Vascular Oxidative Stress and Endothelial Dysfunction in Patients With Chronic Heart Failure

Role of Xanthine-Oxidase and Extracellular Superoxide Dismutase

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Background—Impaired flow-dependent, endothelium-mediated vasodilation (FDD) in patients with chronic heart failure (CHF) results, at least in part, from accelerated degradation of nitric oxide by oxygen radicals. The mechanisms leading to increased vascular radical formation, however, remain unclear. Therefore, we determined endothelium-bound activities of extracellular superoxide dismutase (ecSOD), a major vascular antioxidant enzyme, and xanthine-oxidase, a potent radical producing enzyme, and their relation to FDD in patients with CHF.

Methods and Results—ecSOD and xanthine-oxidase activities, released from endothelium into plasma by heparin bolus injection, were determined in 14 patients with CHF and 10 control subjects. FDD of the radial artery was measured using high-resolution ultrasound and was assessed before and after administration of the antioxidant vitamin C (25 mg/min; IA). In patients with CHF, endothelium-bound ecSOD activity was substantially reduced (5.0±0.7 versus 14.4±2.6 U·mL⁻¹·min⁻¹; \(P<0.01\)) and closely related to FDD (\(r=0.61\)) and inversely related to FDD (\(r=-0.35\)) in patients with CHF. In patients with low ecSOD and high xanthine-oxidase activity, a greater benefit of vitamin C on FDD was observed, ie, the portion of FDD inhibited by radicals correlated negatively with ecSOD (\(r=-0.71\)) but positively with xanthine-oxidase (\(r=0.75\)).

Conclusions—These results demonstrate that both increased xanthine-oxidase and reduced ecSOD activity are closely associated with increased vascular oxidative stress in patients with CHF. This loss of vascular oxidative balance likely represents a novel mechanism contributing to endothelial dysfunction in CHF. (Circulation. 2002;106:3073-3078.)

Key Words: endothelium ■ free radicals ■ heart failure

Patients with chronic heart failure (CHF) are characterized by an increased systemic vasomotor tone and a concomitantly reduced peripheral perfusion. An important role of the endothelium in regulating local vasomotor tone and tissue perfusion has now been recognized. Endothelial dysfunction has been documented in peripheral and coronary arteries in patients with CHF. An important consequence of endothelial dysfunction is the inability of a vessel to dilate in response to physiological stimuli, such as increases in blood flow, reflecting impaired flow-dependent, endothelium-mediated vasodilation (FDD).

The impairment of FDD in patients with CHF is largely the result of a reduced nitric oxide (NO) bioavailability. Short-term and long-term administration of the antioxidant vitamin C improves FDD in patients with CHF as the result of increased NO bioavailability, suggesting that endothelial dysfunction is, at least in part, attributable to accelerated degradation of NO by oxygen radicals. This concept is additionally supported by the experimental observation that treatment with superoxide dismutase can restore endothelium-dependent vasodilation in rats with heart failure. These findings raise the question of what mechanisms lead to increased vascular oxidative stress in patients with CHF.

Recently, extracellular superoxide dismutase (ecSOD) has been reported to be a major form of SOD in the vascular wall and as such represents an important vascular enzymatic antioxidant defense system. Vascular SOD levels are critical for the ability of NO to modulate vascular tone. In the present study, we therefore analyzed endothelium-bound ecSOD activity and its relation to endothelium-dependent vasodilation in patients with CHF.

In addition, there are several potential sources of superoxide within the human arterial wall. Treatment of human internal

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mammary arteries with the xanthine-oxidase inhibitor allopurinol caused a marked reduction of superoxide production, suggesting that xanthine-oxidase represents an important source of superoxide in human vessels. Furthermore, serum levels of uric acid, the product of xanthine-dehydrogenase/xidase, are elevated in patients with CHF. This enzyme is synthesized as xanthine-dehydrogenase using NAD as electron acceptor but can be readily converted to xanthine-oxidase that uses molecular oxygen as preferred electron acceptor. Accordingly, we analyzed endothelium-bound xanthine-oxidase activity and its relation to endothelium-dependent vasodilation in patients with CHF. Furthermore, the relationship between endothelium-bound xanthine-oxidase activity and the portion of FDD inhibited by oxygen free radicals (ie, recovered by the antioxidant vitamin C) was determined in patients with CHF.

