Increased superoxide production causes coronary endothelial dysfunction and depressed oxygen consumption in the failing heart

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Chen, Yingjie, Mingxiao Hou, Yunfang Li, Jay H. Traverse, Ping Zhang, Daniela Salvemini, Tohru Fukai, and Robert J. Bache. Increased superoxide production causes coronary endothelial dysfunction and depressed oxygen consumption in the failing heart. Am J Physiol Heart Circ Physiol 288: H133–H141, 2005; doi: 10.1152/ajpheart.00851.2003.—This study examined whether increased superoxide (O2−·) production contributes to coronary endothelial dysfunction and depressed coronary blood flow (CBF) in congestive heart failure (CHF). To test this hypothesis, the effects of the low-molecular-weight SOD mimetic M40401 on CBF and myocardial oxygen consumption (MVO2) were examined in dogs during normal conditions and after CHF was produced by 4 wk of rapid ventricular pacing. The development of CHF was associated with decreases of left ventricular (LV) systolic pressure, maximum first derivative of LV pressure, MVO2, and CBF at rest and during treadmill exercise as well as endothelial dysfunction with impaired vasodilation in response to intracoronary acetylcholine. M40401 increased CBF (18 ± 5%, P < 0.01) and MVO2 (14 ± 6%, P < 0.01) in CHF dogs and almost totally reversed the impaired CBF response to acetylcholine. M40401 had no effect on acetylcholine-induced coronary vasodilation, CBF, or MVO2 in normal dogs. Western blot analysis demonstrated that extracellular SOD (EC-SOD) was significantly decreased in CHF hearts, whereas mitochondrial Mn-containing SOD was increased. Cytosolic Cu/Zn-containing SOD was unchanged. Both increased O2−· production and decreased vascular O2−· scavenging ability by EC-SOD could have contributed to endothelial dysfunction in the failing hearts.

free radicals; myocardium; nitric oxide

CONGESTIVE HEART FAILURE (CHF) is associated with increased oxidative stress (11, 19). Several investigators have reported that superoxide (O2−·) production is increased both in myocardial mitochondria (19) and in coronary arteries (3, 4, 42) from failing hearts. In physiological circumstances, superoxide (O2−·) can function as a messenger intermediate in signal transduction (22), but high concentrations of O2−· can result in cell damage and tissue injury. O2−· reacts avidly with nitric oxide (NO) to form peroxynitrite (ONOO−), a strong oxidant and nitrating species known to promote oxidative damage (5). In low concentrations, however, ONOO− may play a regulatory role in mitochondrial physiology (5, 16). Because O2−· has low membrane permeability, reactions with this molecule occur mainly in the compartment in which it is generated. Therefore, O2−· produced in vessels can react locally with endothelium-derived NO, thereby decreasing NO bioavailability and contributing to the endothelial dysfunction seen in CHF.

(3, 4). O2−· produced in mitochondria can react with NO to form ONOO−, which may have the potential to alter mitochondrial respiration both directly by inactivation of mitochondrial complexes I, II, and V and by removing the inhibitory effect of NO on cytochrome-c oxidase (5, 8, 16, 31). Thus increased O2−· production has the potential to alter both vascular and mitochondrial function in the failing heart.

M40401 is a new synthetic low-molecular-weight S2−/S2−-dimethyl-substituted bis(cyclohexylpyridine) manganese-based SOD mimetic with high tissue permeability that is stable in vivo, possesses high activity (at pH 7.4, >1 × 109 M−1 s−1) comparable to the native Cu/Zn-containing SOD (Cu/Zn-SOD) enzyme, and is selective for O2−· with no activity toward H2O2, ONOO−, NO, or OCl− (34). This novel selectivity resides in the nature of the manganese center. The resting redox state of M40401 is the reduced state, Mn(II); as a consequence, the complex has no reactivity for reducing agents until it is oxidized to Mn(III) by O2−· (9, 34). Moreover, M40401 is relatively difficult to oxidize [+0.75 v (standard hydrogen electrode)], so that many oxidants including NO and oxygen will not oxidize the complex (34). Because M40401 operates via a facile one-electron oxidation pathway, two-electron non-radical oxidants are also not able to oxidize the Mn(II) complex, e.g., ONOO−, OCl−. The unique selectivity of this agent makes it possible to dissect the role of O2−· in the presence of other reactive oxygen species (ROS).

