Carvedilol Decreases Elevated Oxidative Stress in Human Failing Myocardium

Kazufumi Nakamura, MD, PhD; Kengo Kusano, MD, PhD; Yoichi Nakamura, MD; Mikio Kakishta, MD, PhD; Keiko Ohta, MD; Satoshi Nagase, MD; Mika Yamamoto, MD; Katsumasa Miyaji, MD; Hironori Saito, MD; Hiroshi Morita, MD, PhD; Tetsuro Emori, MD; Hiromi Matsubara, MD, PhD; Shinya Toyokuni, MD, PhD; Tohru Ohe, MD, PhD

Background—Oxidative stress has been implicated in the pathogenesis of heart failure. However, direct evidence of oxidative stress generation in the human failing myocardium has not been obtained. Furthermore, the effect of carvedilol, a vasodilating β-blocker with antioxidant activity, on oxidative stress in human failing hearts has not been assessed. This study was therefore designed to determine whether levels of lipid peroxides are elevated in myocardia of patients with dilated cardiomyopathy (DCM) and whether carvedilol reduces the lipid peroxidation level.

Methods and Results—Endomyocardial biopsy samples obtained from 23 patients with DCM and 13 control subjects with normal cardiac function were studied immunohistochemically for the expression of 4-hydroxy-2-nonenal (HNE)-modified protein, which is a major lipid peroxidation product. Expression of HNE-modified protein was found in all myocardial biopsy samples from patients with DCM. Expression was distinct in the cytosol of cardiac myocytes. Myocardial HNE-modified protein levels in patients with DCM were significantly increased compared with the levels in control subjects (P<0.0001). Endomyocardial biopsy samples from 11 patients with DCM were examined before and after treatment (mean, 9±4 months) with carvedilol (5 to 30 mg/d; mean dosage, 22±8 mg/d). After treatment with carvedilol, myocardial HNE-modified protein levels decreased by 40% (P<0.005) along with amelioration of heart failure.

Conclusions—Oxidative stress is elevated in myocardia of patients with heart failure. Administration of carvedilol resulted in a decrease in the oxidative stress level together with amelioration of cardiac function. (Circulation. 2002;105:2867-2871.)

Key Words: cardiomyopathy ■ immunohistochemistry ■ myocardium
Medical treatment consisted of administration of ACE inhibitors (n=18), angiotensin II receptor blockade (n=4), digitalis (n=6), and diuretics (n=17).

Endomyocardial biopsy samples obtained from 13 patients (9 men and 4 women; mean age, 51 ± 12 years) with primary arrhythmia (idiopathic ventricular tachycardia, n=5; idiopathic ventricular fibrillation, n=2; lone atrial fibrillation, n=5; atrial tachycardia, n=1) and with LVEF > 60% but without histological evidence of myocardial inflammation, hypertrophy, or fibrosis were used as control samples in immunohistochemical examination.

Study Protocol
After cardiac catheterization, carvedilol treatment was started at a dosage of 1 mg/d in all patients with DCM. The dosage gradually was increased to a maximum of 5 to 30 mg/d. In 11 patients, cardiac catheterization, including endomyocardial biopsy, was performed during carvedilol treatment. The mean carvedilol treatment period was 9 ± 4 months, and the mean carvedilol dosage was 22 ± 8 mg/d.

To distinguish DCM from myocarditis according to the Dallas criteria,20 we recommended patients with heart failure of unknown pathogenesis to undergo endomyocardial biopsy twice. Therefore, this study was designed as a substudy of diagnosis. We explained to the DCM patients that we would perform biopsy for diagnosis and for this study. Arrhythmia with unknown pathogenesis is sometimes related to myocarditis and early cardiomyopathy. To obtain a definite diagnosis, we performed biopsies in patients with arrhythmia. In the present study, we used biopsy samples with no histological evidence of myocardial inflammation, hypertrophy, or fibrosis as control samples. We explained to the arrhythmia patients that we would perform biopsy for diagnosis and for this study. We explained the risk of endomyocardial biopsy to all patients and obtained their consent. Written informed consent was obtained from all subjects before each investigation. There were no significant complications and no prolonged clinical stays.