### Methods

Fourteen patients with New York Heart Association functional class III chronic heart failure attributable to idiopathic dilated cardiomyopathy and 10 age-matched control subjects were studied (Table). All patients were treated with digitalis, angiotensin-converting enzyme inhibitors, and diuretics. Seven patients were also on ?-blocker therapy. Patients with diabetes mellitus, current tobacco use, significant valvular heart disease, heparin therapy within the last 24 hours, or any condition that would preclude safely withholding vasoactive medication were excluded from the study. Control subjects had no cardiovascular risk factors (history, physical examination, or laboratory analysis). Written informed consent was obtained for all subjects. The local ethics committee approved the protocol.

#### Measurement of Endothelium-Bound ecSOD Activity In Vivo

ecSOD is rapidly released from the endothelium into plasma by heparin bolus injection, allowing determination of endothelium-bound ecSOD activity in humans in vivo. Plasma Cu,Zn, and Mn-SOD are not affected by heparin. For measurement of endothelium-bound ecSOD, 2 venous blood samples (antecubital vein) were obtained at baseline. Five thousand units of heparin were then injected into the brachial artery of the same arm (nondominant arm), and blood samples were obtained in time intervals from the antecubital vein (1, 3, 5, 7, 10, 15, and 20 minutes after heparin injection) as described in detail previously. Tubes were immediately centrifuged (2000g, 15 minutes, 4°C), and plasma was stored at −80°C.

Activity of SOD in plasma was measured at pH 8.2 by a modified nitrite method. Superoxide generated by hypoxanthine and xanthine-oxidase was changed to nitrite ion by hydroxylamine. Nitrite ion was measured by color densitometry at 550 nm using a coloring reagent. The amount of SOD required to inhibit the rate of nitrite ion generation by 50% was defined as 1 unit of SOD activity, according to McCord and Fridovich. SOD measurements were performed at multiple time points after heparin bolus injection to exclude a significant interference of SOD activity released from lysed erythrocytes. Endothelium-bound ecSOD activity was calculated as area under the curve of the increase of plasma SOD activity after heparin bolus injection (Figure 1). Reagents were from Sigma-Aldrich.

#### Measurement of Endothelium-Bound Xanthine-Oxidase Activity by Electron Spin Resonance Spectroscopy

Xanthine-oxidase, bound to glycosaminoglycans on the endothelial cell surface, is rapidly released into plasma after heparin bolus injection, allowing determination of endothelium-bound xanthine-oxidase activity in vivo. For measurements of endothelium-bound xanthine-oxidase activity, blood samples were obtained at baseline and 5 minutes after heparin bolus injection (5000 U), as described above. Endothelium-bound xanthine-oxidase activity was calculated as difference between plasma xanthine-oxidase activity after heparin injection and at baseline. In preliminary studies, we found that maximal increase of xanthine-oxidase activity was reached 5 minutes after heparin bolus injection in patients with CHF (data not shown). Activity of xanthine-oxidase in plasma samples was determined by electron spin resonance (ESR) spectroscopy using the spin trap 1-hydroxy-3-carboxy-pyrrolidine (CP-H). ESR measurements were performed at room temperature using an EMX ESR spectrometer (Bruker BioSpin Corporation). ESR spectrometer settings were as follows: field center, 3497 G; field sweep width, 110 G; microwave frequency, 9.82 GHz; microwave power, 20 mW; magnetic field modulation frequency, 100 kHz; modulation amplitude, 2 G; conversion time, 164 ms; detector time constant, 328 ms. Plasma samples were added to sodium phosphate buffer (50 mmol/L; pH 7.4) containing 2 mmol/L DTPA to decrease autoxidation of spin trap by transition metal ions. Xanthine (100 µmol/L) and the spin trap CP-H (5 mmol/L; Alexis Corporation; San Diego) were added. ESR spectra were recorded in 50-µL glass capillaries. Superoxide formation was determined by following the oxidation of CP-H to paramagnetic 3-carboxy-proxyl (CP·) The intensity of ESR spectra was quantified after subtraction of the ESR signal of plasma samples without xanthine (obtained for each sample). The xanthine-driven ESR signal in plasma samples was completely inhibited by oxyphenyl (1 mmol/L) or superoxide dismutase (50 U).

