Dimethylarginine dimethylaminohydrolase and endothelial dysfunction in failing hearts

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Chen, Yingjie, Yunfang Li, Ping Zhang, Jay H. Traverse, Mingxiao Hou, Xin Xu, Masumi Kimoto, and Robert J. Bache. Dimethylarginine dimethylaminohydrolase and endothelial dysfunction in failing hearts. Am J Physiol Heart Circ Physiol 289: H2212–H2219, 2005. First published July 15, 2005; doi:10.1152/ajpheart.00224.2005.—Congestive heart failure (CHF) is associated with impaired endothelium-dependent nitric oxide (NO)-mediated vasodilation (endothelial dysfunction). We hypothesized that coronary endothelial dysfunction in CHF may be due in part to decreased dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades endogenous inhibitors of NO synthase (NOS), including asymmetric dimethylarginine. Coronary blood flow and the endothelium-dependent vasodilator response to acetylcholine were studied in dogs in which CHF was produced by rapid ventricular pacing for 4 wk. Coronary flow and myocardial O2 consumption at rest and during treadmill exercise were decreased after development of CHF, and the vasodilator response to intracoronary acetylcholine (3.75–75 μg/min) was decreased by 10.2 ± 0.3%/min, whereas acetylcholine-induced vasodilation was increased by 10.2 ± 0.3%/min. DDAH activity and DDAH isoform 2 (DDAH-2) protein content were decreased by 53 ± 13% and 58 ± 14%, respectively, in hearts with CHF, whereas endothelial NOS and DDAH isoform 1 (DDAH-1) were increased. Caveolin-1 and protein arginine N-methyltransferase 1, the enzyme that produces asymmetric dimethylarginine, were unchanged. Immunohistochemical staining showed DDAH-1 strongly expressed in coronary endothelium and smooth muscle and in the sarcolemma of cardiac myocytes. In cultured human endothelial cells, DDAH-1 was uniformly distributed in the cytosol and nucleus, whereas DDAH-2 was found only in the cytosol. Decreased DDAH activity and DDAH-2 protein expression may cause accumulation of endogenous inhibitors of endothelial NOS, thereby contributing to endothelial dysfunction in the failing heart.

nitric oxide; pacemaker; heart failure; exercise

DIMETHYLARGININE DIMETHYLAMINOHYDROLASE 1 (DDAH-1) and 2 (DDAH-2) are enzymes that hydrolyze asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthase (NOS) (24). Increased DDAH activity would be expected to augment ADMA degradation, thereby enhancing NO production, whereas decreased DDAH would decrease NO activity and NO bioavailability (24, 31). Congestive heart failure (CHF) is associated with impaired endothelium-dependent, NO-mediated coronary vasodilation (endothelial dysfunction); however, no consistent change in endothelial NOS (eNOS) protein has been reported in the failing heart (13, 34), therefore it is possible that the endothelial dysfunction could be related to decreased DDAH protein expression and/or depressed DDAH activity. This hypothesis is supported by reports that plasma ADMA levels are increased in the setting of CHF (11, 25, 32). Consequently, this study was carried out to determine DDAH activity, protein content, and the cellular distribution of DDAH-1 and DDAH-2 in the normal and failing heart.

METHODS

Studies were performed in 11 adult mongrel dogs weighing 20–26 kg and trained to run on a treadmill. All experiments were performed in accordance with the “Guiding Principles in the Care and Use of Laboratory Animals” approved by the council of the American Physiological Society and with the prior approval of the University of Minnesota Animal Care Committee.

Surgical preparation. Animals were anesthetized with pentobarbital sodium (25–30 mg/kg), intubated, and ventilated with 1–2% isoflurane supplemented with oxygen. A left thoracotomy was performed, and polyvinyl chloride catheters (3.0 mm OD) were inserted into the descending aorta and the left ventricle (LV). A solid-state micromanometer (Konigsberg Instruments, Pasadena, CA) was introduced into the LV at the apex. A final catheter was introduced into the right atrial appendage and advanced through the coronary sinus until the tip could be palpated at the anterior interventricular vein to allow selective sampling of blood draining the myocardium perfused by the left anterior descending coronary artery (LAD). A Doppler velocity probe (Craig Hartley, Houston, TX) was positioned on the LAD for measurement of coronary blood flow (CBF), and a silicone catheter (0.3 mm ID) was introduced into the LAD distal to the velocity probe. An epicardial pacing electrode was screwed into the mid-right ventricular free wall. The catheters and leads were tunneled to exit at the base of the neck, and the catheters were flushed daily to maintain patency. Postoperative analgesia was provided with butorphanol (0.4 μg/kg, every 4 to 6 h subcutaneously).