Immunohistochemistry
Endomyocardial biopsy samples were fixed in 10% formalin and embedded in paraffin. Each human tissue sample was serially cut into 5-μm-thick sections. Immunoenzymatic staining was performed using a DAKO LSAB System (Dako) according to the manufacturer’s instructions. Briefly, the heart sections embedded in paraffin were deparaffinized in xylene, rehydrated through a graded ethanol series, and then immersed in 12% hydrogen peroxide solution for 20 minutes to block nonspecific reactions. Mouse monoclonal anti-HNE-modified protein antibody (1:50 dilution, NOF Medical Department) was added, and the sections were incubated at 4°C overnight. The sections were then incubated with biotinylated anti-mouse immunoglobulin for 20 minutes and subsequently with horseradish peroxidase labeled streptavidin solution for 20 minutes. The slides were rinsed in cold tris-buffered saline after each step of incubation. Peroxidase activity was visualized with diaminobenzidine tetrahydrochloride solution.

Quantitative Analysis of Stained Areas
Myocardial HNE-modified protein content was measured by a semiquantitative analysis of HNE-modified protein-stained myocardial tissue. Digital images of stained sections were taken with a Fujix Digital Camera HC-300/OL mounted on an Olympus BH-2 microscope. Color images from 5 randomly selected separate high-power fields (×200) in 3 or 4 sections per patient were obtained. Each image covered an area of ~340 × 270 μm². Staining was analyzed using NIH Image 1.56 software. Both intensity level and area were analyzed essentially by the method of Matsuo et al.21 According to the method of Nagahashi et al,22 the results presented in the present report are based on the area of positive staining within the color spectrum for diaminobenzidine of all intensities greater than those found in negative control sections incubated without a primary antibody. All investigators were blinded to the myocardial source in all analyses.
Elevated Levels of HNE-Modified Protein in Myocardia of Patients With DCM
To determine whether oxidative stress is elevated in human failing hearts, we examined levels of HNE-modified protein in myocardia of patients with DCM. Expression of HNE-modified protein was found in all myocardial biopsy samples from patients with DCM. Figure 1B shows a representative immunostaining (brown) for HNE-modified protein in a patient with DCM. Positive immunohistochemical staining for HNE-modified protein was distinct in the cytosol of cardiac myocytes. Table 2 shows the stained areas in the myocardia of all patients. The standard deviation of the stained area in each patient was large. These results show that the hearts exhibit a great deal of heterogeneity in oxidative stress. The mean stained areas were 19,807 ± 1,936 µm² in patients with DCM but 3734 ± 2598 µm² in control subjects (P < 0.0001). These data indicate that levels of oxidative stress were elevated in myocardia of patients with DCM.

Amelioration of Cardiac Function and Diminution of HNE-Modified Protein After Treatment With Carvedilol
Treatment with carvedilol reduced heart rate (P < 0.0001), NYHA class (P < 0.05), and left ventricular end-diastolic diameter (P < 0.005), and it ameliorated LVEF (P < 0.005) (Table 3). In 11 patients, endomyocardial biopsy was performed after treatment with carvedilol (5 to 30 mg/d; mean dosage, 22 ± 8 mg/d). The HNE-positive area decreased by 40% during treatment with carvedilol (18,499 ± 5,124 µm² before and 11,181 ± 5,951 µm² after carvedilol administration, P < 0.005) (Figure 2). Figures 1B and 1D show representative staining patterns in myocardial biopsy samples from the same DCM patient before and 9 months after the beginning of carvedilol treatment. They show that the HNE-positive area decreased. These findings indicate that carvedilol caused reduction in the oxidative stress level in the myocardium together with amelioration of heart failure.

