

# Carvedilol Decreases Elevated Oxidative Stress in Human Failing Myocardium

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**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  $\beta$ -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,  $9\pm 4$  months) with carvedilol (5 to 30 mg/d; mean dosage,  $22\pm 8$  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

Heart failure is a major and escalating public health problem in many countries. Despite advances in various treatments, the number of patients with chronic heart failure is increasing.<sup>1</sup> Therefore, elucidation of the fundamental mechanisms responsible for the development of heart failure is important.

Recently, several investigators have reported that oxidative stress is implicated in the pathogenesis of heart failure. Experimental studies have revealed that reactive oxygen species (ROS) are produced in the failing myocardium and that ROS cause injury in cardiac myocytes.<sup>2,3</sup> Furthermore, we and other investigators have reported that ROS have important functions in apoptosis and cytokine-stimulated hypertrophy of cardiac myocytes.<sup>4,5</sup> Clinical studies have also revealed that lipid peroxides and 8-iso-prostaglandin F<sub>2</sub> $\alpha$ , which are the major biochemical products of ROS generation, are elevated in plasma and pericardial fluid of patients with

heart failure.<sup>6–9</sup> However, direct evidence of oxidative stress elevation in the human myocardium has not been obtained.

ROS cause damage to lipid cell membranes in the process of lipid peroxidation. In this process, several aldehydes, including 4-hydroxy-2-nonenal (HNE), are generated as final products. HNE disrupts protein function via its facile reactivity with amino acids such as histidine.<sup>10,11</sup> HNE is recognized as the most reliable marker of lipid peroxidation.<sup>10,11</sup> Therefore, to determine whether oxidative stress is elevated in human failing hearts, we examined levels of HNE-modified protein in myocardia of patients with dilated cardiomyopathy (DCM).

Several experimental studies have shown that catecholamine induces oxidative stress in the heart.<sup>12–15</sup> It has been shown that isoproterenol induces lipid peroxidation and that norepinephrine generates hydroxyl free radicals in animal hearts.<sup>12,13</sup> Therefore,  $\beta$ -blocker therapy may prevent oxida-

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**TABLE 1. Clinical Characteristics of 23 Patients With DCM**

Age, y	50±11
Sex, male/female	15/8
Height, cm	163±9
Weight, kg	59±10
NYHA functional class	2.3±0.7
Cardiothoracic ratio, %	58±7
Laboratory data	
WBC per $\mu$ L	5900±1200
CRP, mg/dL	0.3±0.9
BUN, mg/dL	17.1±5.4
Creatinine, mg/dL	0.8±0.4
Echocardiographic data	
LVDd, mm	63±8
LVDs, mm	53±9
FS, %	17±5
Hemodynamic data	
LVEF, %	30±14
CI, L/min per $m^2$	2.5±0.5
PCWP, mm Hg	7±4

WBC indicates white blood cell; CRP, C-reactive protein; BUN, blood urea nitrogen; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; FS, fractional shortening; LVEF, left ventricular ejection fraction; CI, cardiac index; and PCWP, pulmonary capillary wedge pressure.

Data are given as mean±SD.

tive stress in patients with heart failure. Actually, Kukin et al<sup>16</sup> reported that  $\beta$ -blockers such as carvedilol and metoprolol reduced plasma thiobarbituric acid–reactive substances, a marker of lipid peroxidation, in patients with heart failure. Carvedilol is known not only as a vasodilating  $\beta$ -blocker but also as an antioxidant. Carvedilol inhibits  $Fe^{2+}$ -initiated lipid peroxidation and partially prevents hydroxyl radical–induced cardiac contractile dysfunction.<sup>17,18</sup> Carvedilol may reduce the oxidative stress level in the human failing myocardium via such various mechanisms. This study therefore was designed to determine whether carvedilol reduces the levels of HNE-modified protein in myocardia of patients with DCM.

## Methods

### Subjects

The patient population studied comprised 23 consecutive patients (15 men and 8 women; mean age, 50±11 years) admitted to Okayama (Japan) University Hospital between 1998 and 2001 (Table 1). DCM was diagnosed according to the criteria of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force.<sup>19</sup> None of the patients showed any evidence of coronary artery disease, systemic hypertension, valvular heart disease, or pericardial heart disease.

Cardiac catheterization, including coronary angiography, was performed by the Judkins technique in all patients. Endomyocardial biopsy samples (3 or 4 per patient) were obtained from the right ventricles of all patients by the internal jugular approach. The pathological changes were nonspecific but consisted of hypertrophy of myocardial fibers, thickening of the endocardium, and interstitial fibrous replacement.

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.