Coronary endothelium-dependent vasodilation. The increase in CBF produced by intracoronary acetylcholine (3.75–75 μg/min) was observed in the same group of animals under normal conditions, after 2 wk of pacing, and after 4–5 wk of pacing when clear evidence of CHF was present.

Measurements at rest and during exercise. Studies were begun 2 wk after surgical instrumentation in the awake state before pacing was started so that each animal could serve as its own control. Myocardial O2 consumption (MV̇O2) and hemodynamics were measured during normal control conditions at rest and during a two-stage treadmill exercise protocol (3.2 km/h and 6.4 km/h, both at 0° grade) as previously described (6). Hemodynamic and MV̇O2 measurements were subsequently performed in the same group of animals at rest and during the two exercise stages after development of heart failure.

Production of CHF. CHF was produced by rapid ventricular pacing (6). After studies during normal conditions were completed, 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

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the evidence for development of CHF. Weekly resting hemodynamic and MV\textsubscript{O}₂ measurements were performed to monitor the development of CHF. CHF was deemed to have developed when resting LV end-diastolic pressure was \(>20\) mmHg.

**Hemodynamic measurements.** LV pressure was measured with the micromanometer calibrated to the fluid-filled LV catheter; the maximal first derivative of LV pressure (dp/dt\textsubscript{max}) was obtained via electrical differentiation. Coronary blood velocity was measured with a Doppler flowmeter (Craig Hartley). Data were recorded on an eight-channel recorder.

**Myocardial oxygen consumption.** PO\textsubscript{2}, PCO\textsubscript{2}, and pH were measured with a blood gas analyzer (model 113, Instrumentation Laboratory, Lexington, MA). Hemoglobin was determined by the cyanmethemoglobin method. Hemoglobin oxygen saturation was calculated from the blood PO\textsubscript{2}, pH, and temperature with the use of the oxygen dissociation curve for canine blood. Blood O\textsubscript{2} content was computed as (hemoglobin \(\times 1.34 \times \%\text{O}_2\) saturation) + (0.0031 \(\times\) PO\textsubscript{2}). MV\textsubscript{O}₂ was calculated as the product of the LAD blood flow and the aortic-coronary vein O₂ content difference.

Effects of ADMA on endothelium-dependent coronary vasodilation. Endothelium-dependent coronary vasodilation was examined in the normal animals before beginning pacing. Coronary vasodilation in response to intracoronary acetylcholine (3.75–37.5 μg/min) was determined during vehicle infusion and during ADMA infusion at a dose calculated to produce a coronary blood level of 35 μmol/l. This infusion was chosen on the basis of the report of Achan et al. (1) that administration of ADMA in a dose of 3 mg/kg to normal human subjects (equivalent to 56 μmol/l of extracellular water) caused only a 1.47 μmol/l increase of plasma ADMA concentration (a 38-fold difference from that calculated for extracellular water). If we assume the volume of distribution reported by Achan et al. (1), our infusion rate would have caused an increase in plasma concentration of \(~1\) μmol/l. However, because we infused ADMA by the intracoronary route, coronary plasma levels were likely higher than this. Cytosolic levels are dependent on the cationic amino acid transporter, which transfers ADMA across the cell membrane in competition with \(\text{L-arginine}\) (8). In normal rabbit carotid arteries, cytosolic ADMA levels were reported to be substantially higher than in plasma \((\sim 5\) μmol/l), implying that ADMA is concentrated in the endothelium (21). Although the above considerations prevent estimation of the increase of cytosolic ADMA levels produced by our infusion in the present study, the ability to depress the response to acetylcholine without altering basal coronary flow suggests that a pathophysiologically relevant concentration was achieved.