Discussion
Two major new findings were obtained in the present study. First, lipid peroxidation was elevated in myocardia of patients with DCM. There are several reports on elevated circulating levels of lipid peroxides in patients with heart failure. However, to our knowledge, this is the first report of raised levels of lipid peroxides in myocardia of patients with DCM. This finding indicates that oxidative stress is elevated in the human failing myocardium. Second, carvedilol reduced the level of lipid peroxide with amelioration of heart failure. Carvedilol reduced the oxidative stress level in the failing myocardium, although the precise mechanism underlying this effect of carvedilol remains unclear.

HNE is recognized not only as a reliable marker of lipid peroxidation but also as a toxic product to many kinds of cells. HNE exhibits cytopathological effects, such as enzyme inhibition and inhibition of DNA, RNA, and protein synthesis. Administration of HNE causes contractile failure and elicits proarrhythmic effects in hearts. Anti-ischemic agents are useful for catecholamine-induced oxidative stress. Actually, Kukin et al. reported that not only carvedilol but also metoprolol, a β₁-selective blocker, reduced plasma lipid peroxidation in patients with heart failure. Anti-ischemic
properties, including negative chronotropic effects via β receptor, may also be important, because ischemia has been shown to increase HNE formation in the heart.26 Second, the direct antioxidative property of carvedilol may contribute to the reduction of oxidative stress. Carvedilol inhibited Fe2+-initiated lipid peroxidation in vitro, but propranolol did not.17 The mechanism of inhibition is via scavenging free radicals.17

Table 3: Amelioration of Cardiac Function by Carvedilol

<table>
<thead>
<tr>
<th>Control Subjects</th>
<th>DCM Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>HNE, µm²</td>
</tr>
<tr>
<td>1</td>
<td>Idiopathic VT</td>
</tr>
<tr>
<td>2</td>
<td>Idiopathic VF</td>
</tr>
<tr>
<td>3</td>
<td>Idiopathic VT</td>
</tr>
<tr>
<td>4</td>
<td>Idiopathic VT</td>
</tr>
<tr>
<td>5</td>
<td>Idiopathic VF</td>
</tr>
<tr>
<td>6</td>
<td>Idiopathic VT</td>
</tr>
<tr>
<td>7</td>
<td>Lone AF</td>
</tr>
<tr>
<td>8</td>
<td>Lone AF</td>
</tr>
<tr>
<td>9</td>
<td>Lone AF</td>
</tr>
<tr>
<td>10</td>
<td>Lone AF</td>
</tr>
<tr>
<td>11</td>
<td>AT</td>
</tr>
<tr>
<td>12</td>
<td>Lone AF</td>
</tr>
<tr>
<td>13</td>
<td>Idiopathic VT</td>
</tr>
</tbody>
</table>

Mean 3734±2598 Mean 19 807±4736

VT indicates ventricular tachycardia; VF, ventricular fibrillation; AF, atrial fibrillation; and AT, atrial tachycardia.

Table 2: Expression Levels of HNE-Modified Protein

<table>
<thead>
<tr>
<th>Control Subjects</th>
<th>DCM Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>HNE, µm²</td>
</tr>
<tr>
<td>1</td>
<td>Idiopathic VT</td>
</tr>
<tr>
<td>2</td>
<td>Idiopathic VF</td>
</tr>
<tr>
<td>3</td>
<td>Idiopathic VT</td>
</tr>
<tr>
<td>4</td>
<td>Idiopathic VT</td>
</tr>
<tr>
<td>5</td>
<td>Idiopathic VF</td>
</tr>
<tr>
<td>6</td>
<td>Idiopathic VT</td>
</tr>
<tr>
<td>7</td>
<td>Lone AF</td>
</tr>
<tr>
<td>8</td>
<td>Lone AF</td>
</tr>
<tr>
<td>9</td>
<td>Lone AF</td>
</tr>
<tr>
<td>10</td>
<td>Lone AF</td>
</tr>
<tr>
<td>11</td>
<td>AT</td>
</tr>
<tr>
<td>12</td>
<td>Lone AF</td>
</tr>
<tr>
<td>13</td>
<td>Idiopathic VT</td>
</tr>
</tbody>
</table>

Mean 3734±2598 Mean 19 807±4736

VT indicates ventricular tachycardia; VF, ventricular fibrillation; AF, atrial fibrillation; and AT, atrial tachycardia.