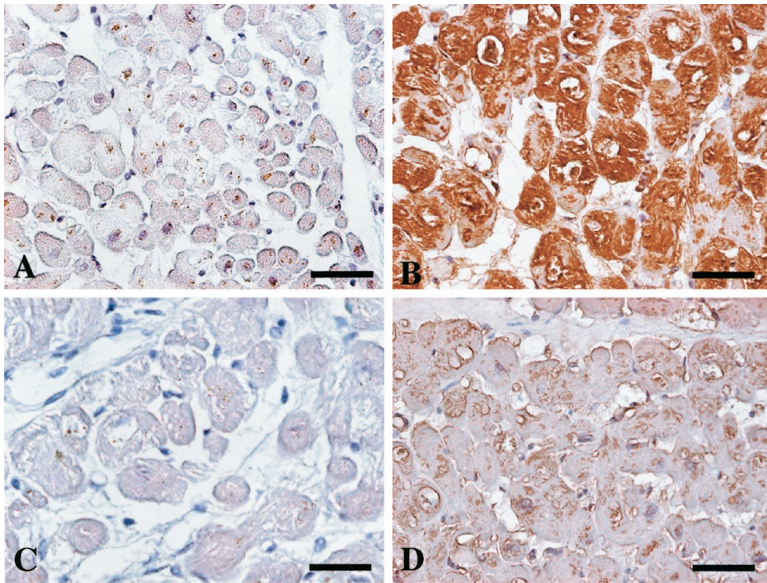
To distinguish DCM from myocarditis according to the Dallas criteria,<sup>20</sup> we recommended patients with heart failure of unknown pathogenesis to undergo endomyocardial biopsy twice. Therefore, this study was designed as a substudy of diagnosis. We explained to the DCM patients that we would perform 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- $\mu$ m–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 preincubated with 1.5% hydrogen peroxide and normal BSA 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 ( $\times 200$ ) in 3 or 4 sections per patient were obtained. Each image covered an area of  $\approx 340 \times 270 \mu m^2$ . Staining was analyzed using NIH Image 1.56 software. Both intensity level and area were analyzed essentially by the method of Matsuo et al.<sup>21</sup> According to the method of Nagueh et al.,<sup>22</sup> 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.



**Figure 1.** Immunohistochemical examination of HNE-modified protein in myocardial biopsy samples. A, Representative immunohistochemical staining in a myocardial biopsy sample from a control subject. There is no positive staining in cardiac myocytes. B, Representative staining (brown) in a myocardial biopsy sample from a DCM patient before carvedilol treatment. Positive staining (brown) for HNE-modified protein was distinct in the cytosol of cardiac myocytes. C, Negative control sections incubated without a primary antibody. D, Representative staining in a myocardial biopsy sample from the same DCM patient 9 months after the beginning of carvedilol treatment. HNE-positive area is diminished. Bar=50  $\mu$ m.

### Statistical Analysis

All data are expressed as mean $\pm$ SD. Statistical significance for comparison between 2 measurements was determined using Student's *t* test. Values of  $P<0.05$  were considered significant.

## Results

### 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  $19\,807\pm4737\ \mu\text{m}^2$  in patients with DCM but  $3734\pm2598\ \mu\text{m}^2$  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 ( $P<0.05$ ) (Table 3). In 11 patients, endomyocardial biopsy was performed after treatment with carvedilol (5 to 30 mg/d; mean dosage,  $22\pm8$  mg/d). The HNE-positive area decreased by 40% during treatment with carvedilol ( $18\,499\pm5124\ \mu\text{m}^2$  before and  $11\,181\pm5951\ \mu\text{m}^2$  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.<sup>6–8</sup> 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.<sup>10,23</sup> Administration of HNE causes contractile failure and elicits proarrhythmic effects in hearts.<sup>24,25</sup> In this study, we found that levels of HNE were raised in failing human hearts. HNE may thus play a critical role in the pathogenesis of heart failure.

We also found that lipid peroxidation in human failing hearts was reduced by administration of carvedilol. This reduction could be caused by several possible mechanisms. First,  $\beta$ -blocking effects of carvedilol may be important. Isoproterenol induces lipid peroxidation and norepinephrine increases hydroxyl free radicals and oxidized glutathione in animal hearts.<sup>12–14</sup> Therefore, adrenergic receptor blockers are useful for catecholamine-induced oxidative stress. Actually, Kukin et al<sup>16</sup> reported that not only carvedilol but also metoprolol, a  $\beta_1$ -selective blocker, reduced plasma lipid peroxidation in patients with heart failure. Anti-ischemic