**Immunohistochemistry.** Frozen LV myocardial sections (8 μm) were fixed in 4% paraformaldehyde at room temperature for 15 min and permeabilized in 0.3% Triton X-100 at room temperature for 15 min. Endogenous peroxidase activity was inactivated with 3% H\textsubscript{2}O\textsubscript{2} for 10 min. The sections were then blocked with 5% bovine serum and incubated for 2 h with mouse monoclonal anti-DDAH-1 (1:100, produced in our laboratory) (14) and rabbit anti-caveolin-1 (1:400, Sigma) or anti-caveolin-3 (1:1,000, Santa Cruz Biotechnology) antibodies in 5% normal bovine serum and PBS. The sections were washed five times in 0.1% Tween in PBS and incubated with Cy2-conjugated donkey anti-mouse IgG (1:500, Molecular Probes) and rhodamine-labeled goat anti-rabbit IgG (1:500, Sigma) in 5% normal bovine serum and PBS for 30 min at room temperature. The positive control slides were incubated with corresponding mouse and rabbit IgG instead of the specific antibodies. The sections were washed five times for 5 min with 0.1% Tween-PBS and observed with a laser confocal microscope. To determine whether DDAH-1 was located in mitochondria, the slides were costained for DDAH-1 and cytochrome c, a specific marker for mitochondria.

**Subcellular distribution of DDAH-1 and DDAH-2 in cultured endothelial cells.** To further determine the subcellular distribution of DDAH-1, cultured human microvascular endothelial cells (a gift from Dr. R. Hebel, University of Minnesota) and human umbilical vein endothelial cells were stained for DDAH-1 (monoclonal antibody produced in our laboratory was (14) and DDAH-2 (Abcam). Costaining of DDAH-1 with caveolin-1 was also performed.

**Western blot analysis.** Tissue homogenates of LV myocardium from six normal dogs and seven dogs with pacing-induced CHF were separated on 12% SDS-PAGE, transferred onto nitrocellulose membrane, and followed by routine Western blot analysis. Antibodies against DDAH-2, eNOS, caveolin 1, and caveolin-3 were purchased from Abcam, BD Transduction Laboratories, and Santa Cruz Biotechnology, respectively. The antibody against protein arginine N-methyltransferase 1 (PRMT-1) was purchased from Sigma. In studies in which selective DDAH-1 gene silencing was used in our laboratory, we found that commercially available DDAH-1 antibody (Abcam) did not detect DDAH-1 protein. Consequently, DDAH-1 antibody was produced in our laboratory (M. Kimoto).

**Measurement of DDAH activity.** DDAH activity was assayed as previously described (24, 31). Briefly, the tissue homogenates or cell lysate was incubated with 4 mmol/l ADMA-0.1 mol/l sodium phosphate (pH 7.4). The reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid, and the supernatant was boiled with diacetyl monoxime (0.8% wt/vol in 5% acetic acid) and antipyrine (0.5% wt/vol in 50% sulfuric acid). The amounts of L-citrulline formed were determined with the spectrophotometric analysis at 466 nm. As the assay blank, the enzyme preparations heated in a boiling water bath were subjected to the determinations of the activity.

**Measurement of ADMA.** Plasma ADMA concentration was determined with an ELISA kit (DLD Diagnostika, Hamburg, Germany).

**Real-time quantitative PCR.** One microgram of total RNA was reverse-transcribed with the use of random hexamers and Moloney murine leukemia virus reverse transcriptase (Life Technologies). Oligonucleotide primer sequences of DDAH-1 were designed according to a previous report (17) as follows: sense, 5′-GCAAGGAAGAGGTGTGACATGA; and antisense, 5′-GACCCAAATTCGATCAGGT (17). Oligonucleotide primers of protein arginine N-methyltransferase 1 were designed according to the corresponding canine cDNA sequences in the GenBank (access number BQ-426207) as follows: sense, 5′-GACCCAAATTCGATCAGGT; and antisense, 5′-TAACGACCTGAACACACC. Primer sequences of GAPDH were as follows: sense, 5′-ACCCACAGTCCATGCCATCAC; and antisense, 5′-TCCACACCCGTGTCGTTGTA. The mRNA levels were compared by real-time quantitative RT-PCR analysis with the use of the Light Cycler Thermocycler (Roche Diagnostics). The 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 the cycle numbers obtained at this point were plotted against a standard curve prepared from serially diluted control samples. The results were normalized to \(\beta\)-actin expression levels.