Data are given as mean±SD.

*P<0.0001, control subjects vs DCM patients.

Figure 2. Decrease in HNE-positive area attributable to treatment with carvedilol. During treatment with carvedilol, the HNE-positive area decreased by 40% (18 499±5124 µm² after carvedilol administration; P<0.005).

Carvedilol prevented hydroxyl radical–induced cardiac contractile dysfunction in human myocardial tissue, but metoprolol did not.18 These results suggest the possible importance of the use of carvedilol. Third, a recent study has revealed that carvedilol inhibits ROS generation by leukocytes.27 These mechanisms may contribute to the reduction of HNE in human failing hearts. We cannot identify the most important of these direct or indirect antioxidative mechanisms, because we cannot compare results of treatments of patient groups with other β-blockers such as metoprolol to the results of treatment with carvedilol in this study. Additional studies using other β-blockers are needed.

The mean daily dose of carvedilol in this study (22 mg) was lower than the dose in the United States Carvedilol Heart Failure Study28 (45 mg) and that in the Carvedilol Prospective Randomized Cumulative Survival (COPERNICUS)29 trial (37 mg). The dose of carvedilol used in Japan is generally lower than that used in other countries. Up to 20 mg of carvedilol is considered to be tolerable in most Japanese patients with heart failure.30 Carvedilol at a dose of 10 to 20 mg once daily is considered to be an effective and safe treatment for Japanese patients with essential hypertension.31 Therefore, the mean dose in this study was not too low for Japanese patients with heart failure.

Oxidative stress causes a progression of heart failure.2–9 Experimental studies have shown the beneficial effects of antioxidants for prevention of heart failure,5,14,32 However, the effectiveness of antioxidants for human patients with heart failure has not been clarified. Keith et al33 reported that supplementation with vitamin E did not result in any significant improvement in patients with heart failure. In this study, we have reported the reduction of harmful HNE by carvedilol treatment. Not only scavenging free radicals but also inhibition of the oxidative stress source, eg, catecholamines and ischemia, may be useful in treatment of human failing hearts. Inhibition of the other oxidative stress sources, eg, angiotensin II or tumor necrosis factor-α,3 is also therefore expected to be effective in reducing oxidative stress.
Study Limitations

The present study was limited by the absence of a control group of subjects without heart disease and a group of patients treated with other \( \beta \)-blockers such as metoprolol. We decided against the use of a control group for ethical reasons. Instead, biopsy samples obtained from primary arrhythmia patients that showed no histological evidence of myocardial inflammation, hypertrophy, or fibrosis were used as control samples. Because we did not compare the effects of other \( \beta \)-blockers, this study did not show whether the reduction of HNE-modified protein in the myocardium is specific to carvedilol treatment. The effects of various \( \beta \)-blockers are not clear. Additional studies using various \( \beta \)-blockers are therefore needed.

Conclusion

Oxidative stress is elevated in myocardia of patients with heart failure, and administration of carvedilol causes a reduction in the oxidative stress level together with amelioration of cardiac function.

Acknowledgment

The authors thank Kaoru Kobayashi for excellent technical assistance.

References

Carvedilol Decreases Elevated Oxidative Stress in Human Failing Myocardium
Kazufumi Nakamura, Kengo Kusano, Yoichi Nakamura, Mikio Kakishita, Keiko Ohta, Satoshi Nagase, Mika Yamamoto, Katsumasa Miyaji, Hironori Saito, Hiroshi Morita, Tetsuro Emori, Hiromi Matsubara, Shinya Toyokuni and Tohru Ohe

*Circulation*. 2002;105:2867-2871; originally published online May 28, 2002; doi: 10.1161/01.CIR.0000018605.14470.DD
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 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/105/24/2867

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/