We hypothesized that increased endothelial O2−· production contributes to endothelial dysfunction in the failing heart, so that scavenging O2−· would increase NO bioavailability in the coronary vessels, thereby enhancing endothelium-dependent vasodilation. In addition, we hypothesized that increased O2−· production by myocardial mitochondria impairs respiration in the failing heart, so that scavenging O2−· with M40401 would cause an increase of oxygen uptake. The myocardial protein content of Cu/Zn-SOD, mitochondrial Mn-containing SOD (Mn-SOD), and extracellular SOD (EC-SOD) were also measured to determine whether a decrease of these enzymes might contribute to increased oxidative stress in the failing heart.

METHODS

Studies were performed in 12 adult mongrel dogs, weighing 20–26 kg, trained to run on a treadmill. All experiments were performed in accordance with the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society, with prior approval of the University of Minnesota Animal Care Committee.

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Surgical preparation. Animals were anesthetized with pentobarbital sodium (25–30 mg/kg), intubated, and ventilated with 1–2% isoflurane. A left thoracotomy was performed, and polyvinyl chloride catheters (3.0-mm outer diameter) were inserted into the ascending aorta and the left ventricle (LV). A solid-state micromanometer (Konigsberg, Pasadena, CA) was introduced into the LV. A final catheter was introduced through the right atrial appendage into the coronary sinus to the origin of the anterior interventricular vein to allow selective sampling of blood draining the myocardium perfused by the left anterior descending coronary artery (LAD). A Doppler flow probe (Craig Hartley, Houston, TX) was positioned on the LAD for measurement of coronary blood flow (CBF), and a silicone microcatheter (0.3-mm inner diameter) was introduced into the LAD. Catheters were flushed daily to maintain patency.

Effect of M40401 in normal heart. The SOD mimetic M40401 was studied in seven normal dogs 10–14 days after surgery. Resting hemodynamics were recorded, and 2 ml of blood were withdrawn from the aortic and coronary venous catheters for blood gas analysis. Subsequently, a three-stage treadmill exercise protocol was begun (stage 1: 3.2 km/h at 0% grade; stage 2: 6.4 km/h at 0% grade; stage 3: 6.4 km/h at 5% grade). Each exercise stage was 3 min in duration; aortic and coronary venous blood samples were withdrawn during the last 30 s of each exercise stage. After a 10-min rest period, M40401 (1.5 mg/kg iv) was infused over 10 min. Forty minutes after M40401, all measurements were repeated at rest and during exercise.

Coronary endothelium-dependent vasodilation in normal dogs. The effects of M40401 and the NO synthase (NOS) inhibitor N\textsuperscript{G}-nitro-L-arginine (L-NNA) were examined in five normal dogs. The increases in CBF produced by intracoronary acetylcholine (3.75–75 μg/min) were observed under control conditions, after M40401 (1.5 mg/kg intracoronary), and after the addition of L-NNA (1.5 mg/kg iv).

Production of CHF. CHF was produced by rapid ventricular pacing (7, 9). After completion of studies during normal conditions, the pacemaker was activated at 230 beats/min; pacing was continued at this rate or adjusted upward to a maximum of 250 beats/min based on weekly assessments of hemodynamics obtained 30 min after deactivating the pacemaker. CHF was deemed to have developed when resting LV end-diastolic pressure (LVEDP) was >20 mmHg.

Effect of M40401 in CHF dogs. The effect of M40401 was examined in eight CHF dogs during sinus rhythm beginning 30 min later rest and during two stages of treadmill exercise; animals with CHF had exercise intolerance and were unable to perform the third exercise stage. M40401 (1.5 mg/kg iv) was then infused over 10 min, and 40 min later rest and exercise measurements were repeated. In three additional animals, the effect of a higher dose of M40401 was examined. In these three animals, resting studies were performed as described above during control conditions, after administration of M40401 (1.5 mg/kg iv), and then after administration of an additional dose of M40401 of 3.5 mg/kg intravenously (total dose 5 mg/kg), and all measurements were repeated.