**Data analysis.** Hemodynamic variables were measured from the chart recordings. Coronary blood velocity was measured with a Doppler flowmeter (Craig Hartley). Statistical analysis was performed with the use of two-way (exercise level and treatment) ANOVA for repeated measures. Comparisons within groups were made with the use of one-way ANOVA followed by Scheffe’s post hoc test. Comparisons between groups were made with the use of Student’s independent \(t\)-test. Significance was accepted at \(P < 0.05\). Data are presented as means ± SE.

**RESULTS**

**Hemodynamics and MV\textsubscript{O}₂.** Hemodynamic data at rest and during exercise in 8 dogs during normal control conditions and during the development of CHF are shown in Table 1 and Fig. 1. The development of CHF was associated with decreases of LV systolic pressure, LV dp/dt\textsubscript{max}, aortic pressure, rate-pressure product, MV\textsubscript{O}₂, and CBF at rest and during exercise.
Further depressed as pacing continued, so that after the development of CHF the increase of coronary flow in response to acetylcholine decreased ~39%.

**Effect of ADMA on endothelium-dependent coronary vasodilation.** The effect of ADMA on acetylcholine-induced coronary vasodilation was examined in four normal dogs. Intracoronary ADMA at a dose of 0.89 μg·min^{-1}·ml^{-1} of coronary flow caused no change in heart rate, arterial pressure, LV systolic pressure, or LV end-diastolic pressure. In these normal dogs, whereas LV end-diastolic pressure was increased. The resting heart rate was increased after the development of CHF, whereas the heart rate during exercise was unchanged. Coronary venous oxygen tension did not change with the development of CHF.

**Endothelium-dependent coronary vasodilation.** CBF responses to acetylcholine in the same nine animals before and after pacing are shown in Fig. 2. Intracoronary acetylcholine had no effect on heart rate or aortic pressure. Under control conditions, coronary flow increased from 37 ± 2.4 ml/min at baseline to 101 ± 7.1 ml/min during the maximum acetylcholine dose (75 μg/μm). Both CBF and the increase of coronary flow caused no change in heart rate, arterial pressure, LV systolic pressure, or LV end-diastolic pressure. In these normal dogs, whereas LV end-diastolic pressure was increased. The resting heart rate was increased after the development of CHF, whereas the heart rate during exercise was unchanged. Coronary venous oxygen tension did not change with the development of CHF.

**Table 1. Hemodynamic alterations produced by rapid, ventricular pacing in chronically instrumented dogs**

<table>
<thead>
<tr>
<th>Hemodynamic Measure</th>
<th>Rest</th>
<th>Stage 1, 3.2 km/h, 0°</th>
<th>Stage 2, 6.4 km/h, 0°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean aortic pressure, mmHg</td>
<td>Normal</td>
<td>111 ± 5.4</td>
<td>118 ± 5.7*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>Normal</td>
<td>130 ± 3.6</td>
<td>164 ± 8.8*</td>
</tr>
<tr>
<td>LV systolic pressure, mmHg</td>
<td>Normal</td>
<td>131 ± 5.7</td>
<td>146 ± 7.6*</td>
</tr>
<tr>
<td>CBF rate-pressure product, mmHg·beats^{-1}·min^{-1}</td>
<td>Normal</td>
<td>17.0 ± 0.7</td>
<td>23.7 ± 1.8*</td>
</tr>
<tr>
<td>LV dp/dmax, mmHg/s</td>
<td>Normal</td>
<td>2734 ± 242</td>
<td>3734 ± 420*</td>
</tr>
<tr>
<td>Coronary blood flow, ml/min</td>
<td>Normal</td>
<td>36.7 ± 2.4</td>
<td>44.0 ± 3.6*</td>
</tr>
<tr>
<td>Coronary vein PO2, mmHg</td>
<td>Normal</td>
<td>21.5 ± 2.5</td>
<td>18.4 ± 2.6</td>
</tr>
<tr>
<td>MVO2, ml/min</td>
<td>Normal</td>
<td>4.8 ± 0.6</td>
<td>6.4 ± 0.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 dogs. *P < 0.05 compared with corresponding resting condition; †P < 0.05 compared with corresponding normal control conditions; ‡Congestive heart failure present. LV, left ventricular; dp/dmax, maximal first derivative of LV pressure; MVO2, myocardial O2 consumption.