### Characteristics of Patients With CHF and Control Subjects

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<thead>
<tr>
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<th>Controls</th>
<th>CHF</th>
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<tr>
<td><strong>(n=10)</strong></td>
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<tr>
<td><strong>(n=14)</strong></td>
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<tr>
<td>Age, y</td>
<td>48±4</td>
<td>51±3</td>
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<tr>
<td>Male/female</td>
<td>9/1</td>
<td>10/4</td>
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<tr>
<td>Mean blood pressure, mm Hg</td>
<td>90±2</td>
<td>93±4</td>
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<tr>
<td>Left ventricular ejection fraction, %</td>
<td>61±1</td>
<td>25±2*</td>
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<tr>
<td>LDL cholesterol, mg/dL</td>
<td>120±3</td>
<td>129±10</td>
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<tr>
<td>Creatinine, mg/dL</td>
<td>0.9±0.1</td>
<td>1.0±0.1</td>
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<tr>
<td>Body weight, kg</td>
<td>78±2</td>
<td>84±5</td>
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<tr>
<td>Height, cm</td>
<td>176±2</td>
<td>174±3</td>
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*P<0.01 vs control subjects.
Measurement of FDD
Radial artery diameters were measured using a high-resolution ultrasound system (ASULAB). This method is well established in our laboratory, has an excellent reproducibility and variability, and was used as described in detail previously. Vasoactive medications were withheld and alcohol and caffeine were prohibited for at least 12 hours before the study.

Blood flow velocity was recorded continuously, and radial artery diameter was determined every 30 seconds until stable baseline conditions were obtained (~30 minutes). Then a wrist arterial occlusion (8 minutes) was performed and FDD in response to reactive hyperemic blood flow was assessed. When radial artery diameter and blood flow had returned to baseline values, the antioxidant vitamin C was infused (25 mg/min; 10 minutes; brachial artery) followed by determination of FDD.

Statistical Analysis
All data are expressed as mean±SEM. To compare data between different groups, ANOVA was used; to compare repeated measurements within one group of patients, a one-way ANOVA for repeated measures was performed followed by Student-Newman Keuls test. Linear regression analysis was used to analyze the relation between endothelium-bound ecSOD and xanthine-oxidase activity and FDD. A value of $P<0.05$ was considered to be statistically significant.

Results
Endothelium-Bound ecSOD Activity in Patients With CHF and Controls
Endothelium-bound ecSOD activity was markedly reduced in patients with CHF compared with controls (CHF versus control subjects, 5.0±0.7 versus 14.4±2.6 U·mL$^{-1}$·min$^{-1}$; $P<0.01$; Figure 1A). The increase of ecSOD activity in plasma after heparin bolus injection was significantly decreased in patients with CHF at all time points studied (Figure 1B). Baseline plasma ecSOD activity was similar in patients with CHF and controls (data not shown).

Endothelium-Bound Xanthine-Oxidase Activity in Patients With CHF and Controls
Endothelium-bound xanthine-oxidase activity as determined by ESR spectroscopy was increased by $>200\%$ in patients with CHF compared with controls (CHF versus control subjects, 38±10 versus 12±4 nmol O$_2^-$·μL$^{-1}$; $P<0.05$; Figure 2A). A representative ESR spectrum demonstrating xanthine-oxidase activity in plasma after heparin bolus injection (5000 U) in a patient with CHF compared with a control subject is shown in Figure 2B. Xanthine-dependent O$_2^-$ formation was completely inhibited by oxypurinol (1 mmol/L) or superoxide dismutase (50 U) (data not shown). Plasma xanthine-oxidase activity at baseline was higher in patients with CHF compared with control subjects (CHF versus control subjects, 12±3 versus 6±2 nmol O$_2^-$·μL$^{-1}$; $P<0.05$). In some baseline plasma samples of control subjects, we found no detectable xanthine-oxidase activity.

Flow-Dependent, Endothelium-Mediated Vasodilation
FDD, defined as percent increase in vessel diameter after wrist occlusion, was reduced in patients with CHF compared with controls (Figure 3). A significant improvement of FDD was observed in patients with CHF after intraarterial infusion of the antioxidant vitamin C, but not in controls (Figure 3).
Forearm blood flow at rest (CHF versus controls, 26±7 versus 29±4 mL/min⁻¹) and at maximal reactive hyperemia (91±10 versus 89±5 mL/min⁻¹) was similar in patients with CHF and controls. Systemic blood pressure and heart rate did not change during the experimental protocol (data not shown).