Effect of L-NNA in CHF dogs. To study the effect of NOS blockade in dogs with CHF, L-NNA (1.5 mg/kg intracoronary) was administered to six dogs that previously had received M40401 and all measurements were repeated 40 min later.

Coronary endothelium-dependent vasodilation in CHF dogs. In seven CHF dogs, the increases in CBF produced by intracoronary acetylcholine (3.75–75 μg/min) were observed before and after M40401 (1.5 mg/kg iv). In three animals, dose–response curves to intracoronary acetylcholine were performed after M40401 in an intravenous dose of 1.5 mg/kg and after administration of an additional intravenous dose of M40401 of 3.5 mg/kg (total dose 5 mg/kg). In five animals, the coronary flow responses to acetylcholine were examined after the addition of L-NNA (1.5 mg/kg intracoronary) after M40401.

Experimental measurements. LV pressure was measured with the micromanometer; the first derivative of LV pressure (LV dp/dt) was obtained via electrical differentiation. PO\textsubscript{2}, Pco\textsubscript{2}, and pH were measured with a blood gas analyzer (Instrumentation Laboratory, Lexington, MA). Hemoglobin oxygen saturation was calculated from the blood PO\textsubscript{2}, pH, and temperature (40). Blood O\textsubscript{2} content was computed as (hemoglobin × 1.34 × %O\textsubscript{2} saturation) + (0.0031 × P\textsubscript{O\textsubscript{2}}). Myocardial oxygen consumption (MV\textsubscript{O\textsubscript{2}}) was calculated as the product of LAD blood flow and the aortic-coronary vein O\textsubscript{2} content difference.

Western blotting. Tissue homogenates of LV myocardium were separated on 12% SDS-PAGE and transferred onto nitrocellulose membranes, followed by routine Western blotting. Antibodies against Cu/Zn-SOD and Mn-SOD were purchased from BD Transduction Laboratories and Santa Cruz Biotechnology, respectively. The anti-EC-SOD antibody was produced in our laboratory and has been reported previously (14).

Real-time RT-PCR. One microgram of total RNA was reverse-transcribed with random hexamers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies). Oligonucleotide primers were designed according to the corresponding canine cDNA sequences in the National Institutes of Health GenBank. The primer sequences of Cu/Zn-SOD were sense: 5′-AGTGGGCGTTG-TGTGGGATC and antisense: 5′-AGTACAGTGCCCAGGTTC (PCR product of 189 bp). The primer sequences of GAPDH were sense: 5′-TGCCCCCATGGTGTGATG and antisense: 5′-CCAGC-C CCCAGGCTCTAAAGTG (product of 519 bp). mRNA levels were compared by quantitative real-time RT-PCR analysis with the Light Cycler Thermocycler (Roche Diagnostics). Reactions were prepared in the presence of the fluorescent dye SYBR green I for specific detection of double-stranded DNA. Quantification was performed in the log-linear phase of the reaction, and cycle numbers obtained at this point were plotted against a standard curve prepared from serially diluted control samples. Results were normalized to GAPDH.

Malondialdehyde assay. The malondialdehyde concentration was assayed spectrophotometrically in myocardial tissue according to the method of Buege and Aust (6), which is based on the reaction of the end products of lipid peroxidation with thiobarbituric acid. Results were normalized to the mean values of the normal control hearts.

Data analysis. Coronary flow was computed from the Doppler shift (40). Statistical analysis was performed with two-way (exercise level and treatment) ANOVA for repeated measures. Comparisons within groups were made with one-way ANOVA followed by Scheffe’s post hoc test. Comparisons between groups were made with Student’s independent t-test. Significance was accepted at P < 0.05. Data are presented as means ± SE.

RESULTS

Effect of M40401 in normal dogs. In seven normal animals, M40401 caused no significant hemodynamic changes at rest or during exercise and had no effect on CBF or MV\textsubscript{O\textsubscript{2}} (Table 1). The relationships between CBF or MV\textsubscript{O\textsubscript{2}} and rate-pressure product were unchanged after M40401 (Figs. 1 and 2).

