (57%) treated with bosentan 100 mg/kg survived, as did 9 of 21 mice (43%) treated with 10 mg/kg. Although the survival of the actively treated mice was higher than in the control group (8 of 21 mice, 38%), the differences were not statistically significant.

**Body and Heart Weight**

Although the mean baseline body weight was comparable among the 3 groups (Figure 5, left), at 14 days it was significantly higher in the mice treated with bosentan 100 mg·kg⁻¹·d⁻¹ than in the other groups. Furthermore, the heart weight/body weight ratio was significantly lower in the mice treated with bosentan 100 mg·kg⁻¹·d⁻¹ than in the other groups (8.3±1.8 versus 11.2±2.4 and 10.8±2.4, P<0.05, all data expressed ×10^3, Figure 5, right).

**Myocardial Histology**

The histological scores for myocardial necrosis were 3.0±0.4 in the control group, 2.9±0.3 in mice treated with bosentan 10 mg·kg⁻¹·d⁻¹, and 2.0±0.2 in mice treated with 100 mg·kg⁻¹·d⁻¹. The respective scores for cellular infiltration were 3.3±0.4, 2.9±0.4, and 2.3±0.3. In mice treated with bosentan 100 mg·kg⁻¹·d⁻¹, the histological score was significantly lower (P<0.05) than in the control group (Figure 6).
Effects of Bosentan on Viral Replication and Necrotic Area on Day 5

The virus titer in the heart of the mice treated with bosentan 100 mg/kg was comparable to that measured in the control mice. However, myocardial necrosis in actively treated animals was significantly less ($P<0.05$) than in the control group (Table).

**Discussion**

This report describes the temporal changes in the expression of ECE-1 and prepro-ET-1 mRNA in murine myocarditis. The stimuli responsible for the increased expression of ECE-1 and prepro-ET-1 in this model remain to be identified. Several factors are known to induce the expression of ET-1 in endothelial cells, including transforming growth factor-$\beta$,$^{13}$ interleukin-1,$^{14}$ tumor necrosis factor (TNF)-$\alpha$,$^{15}$ hypoxia,$^{16}$ and shear stress.$^{17}$ Enhanced expression of ET-1 and ECE-1 has also been reported in activated macrophages,$^{10,18}$ and ECE-1 mRNA expression is upregulated by TNF-$\alpha$.$^{19}$ Because the enhanced expression of cytokines was previously observed in our murine model of myocarditis$^{20}$ and hemodynamic stress may be caused by loss of myocytes, several factors may have been responsible for the induction of ET-1.

Plasma and tissue ET-1 levels were also measured in this study. An increase in circulating ET-1 has been reported in patients with congestive heart failure, and higher concentrations were found in the failing left ventricle than in plasma.$^{4,21}$ In this animal model, extensive inflammation develops in the heart, and myocardial and plasma ET-1 levels increased in parallel with the progression of myocardial injury. The finding of higher ET-1 concentrations in myocardium than in plasma suggests that the heart is a major ET-1–producing organ in myocarditis. Our immunohistochemical studies were performed to identify the cellular origin of ET-1. Although we found ET-1 to be localized in failing cardiac myocytes,$^4$ as observed in previous studies, infiltrating mononuclear cells were also positive for ET-1, a finding specific to this model. However, tissue ET-1 had already risen by day 5 after virus inoculation, when few infiltrating cells were present. Therefore, myocytes and endothelial cells seem to produce ET-1 mostly before cellular infiltration occurs. Because myocardial necrosis with calcification appears as early as day $4$, a contribution of heart failure to the increase in ET-1 concentration cannot be excluded at this early stage.