**TABLE 2. Expression Levels of HNE-Modified Protein**

Control Subjects			DCM Patients	
No.	Clinical Diagnosis	HNE, $\mu\text{m}^2$	No.	HNE, $\mu\text{m}^2$
1	Idiopathic VT	1946 $\pm$ 411	1	20 509 $\pm$ 3375
2	Idiopathic VF	1845 $\pm$ 862	2	19 825 $\pm$ 5223
3	Idiopathic VT	2882 $\pm$ 1843	3	26 472 $\pm$ 5709
4	Idiopathic VT	9416 $\pm$ 4011	4	27 492 $\pm$ 1511
5	Idiopathic VF	6221 $\pm$ 2229	5	22 696 $\pm$ 3241
6	Idiopathic VT	1972 $\pm$ 732	6	20 342 $\pm$ 7651
7	Lone AF	989 $\pm$ 669	7	19 251 $\pm$ 2972
8	Lone AF	2660 $\pm$ 681	8	15 556 $\pm$ 6579
9	Lone AF	4979 $\pm$ 4020	9	27 883 $\pm$ 6496
10	Lone AF	4835 $\pm$ 4035	10	24 710 $\pm$ 4583
11	AT	1237 $\pm$ 650	11	17 600 $\pm$ 5518
12	Lone AF	2324 $\pm$ 400	12	20 850 $\pm$ 2930
13	Idiopathic VT	7237 $\pm$ 4798	13	13 809 $\pm$ 3210
			14	21 176 $\pm$ 4156
			15	18 561 $\pm$ 3882
			16	23 269 $\pm$ 6553
			17	14 069 $\pm$ 3172
			18	13 934 $\pm$ 8749
			19	25 570 $\pm$ 1751
			20	14 710 $\pm$ 8222
			21	10 987 $\pm$ 1824
			22	16 405 $\pm$ 8089
			23	19 880 $\pm$ 4023
Mean		3734 $\pm$ 2598	Mean	19 807 $\pm$ 4736*

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

Data are given as mean $\pm$ SD.

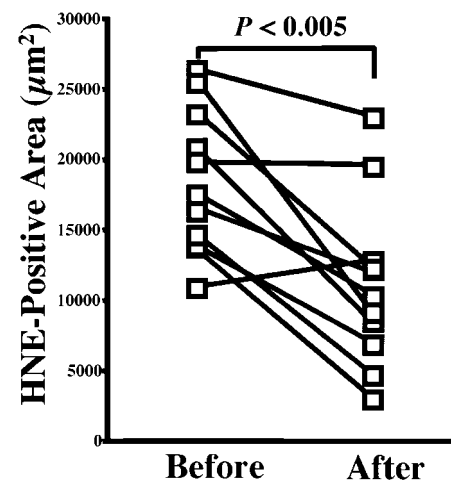
\* $P<0.0001$ , control subjects vs DCM patients.

properties, including negative chronotropic effects via  $\beta$  receptor, may also be important, because ischemia has been shown to increase HNE formation in the heart.<sup>26</sup> Second, the direct antioxidative property of carvedilol may contribute to the reduction of oxidative stress. Carvedilol inhibited  $\text{Fe}^{2+}$ -initiated lipid peroxidation in vitro, but propranolol did not.<sup>17</sup> The mechanism of inhibition is via scavenging free radicals.<sup>17</sup>

**TABLE 3. Amelioration of Cardiac Function by Carvedilol**

	Before	After	P
Systolic BP, mm Hg	127 $\pm$ 19	123 $\pm$ 18	NS
Diastolic BP, mm Hg	77 $\pm$ 13	73 $\pm$ 8	NS
HR per min	81 $\pm$ 13	69 $\pm$ 11	<0.0001
NYHA	2.3 $\pm$ 0.8	1.8 $\pm$ 0.6	<0.05
LVDd, mm	65 $\pm$ 10	61 $\pm$ 10	<0.005
LVDs, mm	55 $\pm$ 10	50 $\pm$ 11	<0.05
LVEF, %	30 $\pm$ 14	38 $\pm$ 10	<0.05
PCWP, mm Hg	7 $\pm$ 5	7 $\pm$ 4	NS

HR indicates heart rate; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; and PCWP, pulmonary capillary wedge pressure. Data are given as mean $\pm$ SD.



**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 $\pm$ 5124  $\mu\text{m}^2$  after carvedilol administration;  $P<0.005$ ).

Carvedilol prevented hydroxyl radical-induced cardiac contractile dysfunction in human myocardial tissue, but metoprolol did not.<sup>18</sup> These results suggest the possible importance of the use of carvedilol. Third, a recent study has revealed that carvedilol inhibits ROS generation by leukocytes.<sup>27</sup> 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  $\beta$ -blockers such as metoprolol to the results of treatment with carvedilol in this study. Additional studies using other  $\beta$ -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 Study<sup>28</sup> (45 mg) and that in the Carvedilol Prospective Randomized Cumulative Survival (COPERNICUS)<sup>29</sup> 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.<sup>30</sup> 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.<sup>31</sup> 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.<sup>2-9</sup> Experimental studies have shown the beneficial effects of antioxidants for prevention of heart failure.<sup>5,14,32</sup> However, the effectiveness of antioxidants for human patients with heart failure has not been clarified. Keith et al<sup>33</sup> 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- $\alpha$ ,<sup>5</sup> 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.

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