Endothelium-dependent coronary vasodilation in normal dogs. Coronary flow responses to acetylcholine are shown in Fig. 3. Intracoronary acetylcholine (3.75–75 μg/min) had no effect on heart rate or aortic pressure. M40401 (1.5 mg/kg) had no effect on either resting coronary flow or the increase in flow produced by acetylcholine. Inhibition of NO production with L-NNA significantly blunted the increase in coronary flow produced by acetylcholine (Fig. 3).

Effect of M40401 in animals with CHF. As shown in Table 1, CHF was associated with increases in resting heart rate and LVEDP and decreases of aortic pressure, LV systolic pressure (LVSP), maximum LV dp/dt (LV dp/dt\textsubscript{max}), CBF, and MV\textsubscript{O\textsubscript{2}} (each P < 0.05). M40401 (1.5 mg/kg) caused a small but significant decrease of LVEDP at rest and during exercise (P <
Table 1. Effects of M40401 on hemodynamics in normal and CHF dogs

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>3.2 km/h, 0% Grade</th>
<th>6.4 km/h, 0% Grade</th>
<th>6.4 km/h, 5% Grade</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>CHF</td>
<td>Normal</td>
<td>CHF</td>
</tr>
<tr>
<td>Mean aortic pressure, mmHg</td>
<td>110±3.1</td>
<td>88±2.9</td>
<td>110±3.4</td>
<td>90±3.6</td>
</tr>
<tr>
<td>M40401</td>
<td>110±2.8</td>
<td>88±2.6</td>
<td>108±4.0</td>
<td>89±3.0</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>126±4.5</td>
<td>138±4.2</td>
<td>157±5.6*</td>
<td>155±3.4*</td>
</tr>
<tr>
<td>LV systolic pressure, mmHg</td>
<td>129±3.5</td>
<td>136±4.1</td>
<td>154±3.5*</td>
<td>151±4.3*</td>
</tr>
<tr>
<td>LV dP/dt max, mmHg/s</td>
<td>5.1±0.8</td>
<td>5.9±1.1</td>
<td>8.0±1.7*</td>
<td>7.2±1.9*</td>
</tr>
<tr>
<td>Coronary blood flow, ml/min</td>
<td>65±5.6</td>
<td>59±4.3</td>
<td>75±5.8*</td>
<td>54±7.4*†</td>
</tr>
<tr>
<td>CS-PO2, mmHg</td>
<td>17±0.71</td>
<td>23±4.8</td>
<td>15±0.83*</td>
<td>20±4.2*</td>
</tr>
<tr>
<td>MVO2, ml/min</td>
<td>8.9±0.74</td>
<td>7.9±0.67</td>
<td>11±0.68*</td>
<td>10±0.70*</td>
</tr>
<tr>
<td>RPP, mmHg × beats/min × 10³</td>
<td>3.0±0.5</td>
<td>3.5±0.8†</td>
<td>4.0±0.6*</td>
<td>4.8±0.8*†</td>
</tr>
<tr>
<td>Control</td>
<td>16±0.61</td>
<td>14±0.69</td>
<td>21±1.3*</td>
<td>17±0.83*</td>
</tr>
<tr>
<td>M40401†</td>
<td>16±0.51</td>
<td>14±0.65</td>
<td>20±1.1*</td>
<td>16±0.85*</td>
</tr>
<tr>
<td>LV systolic pressure, mmHg</td>
<td>2,442±141</td>
<td>1,429±79</td>
<td>2,946±178*</td>
<td>1,684±106*</td>
</tr>
<tr>
<td>M40401</td>
<td>2,442±131</td>
<td>1,494±79</td>
<td>2,915±218*</td>
<td>1,760±97*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 7 (normal) or 9 [congestive heart failure (CHF)] dogs. RPP, rate-pressure product; MVO2; myocardial oxygen consumption; CS-PO2, coronary sinus oxygen tension; LV, left ventricular; LVEDP, LV end-diastolic pressure; LV dP/dt max, maximum first derivative of LV pressure. *P < 0.05 compared with corresponding resting condition; †P < 0.05 compared with control condition (before M40401) (by 2-way ANOVA).