Relation of ecSOD Activity to FDD in Patients With CHF

In patients with CHF, there was a close positive relation between ecSOD activity and FDD \((r=0.61; P<0.05\); Figure 4A). Furthermore, ecSOD activity was negatively related to the portion of FDD inhibited by radicals; ie, the effect of the antioxidant vitamin C on FDD in patients with CHF \((r=-0.71; P<0.05\); Figure 4B).

Relation of Xanthine-Oxidase Activity to FDD in Patients With CHF

Endothelium-bound xanthine-oxidase activity was inversely related to FDD in patients with CHF \((r=-0.35; P<0.05\); Figure 5A). There was a close positive relation between xanthine-oxidase activity and the effect of the antioxidant vitamin C on FDD in patients with CHF \((r=0.75; P<0.05\); Figure 5B).

Discussion

The major findings of the present study are as follows. First, the activity of extracellular superoxide dismutase, a major vascular antioxidant enzyme, bound to the endothelium, is severely reduced in patients with CHF. Furthermore, decreased ecSOD activity is closely related to impairment of endothelium-dependent vasodilation in patients with CHF, suggesting that reduced ecSOD activity contributes to endothelial dysfunction. Second, the activity of the radical-producing enzyme xanthine-oxidase, bound to the endothelium, is increased by >200% in patients with CHF and is inversely related to endothelium-dependent vasodilation. Third, the beneficial effect of the antioxidant vitamin C on endothelium-dependent vasodilation is greater in patients with low ecSOD activity and high xanthine-oxidase activity, suggesting that both reduced ecSOD and increased xanthine oxidase activity contribute to increased vascular oxidative stress in patients with CHF. This impairment of vascular oxidative balance likely represents a novel mechanism contributing to endothelial dysfunction in patients with CHF.

Recent clinical studies have documented endothelial dysfunction in peripheral and coronary arteries of patients with CHF.3–6 Accumulating evidence suggests that endothelial dysfunction contributes to exercise intolerance, impaired myocardial perfusion, and left ventricular remodeling in CHF.31–35 Of note, short-term and long-term treatment with a high dose of the antioxidant vitamin C restored endothelium-dependent, NO-mediated vasodilation in patients with CHF, suggesting that accelerated degradation of NO by oxygen radicals contributes to endothelial dysfunction.8,36 This concept is additionally supported by the experimental observation that treatment with superoxide dismutase (SOD) restored endothelium-dependent vasodilation in rats with heart failure.9

In the present study, we observed a marked reduction of endothelium-bound ecSOD activity, a major antioxidant defense system of the arterial wall,10 in patients with CHF. Human arteries contain exceptionally large amounts of ecSOD that are ∼100 times higher compared with other tissues, such as skeletal muscle or fat tissue, suggesting a special function of this enzyme within the arterial wall.10,37 Furthermore, decreased ecSOD activity was closely related to impairment of endothelium-
dependent vasodilation in patients with CHF, compatible with the concept that reduced ecSOD activity contributes to endothelial dysfunction. Although the observed correlation does not prove a cause and effect relationship, there is evidence to support this concept. Vascular-bound ecSOD has been shown to have a high efficiency in protecting NO bioactivity against inhibitory effects of superoxide. Furthermore, inhibition of vascular SOD activity by diethyl-dithiocarbamate almost completely abolishes endothelium-dependent vasodilation in bovine coronary arteries and rabbit aortas, suggesting that vascular SOD levels are crucial for the ability of NO to modulate vascular tone. This concept is additionally supported by the observation that chronic inhibition of vascular SOD activity by dietary copper restriction resulted in impaired endothelium-dependent vasodilation attributable to increased inactivation of NO.