Fig. 1. Coronary blood flow and maximal first derivative of left ventricular pressure (dp/dmax) plotted as a function of myocardial O2 consumption in dogs before and during pacing-induced heart failure.

Fig. 2. Response of coronary blood flow to acetylcholine during normal control conditions, after rapid ventricular pacing for 2 wk, and after development of congestive heart failure (CHF). *P < 0.05 compared with normal control response.
hearts, ADMA caused no change in basal coronary flow (39 ± 3.2 before and 37 ± 2.9 ml/min after ADMA). However, ADMA attenuated the increase in coronary flow produced by acetylcholine (3.75 μg/min) by 32% (Fig. 3).

ADMA assay. Plasma ADMA concentration was determined in the same group of animals before ventricular pacing (normal) and after development of CHF. Plasma ADMA was 2.13 ± 0.10 μM in normal animals and increased significantly after the development of heart failure (2.78 ± 0.21 μM; *P < 0.01).

Cellular distribution of DDAH-1. Immunohistochemical examination of LV myocardium from normal dogs demonstrated strong DDAH-1 staining on the endothelium of the coronary microvessels and relatively weak staining of the sarcolemma and cytosol of the cardiac myocytes. The ratio of signal intensity between the endothelium of the coronary microvessels and the sarcolemma of the cardiac myocytes was 3.8 ± 0.06. Strong DDAH-1 staining was also noted in the smooth muscle cells and endothelium of the coronary arteries and veins. Colocalization of DDAH-1 and caveolin-1 (an endothelium-specific marker) was noted in the endothelium of the coronary microvessels (Fig. 4). Colocalization of DDAH-1 and caveolin-3 was noted on the sarcolemma of the cardiac myocytes (Fig. 4). There was no colocalization of DDAH-1 and cytochrome c in the cytosol of the cardiac myocytes, indicating that cytosolic DDAH-1 was not associated with mitochondria.

Subcellular distribution of DDAH-1 and DDAH-2 in cultured endothelial cells. During Western blot analysis of canine myocardial tissue, we found that the DDAH-2 antibody reacted not only with protein of the appropriate molecular weight but also reacted nonspecifically to produce several additional bands with molecular weights different from DDAH-2; consequently, it was not possible to do immunohistochemical staining of canine tissue with the available DDAH-2 antibody. For this reason, studies of subcellular localization of DDAH-1 and DDAH-2 were done with the use of human endothelial cells. DDAH-1 was distributed in both the cytosol and nuclei of cultured human microvascular endothelial cells (Fig. 5A) and human umbilical vein endothelial cells, whereas DDAH-2 was distributed only in the cytosol (Fig. 5D). Costaining for caveolin-1 and DDAH-1 showed that caveolin-1 was located mainly on the cell membrane and that DDAH-1 did not colocalize with caveolin-1. There was no colocalization of cytochrome c with...
either DDAH-1 or DDAH-2 (Fig. 5, A–D), indicating that DDAH was not associated with mitochondria of the endothelial cells.

**Western blot analysis.** In comparison with normal myocardium, eNOS was significantly increased, whereas DDAH-2 was significantly decreased (Fig. 6). DDAH-1, PRMT-1, and caveolin-1 were unchanged in the failing heart.

**DDAH activity.** As shown in Fig. 7, in comparison with normal hearts, DDAH activity was significantly decreased in the failing heart.