The recent development of specific ET receptor antagonists allows the study of important physiological and pathophysiological roles of ETs and of their receptors. Studies of ET antagonists in animals have described improvements in functional alterations caused by acute renal failure$^{22}$ and by subarachnoid hemorrhage.$^{23}$ They have also been shown, in the ischemic heart, a reduction in myocardial infarct size.$^{24,25}$ Little information is available, however, regarding the effects of ET-1 receptor antagonists in acute myocarditis. Bosentan, a mixed ET$_{A}$ and ET$_{B}$ receptor antagonist, was chosen for our experiments because of the recent conflicting results reported with the continuous intravenous infusion of BQ-123, which reduced infarct size in dogs,$^{26}$ versus that of FR13937, which had no effects in rabbits.$^{27}$ We hypothesized that mixed ET$_{A}$ and ET$_{B}$ receptor antagonism would be more effective in limiting myocardial injury because both ET$_{A}$ and ET$_{B}$ receptors are present in arterial and venous smooth muscle$^{7}$ and in

Virus Titers and Necrotic Areas in the Heart 5 Days After Virus Inoculation

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Necrotic Area, %</th>
<th>Virus Titer, log pfu/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>17.4±1.9</td>
<td>4.7±0.2</td>
</tr>
<tr>
<td>Bosentan 10 mg · kg$^{-1}$ · d$^{-1}$</td>
<td>8</td>
<td>15.0±2.1</td>
<td>4.7±0.2</td>
</tr>
<tr>
<td>Bosentan 100 mg · kg$^{-1}$ · d$^{-1}$</td>
<td>8</td>
<td>9.3±2.1*</td>
<td>5.0±0.2</td>
</tr>
</tbody>
</table>

* $P<0.05$ vs control.
cardiac tissue and because coronary vasoconstriction via ET₂ receptors was demonstrated. Treatment with bosentan 100 mg · kg⁻¹ · d⁻¹ was associated with lower heart weight/body weight ratio and lower histological scores for myocardial necrosis and cellular infiltration at 14 days after viral inoculation. As previously reported, infected mice do not develop hypertension; therefore, these effects of bosentan are not simply attributable to its blood pressure-lowering effect. The mechanisms of these beneficial effects are unclear, although abnormalities of the coronary microcirculation are present in acute myocarditis, and ET-1-induced vasoconstriction may be a mediator of its pathophysiology.

Despite comparable virus titers in the hearts of the actively treated and control animals, myocardial necrosis was significantly less in the mice treated with bosentan 100 mg · kg⁻¹ · d⁻¹ than in the control group at 5 days after inoculation. Because the concentration of ET-1 had already increased, the beneficial effects of ET-1 receptor blockade appear to take place in the very early stages of infection. Virus-induced necrosis being a specific consequence of viral myocarditis, this reduction in myocardial necrosis is also particular to myocarditis. Previous studies have measured improved survival and hemodynamics by ET antagonism after MI and in congestive heart failure. Our findings suggest that ET antagonism may have salutary effects in myocarditis as well.

The mouse dosages of bosentan chosen in these experiments were based on the observations of equivalence with dosages ≈2-fold and 12-fold lower in rats and humans, respectively, after correction for body surface area. Thus, a 100-mg · kg⁻¹ · d⁻¹ dosage in mice corresponds to 50 mg · kg⁻¹ · d⁻¹ in rats and 8.3 mg · kg⁻¹ · d⁻¹ in humans, a dosage within the range used in previous studies. In our experimental model, ECE-1 mRNA is upregulated. In ischemic renal failure, the ECE-1 inhibitor phosphoramidon exerted greater beneficial effects on renal function and structure than ET₁ receptor antagonism. A comparison of ECE-1 inhibitors versus ET receptor antagonists in the treatment of myocarditis might be of particular interest.

Acknowledgments

This work was supported, in part, by a grant-in-aid for General Scientific Research and for Developmental Scientific Research from the Ministry of Education, Science, and Culture and by a research grant from the Japanese Ministry of Health and Welfare.

References


Contribution of Endothelin-1 to Myocardial Injury in a Murine Model of Myocarditis: Acute Effects of Bosentan, an Endothelin Receptor Antagonist
Koh Ono, Akira Matsumori, Tetsuo Shioi, Yutaka Furukawa and Shigetake Sasayama

_Circulation_. 1999;100:1823-1829
doi: 10.1161/01.CIR.100.17.1823

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/100/17/1823

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/