0.05), whereas other hemodynamic variables were unchanged. M40401 (1.5 mg/kg) caused increases (P < 0.05) in CBF and MVO2 at rest and during exercise in CHF dogs (Figs. 1 and 2), whereas the relationship between MVO2 and CBF was unchanged. In three animals, an additional intravenous dose of M40401 of 3.5 mg/kg (total dose 5 mg/kg) was administered and all measurements were repeated during resting conditions.

In these animals, the additional dose of M40401 caused no change of heart rate, aortic pressure, LVSP, or LVEDP and did not cause a further increase of MVO2 or CBF beyond that produced by the lower dose of M40401.

Effect of L-NNA in animals with CHF. After completion of the M40401 measurements, NOS inhibition with L-NNA (1.5 mg/kg intracoronary) was produced in five dogs with CHF.

![Graph](image-url)
Compared with measurements after M40401, L-NNA caused significant increases of aortic pressure, LVSP, and LVEDP at rest and during exercise, whereas heart rate and LV dP/dtmax were unchanged (Table 2). L-NNA also caused significant increases of MVO₂ and CBF.

**Endothelium-dependent coronary vasodilation in CHF dogs.** Intracoronary acetylcholine had no effect on heart rate or aortic pressure. Compared with normal dogs, acetylcholine-induced coronary vasodilation was significantly attenuated in CHF dogs (Fig. 3). M40401 caused no change of heart rate or aortic pressure. However, M40401 (1.5 mg/kg iv) significantly augmented the increase of coronary flow produced by acetylcholine (Fig. 3). In three animals, administration of an additional dose of 3.5 mg/kg of M40401 caused no further improvement in the coronary flow response to acetylcholine (Fig. 4). As expected, L-NNA inhibited the increase in coronary flow produced by acetylcholine (P < 0.01).

**SOD isoenzyme content.** Western blot analysis demonstrated that, compared with normal dogs, EC-SOD was decreased (1.0 ± 0.1 in normal vs. 0.72 ± 0.10 in CHF), whereas Mn-SOD was increased (1.0 ± 0.08 in normal vs. 1.28 ± 0.07 in CHF) in the failing hearts (each P < 0.05).

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**Fig. 2.** Relationship between rate-pressure product and coronary blood flow at rest and during exercise in normal (left) and CHF (right) dogs. Data were obtained during control conditions and after administration of M40401 (1.5 mg/kg iv). *P < 0.05, comparing control with M40401.

**Fig. 3.** Effect of M40401 (1.5 mg/kg iv) and nitric oxide synthase (NOS) inhibition with N^G^-nitro-L-arginine (L-NNA; 1.5 mg/kg intracoronary) on net increase (top) and %increase (bottom) in coronary blood flow produced by acetylcholine in normal animals (left) and after the development of pacing-induced CHF (right). *P < 0.05 compared with the respective control conditions.
Cu/Zn-SOD was unchanged after development of CHF (Fig. 5).

*Tissue malondialdehyde content.* The myocardial malondialdehyde content was significantly increased in CHF hearts (1.59 ± 0.24) compared with normal control hearts (1.0 ± 0.08; \( P < 0.05 \)).

*Real-time RT-PCR.* The ratio of Cu/Zn-SOD vs. GAPDH in CHF dogs (2.1 ± 0.5) was not different from that in normal dogs (2.0 ± 0.3).

**DISCUSSION**

In considering the results of this study, it is necessary to take into account the effects of both \( \text{O}_2^\cdot \) and NO on endothelial function and myocardial respiration as well as the effect of their reaction product, ONOO\(^{-}\). NO reacts avidly with \( \text{O}_2^\cdot \) to form ONOO\(^{-}\) with two important results. First, ONOO\(^{-}\) has potent biological effects of its own that might contribute to the observed results. Second, the reaction with \( \text{O}_2^\cdot \) removes NO, thereby quenching its biological effects. An additional consideration is that reactions with \( \text{O}_2^\cdot \) are primarily confined to the compartment in which it is generated, so that the source of \( \text{O}_2^\cdot \) affecting endothelial function is likely to be different from the \( \text{O}_2^\cdot \) that might have an effect on mitochondrial respiration.