**Real-time quantitative PCR.** In comparison with normal conditions, the ratio of DDAH-1 versus GAPDH was increased by 4.5-fold in the CHF dogs, whereas the ratio of PRMT-1 versus GAPDH was unchanged.

**DISCUSSION**

Several new findings are reported in this study. First, we observed that DDAH-1 in the heart is predominantly distributed in coronary endothelial cells, with weaker staining of the sarcolemma of cardiac myocytes. This is the first demonstration of predominant expression of DDAH-1 in coronary endothelial cells. Second, in subcellular localization studies, we found that DDAH-1 was almost equally distributed between the cytosol and nuclei of cultured human microvascular endothelial cells and umbilical vein endothelial cells, whereas DDAH-2 appeared only in the cytosol. To the best of our knowledge, this is the first report of differing subcellular distributions of the two DDAH isoforms in endothelial cells, suggesting different physiological functions for DDAH-1 and DDAH-2. Third, DDAH-2 protein and DDAH activity were decreased in the failing heart, whereas DDAH-1 protein was unchanged. These findings indicate differing responses of DDAH-1 and DDAH-2 during the development of heart failure. In the setting of increased eNOS with unchanged caveolin-1, DDAH-1, and PRMT-1 protein, the depressed expression of DDAH-2 protein and decreased DDAH activity may have contributed to the endothelial dysfunction in the failing heart.

**NO regulation of CBF.** Stimulation of endogenous coronary NO production (as with acetylcholine) results in coronary vasodilation with an increase of blood flow that is blunted in failing hearts, indicative of endothelial dysfunction (15, 27, 31). The progressive depression of acetylcholine-induced coronary vasodilation with pacing observed in the present study implies that endothelial dysfunction occurs progressively during the development of heart failure. Several abnormalities in the failing heart could contribute to the depressed vasodilator response in the setting of CHF. First, a decrease of eNOS protein expression or activity could limit the response to endothelium-dependent, NO-mediated vasodilators. Although almost all studies have demonstrated coronary endothelial dysfunction in the failing heart, conflicting data exist with regard to eNOS expression and activity. Stein et al. (28) reported increased myocardial eNOS expression but no in-

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**Fig. 5.** Different subcellular distributions of DDAH-1 and DDAH-2 in cultured human microvascular endothelial cells: A: DDAH-1; B: cytochrome C; C: merged image of A and B; and D: DDAH-2. Results demonstrate distinct subcellular distributions of the two DDAH isoforms. Monoclonal anti-DDAH-1 antibody produced from our laboratory was used for DDAH-1 staining (11). Polyclonal anti-DDAH-2 (Abcam) was used for DDAH-2 staining.

**Fig. 6.** Protein expression of endothelial nitric oxide synthase (eNOS, Santa Cruz), DDAH-1, DDAH-2 (Abcam), and PRMT-1 (Sigma) in normal and CHF myocardium. A: western blot analysis. B: summary data. *P < 0.05 compared with normal controls.

**Fig. 7.** DDAH activity in normal and CHF hearts (results were normalized to normal hearts). *P < 0.05 compared with normal controls.
crease in eNOS activity in patients with end-stage CHF. Bauersachs et al. (3) reported that vasodilatation and NO production in response to acetylcholine or bradykinin were decreased in isolated coronary vessels from the failing heart despite increased eNOS expression. Studies (33, 34) from other laboratories reported decreased eNOS mRNA and protein expression in coronary microvessels and aortic endothelial cells from dogs with pacing-induced CHF. In contrast, using a similar animal model, Hare et al. (13) failed to find a decrease of eNOS protein in dogs with CHF. In the present study we found that eNOS protein content was increased in animals with pacing-induced CHF, despite the presence of clear endothelial dysfunction. Differences in eNOS expression between previous reports may have been the result of technical differences, i.e., in vivo versus in vitro studies, differing methods of collecting tissues, and different methods of analysis. Furthermore, it is possible that CHF would produce differing responses of eNOS expression in myocardial and vascular tissue. Because Western blot analyses were performed on myocardial lysates, the present data are unable to distinguish between changes in eNOS expression in vascular tissue versus cardiac myocytes.