The present study was not designed to determine mechanisms leading to reduced endothelium-bound ecSOD activity in patients with CHF. Several recent experimental studies, however, have focused on regulation of ecSOD expression in vascular smooth muscle cells (VSMCs), the likely source of endothelium-bound ecSOD, because endothelial cells do not express this enzyme. It was shown that exposure of VSMCs to tumor necrosis factor (TNF)-α resulted in a marked and progressive downregulation of ecSOD expression. This observation is relevant to the present study, because elevated circulating levels of TNF-α have been documented in patients with CHF. In addition, it has recently been shown that NO potently induces ecSOD expression in VSMCs, whereas lack of endothelial NO production reduces vascular ecSOD expression. Therefore, reduced vascular NO availability may augment reduction of vascular ecSOD activity in patients with CHF.

The present study additionally demonstrates a substantial increase of endothelium-bound xanthine-oxidase activity, a potent radical forming enzyme, in patients with CHF. This observation is in line with the recent finding that serum levels of uric acid, the product of xanthine-dehydrogenase/xidase, are elevated in patients with CHF. This enzyme is present in two forms in mammals. It is synthesized as xanthine-dehydrogenase, which uses NAD⁺ as an electron acceptor. A variety of stimuli, including cysteine oxidation and proteolytic cleavage, results in conversion of xanthine-dehydrogenase to xanthine-oxidase, which uses molecular oxygen as an electron acceptor, resulting in superoxide formation. Of note, rapid conversion of xanthine-dehydrogenase to its oxidase form has been demonstrated after exposure of endothelial cells to cytokines, such as TNF-α, that may contribute to endothelial xanthine-oxidase activation in patients with CHF. Furthermore, experimental studies suggest that chronic activation of the renin-angiotensin system may contribute to vascular xanthine-oxidase activation, because inhibition of xanthine-oxidase improved endothelium-dependent vasodilation in renin/angiotensinogen overexpressing rats. Patients with CHF are characterized by a chronically activated renin-angiotensin system that may contribute to activation of endothelium-bound xanthine-oxidase activity. In addition, several recent studies have suggested that circulating xanthine-oxidase may represent a source of increased endothelium-bound xanthine-oxidase activity besides increased local expression and activation of the enzyme. It is conceivable that this plays a role in patients with CHF, because we observed an increased baseline circulating xanthine-oxidase activity in these patients.

Houston et al have recently demonstrated by using an in vitro model that increased xanthine-oxidase binding to endothelial cells causes a marked reduction of NO bioactivity. In line with this concept, we observed an inverse relation between endothelium-bound xanthine-oxidase activity and FDD in patients with CHF, suggesting that increased xanthine-oxidase activity contributes to endothelial dysfunction in patients with CHF. This concept is additionally supported by the recent observation of Doehner et al that treatment with allopurinol, a xanthine-oxidase/dehydrogenase inhibitor, had a beneficial effect on endothelium-dependent vasodilation in patients with CHF. In addition, the observation of the present study that endothelium-bound xanthine-oxidase activity is closely related to the effect of vitamin C on NO-mediated vasodilation suggests that increased oxygen radical production by xanthine oxidase contributes to increased inactivation of NO in patients with CHF.

Of note in this respect, several recent studies have found increased myocardial xanthine-oxidase levels in patients with CHF and in experimental heart failure. Furthermore, xanthine-oxidase inhibition with allopurinol lowered myocardial oxygen consumption and improved myocardial efficiency both in experimental heart failure and in patients with CHF, suggesting that xanthine-oxidase activation may have detrimental myocardial effects in heart failure.

With respect to therapeutic considerations, it is of note that several recent clinical trials have failed to demonstrate a beneficial effect of vitamin supplementation (mostly vitamin E) on cardiovascular events in patients with coronary disease. As has been pointed out recently, however, antioxidants such as vitamin E become a radical (ie, tocopheroxyl radical) after scavenging a radical and may under certain circumstances even enhance oxidative processes. Therefore, inhibition of relevant vascular radical producing enzymes, such as xanthine-oxidase inhibition by allopurinol, may represent a more effective and promising approach to truly reduce vascular oxidative events in patients compared with the approach using oral vitamins to scavenge radicals.

In summary, the present study demonstrates that both decreased ecSOD activity and increased xanthine-oxidase activity bound to the endothelium are closely associated with increased vascular oxidative stress in patients with CHF. This loss of vascular oxidative balance likely represents a novel mechanism underlying endothelial dysfunction in patients with CHF.

References
Vascular Oxidative Stress and Endothelial Dysfunction in Patients With Chronic Heart Failure: Role of Xanthine-Oxidase and Extracellular Superoxide Dismutase
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