![Fig. 4. Effect of low (1.5 mg/kg)- and high (5 mg/kg)-dose M40401 on coronary vasodilation produced by intracoronary acetylcholine in 3 animals with pacing-induced CHF.](http://ajpheart.physiology.org/)
Endothelial dysfunction and vascular $O_2^\cdot$ in CHF. In the present study, the increase of coronary flow in response to acetylcholine was blunted after the development of heart failure, in agreement with previous reports that CHF is associated with endothelial dysfunction (41). Because endothelium-derived NO is an important contributor to acetylcholine-induced vasodilation (1), decreased NO bioavailability could be responsible, at least in part, for the impaired acetylcholine response. A decrease of NO bioavailability could result from either decreased production or increased inactivation of NO. Although no data are available from coronary resistance vessels, endothelial NOS (eNOS) protein expression was increased (not decreased) in aortas from rats with LV dysfunction secondary to myocardial infarction (4). The presence of normal or increased vascular eNOS suggests that endothelial dysfunction was the result of augmented NO degradation. This concept is supported by the present finding that the SOD mimic increased acetylcholine-induced coronary vasodilation. There are several possible endothelial sources of $O_2^\cdot$, including NADPH oxidase (25) and xanthine oxidase (24), and there is evidence that each of these sources may be increased in CHF (24, 25). In addition, increased free radical production by the failing heart might be expected to oxidize tetrahydrobiopterin to result in uncoupling of NOS to produce $O_2^\cdot$ rather than NO (35).

The present data demonstrating $O_2^\cdot$ effects on resistance vessel function in the setting of CHF are similar to previous studies using isolated aortic ring preparations that demonstrated impaired conduit vessel endothelial function in CHF animals that was associated with increased $O_2^\cdot$ and ONOO$^-$ production (35, 42). In those studies, treatment with SOD improved endothelium-dependent vasodilation and normalized cGMP responses to the NO donor sodium nitroprusside, indicating that endothelial dysfunction resulted from inactivation of NO by $O_2^\cdot$ (3). Wiemer et al. (42) recently reported that calcium ionophore-induced NO release (assessed with a NO microsensor) was reduced in aortic endothelial cells from rats after the development of heart failure secondary to myocardial infarction, whereas $O_2^\cdot$ and ONOO$^-$ production were increased. Arimura et al. (2) reported that the $O_2^\cdot$ scavenger Tiron improved endothelium-dependent coronary vasodilation in anesthetized open-chest dogs with CHF. Although anesthetic agents have been demonstrated to cause increased production of ROS in the heart (28), the present findings indicate that scavenging $O_2^\cdot$ also improved coronary endothelial dysfunction in the unanesthetized animals with CHF. In the present study, the SOD mimetic M40401 significantly enhanced acetylcholine-induced coronary vasodilation in dogs with CHF but had no effect in normal dogs. These findings suggest that increased $O_2^\cdot$ production is, at least in part, responsible for the blunted increase in CBF to acetylcholine in the failing heart. Although tissue content of M40401 in resistance vessels has not been measured, this compound has been demonstrated to penetrate into all organs examined including the heart (27) and has been found to improve endothelial function in apoprotein E-deficient mice, implying that the agent does diffuse into vascular tissue (20). The addition of l-NNa after M40401 dramatically attenuated the acetylcholine-induced coronary vasodilation, supporting the concept that the enhanced coronary vasodilation after M40401 in CHF animals was due to an increase of NO bioavailability in the coronary resistance vessels. The decreased EC-SOD protein expression in the present study suggests that attenuated $O_2^\cdot$ scavenging ability also could have contributed to endothelial dysfunction in the failing hearts.

Oxygen consumption in failing heart. Hasenfuss et al. (17) reported that both tension-dependent (actin-myosin cross bridging) and tension-independent (calcium cycling during contraction-relaxation) heat liberation were decreased in muscle strips from failing human hearts, suggesting downregulation of energy-utilizing processes. Oxygen consumption was decreased in saponin-skinned myocardial muscle bundles (36) as well as in isolated mitochondria (39) from failing hearts. In the in vivo situation, alterations in substrate preference in the failing heart, with decreased free fatty acid uptake and increased glucose utilization, could contribute to decreased oxygen utilization (10). In several previous reports pacing-induced CHF was associated with decreased $M\overline{O}_2$ (7, 32, 40), although Shen et al. (37) found an increase in $M\overline{O}_2$ after development of CHF. These differing results may be related to differences in the duration and severity of CHF, as well as the specific protocols used to produce heart failure.

$NO$ regulation of $M\overline{O}_2$. At physiological concentrations NO can modulate mitochondrial respiration and ATP production through reversible binding to the oxygen-binding site of cytochrome oxidase (5). Blockade of NO production with nonselective NOS inhibitors increased $M\overline{O}_2$ in normal animals at rest and during exercise (1, 7). Conversely, stimulating endogenous endothelial NO production with bradykinin or administering an NO donor decreased oxygen consumption in isolated, perfused rat hearts (29). Kelm et al. (21) demonstrated that addition of an NO donor to the perfusate of isolated guinea pig hearts resulted in reversible decreases of $M\overline{O}_2$, myocardial...
phosphocreatine, ATP, and cardiac contractility, suggesting that NO can directly regulate MV\(\text{O}_2\) and ATP production. In dogs with pacing-induced heart failure we previously observed (7, 40) that blockade of NO production with the nonselective NOS inhibitor L-NNA or the selective inducible NOS inhibitor S-methylisothiourea resulted in significant increases of MV\(\text{O}_2\) at rest and during exercise. In addition to the inhibitory action of physiological concentrations of NO on mitochondrial respiration, high concentrations of NO can inhibit respiration by nitrosylating complexes I and II of the electron transport chain (5, 33) and have been shown to increase \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) production in isolated mitochondria (5) and in perfused rat hearts (29). Unlike the rapidly reversible effects of NO on cytochrome oxidase, the protein modifications produced by supraphysiological concentrations of NO are long-lasting.

**Oxidative stress in CHF.** Heart failure of several etiologies has been reported to be associated with increased myocardial free radical formation and increased products of oxygen free radical reactions (such as lipid peroxides) (11, 19). In agreement with this, malondialdehyde levels were increased in myocardium from animals with CHF compared with normal hearts in the present study. Ide et al. (19) reported that \(\text{O}_2^-\) production was increased in mitochondria and submitochondrial particle fractions from pacing-induced failing canine hearts. In isolated bovine LV muscle strips, pyrogallol, which generates \(\text{O}_2^-\) through autoxidation, caused respiratory inhibition that was partially reversed after washout of the pyrogallol (43). The effect of pyrogallol was inhibited by the \(\text{O}_2^-\) scavenger Tiron but not by the ONOO\(^{-}\) scavenger urate, indicating that \(\text{O}_2^-\) can directly inhibit respiration without conversion to ONOO\(^{-}\). The combination of pyrogallol and the NO donor \(\text{S}-\text{nitroso-}N\text{-acetyl-penicillamine (SNAP)}\) resulted in a greater inhibition of respiration than either pyrogallol or NO alone (43). Furthermore, the \(\text{O}_2^-\) scavenger Tiron attenuated the inhibition produced by the combination of pyrogallol and SNAP (43), implying that \(\text{O}_2^-\) can enhance the NO inhibition of respiration by formation of ONOO\(^{-}\). Because these effects occurred in nonworking muscle, they imply free radical-mediated inhibition of mitochondrial respiration. Although Mn-SOD was increased in the failing hearts in the present study, this was apparently not sufficient to compensate for increased mitochondrial \(\text{O}_2^-\) production (19).

In addition to effects on mitochondria, free radicals have the potential to impair contractile function, possibly with a secondary decrease of myocardial energy utilization. The \(\text{O}_2^-\) scavenger Tiron (12) attenuated cytokine-induced myocardial failure, implicating a role for \(\text{O}_2^-\) in the contractile dysfunction. In open-chest dogs with pacing-induced CHF, Arimura et al. (2) reported that intracoronary infusion of the \(\text{O}_2^-\) scavenger Tiron improved contractile function. In a previous study we found (7, 40) that the depressed MV\(\text{O}_2\) in dogs with pacing-induced heart failure was strongly correlated with the decrease of LV dP/d\(\text{d}t\)\(_{\text{max}}\). However, in the present study M40401 did not increase LV dP/d\(\text{d}t\)\(_{\text{max}}\) in the failing hearts, possibly because any \(\text{O}_2^-\) and ONOO\(^{-}\)-induced protein modifications of the contractile apparatus would require a longer time to recover.

In the present study, the SOD mimetic M40401 caused significant increases of MV\(\text{O}_2\) and CBF at rest and during exercise in animals with CHF but not in normal dogs, suggesting that \(\text{O}_2^-\) contributed to the depressed MV\(\text{O}_2\) in the failing hearts. Because the increase of CBF after M40401 in the failing hearts was not associated with an increase of coronary venous oxygen tension, as would be observed after administration of a coronary vasodilator, the increase in coronary flow was likely secondary to the increase of MV\(\text{O}_2\). The addition of L-NNA after M40401 caused a further increase of MV\(\text{O}_2\) in the failing hearts. Increased \(\text{O}_2^-\) degradation by M40401 would be expected to increase NO levels and thereby augment mitochondrial respiratory inhibition by NO. Consequently, it was not unexpected that after M40401 the subsequent inhibition of NO synthesis with L-NNA caused a further increase in MV\(\text{O}_2\).

**SODs in CHF.** Three SOD isozymes have been identified in the heart: Cu/Zn-SOD, which is primarily cytosolic in location, mitochondrial Mn-SOD, and EC-SOD (13). A decrease in SOD activity and an increase in lipid peroxides have been reported in volume-overload heart failure in dogs (30), pressure-overload heart failure in guinea pigs (11), and myocardial infarct-induced heart failure in rats (18). Up to one-half of the total SOD in vessels is EC-SOD, and EC-SOD has been implicated as a regulator of endothelium-derived NO bioavailability (13, 14). In addition, Landmesser et al. (24) reported that EC-SOD was significantly decreased in coronary arteries of patients with CHF, whereas Mn-SOD and Cu/Zn-SOD were unchanged. Furthermore, they found that depression of endothelium-dependent coronary vasodilation was strongly correlated with the decrease of endothelium-bound EC-SOD. Consistent with this, EC-SOD was significantly decreased in the failing hearts in the present study whereas Cu/Zn-SOD protein content was unchanged. Although the mechanisms responsible for the decrease in EC-SOD expression in the failing heart remain unknown, it is conceivable that it could be due to either increased tumor necrosis factor-\(\alpha\), which has been shown to decrease EC-SOD in vascular smooth muscle (38), or to decreased NO bioavailability, which could result in a decrease in EC-SOD expression (15). Furthermore, the decreased EC-SOD could further decrease coronary NO bioavailability by increasing \(\text{O}_2^-\) in the failing heart. It must be acknowledged, however, that the present findings were obtained with homogenates of whole heart tissue and may not necessarily reflect changes that occurred in the coronary resistance vessels where blood flow is regulated.

**Limitations of study.** The dose of M40401 (1.5 mg/kg) was determined from published in vivo studies (9, 26) as well as from unpublished data from the compound provider. However, when a larger dose of M40401 was used, no further improvement of the coronary vasodilator response to acetylcholine was observed, implying that the dose of M40401 used was adequate to substantially eliminate the effects of \(\text{O}_2^-\) in the failing hearts. \(\text{O}_2^-\) and ONOO\(^{-}\) could induce protein modifications that would take a longer time period to recover and would not be revealed by the acute treatment with M40401 used in the present study. Future studies will be needed to examine the effects of prolonged \(\text{O}_2^-\) scavenging on endothelial, mitochondrial, and contractile function in the failing heart.

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