Second, alterations of caveolin-1 have the potential to alter eNOS activity. In the heart, caveolin-1 and caveolin-3 are mainly distributed in endothelial cells and cardiac myocytes, respectively. The binding of caveolin-1 to eNOS in endothelial cells is proposed to antagonize calmodulin binding and thereby inhibit enzyme activity (10). Indeed, coexpression of eNOS and caveolin-1 led to a marked inhibition of enzyme activity (22), and a cell-permeable peptide containing the scaffolding domain of caveolin-1 was also able to inhibit NO-mediated vasodilator responses in vivo (5). The unchanged caveolin-1 protein content in the present study is consistent with a previous report (13) and implies that alterations of caveolin-1 are not responsible for the depressed endothelial function in the CHF dogs.

Finally, an increase in extravascular forces acting on the intramural microvessels could act to oppose coronary flow and limit increases in flow in response to vasodilators (30). In the present study, ADMA impaired the coronary flow response to acetylcholine in normal hearts, but this blunting was less than that observed after the development CHF. It is likely that the greater impairment of the coronary response to acetylcholine after the development of CHF can be attributed, at least in part, to the increased extravascular forces in the failing heart. We used an infusion rate of ADMA that did not cause an increase in blood pressure to avoid reflex changes or alterations of mechanical forces that might influence coronary vasomotor responses. However, we do not know the ADMA levels in the coronary resistance vessels where flow responses are determined, so it is possible that cytosolic ADMA levels were less than exist in the chronically failing heart.

Distribution of DDAH in the heart. Previous investigators (19) have reported both DDAH-1 and DDAH-2 mRNA in normal myocardium, with a relatively high abundance of DDAH-2 mRNA. Using microarray technology, we recently (7) found expression of DDAH-1 and DDAH-2 mRNA in failing human hearts, with a relatively higher mRNA abundance of DDAH-1 (the signal intensity ratio of DDAH-1 to DDAH-2 was close to 2), suggesting that DDAH-1 is the dominant isoform in the failing human heart. DDAH-1 and/or DDAH-2 protein expression have also been reported in vascular tissue (2), endothelial cells (3, 4), smooth muscle cells (2, 17), and myocardium (7). Leiper et al. (19) reported that DDAH-1 has an expression pattern similar to that of neuronal NOS, whereas DDAH-2 has an expression pattern similar to that of eNOS. In the present study, we used a monoclonal antibody against DDAH-1 that does not recognize DDAH-2. Although we did find DDAH-1 on the sarcolemma of cardiac myocytes, DDAH-1 was predominantly distributed in vascular endothelial cells, suggesting an expression pattern similar to eNOS in myocardium. We have observed a similar DDAH-1 distribution pattern in human myocardial tissue (unpublished data). The finding of differing subcellular localizations of DDAH-1 and DDAH-2 suggests different physiological functions of these two isoforms. This is supported by the discordant responses of these proteins with the development of heart failure (DDAH-1 was unchanged, whereas DDAH-2 decreased). Further studies will be needed to understand the functional consequences of these changes of DDAH expression in the failing heart.

ADMA, DDAH-1, and PRMT-1. The discovery of ADMA and DDAH provided a potential mechanism to reconcile the finding of endothelial dysfunction in the presence of unchanged or increased eNOS content/activity in the setting of CHF. ADMA, the endogenous NOS inhibitor, is derived from the catabolism of proteins containing methylated arginine residues by the enzyme PRMT (ADMA is not derived from the methylation of free l-arginine). PRMT-1 is the predominant PRMT isoform (29). DDAH removes the inhibitory effect by hydrolyzing ADMA to l-citrulline and dimethylamine. Therefore, either increasing ADMA production through increased PRMT-1 protein/activity or decreasing ADMA degradation through decreased DDAH protein/activity could result in the elevation of ADMA and the inhibition of eNOS activity. Recent studies have reported elevated plasma levels of ADMA in atherosclerosis (23), diabetes mellitus (20), hypertension (11), and heart failure (9, 28). The increased plasma ADMA in the CHF dogs in the present study is consistent with previous reports (9, 32). Because endothelium-dependent vasodilation is mediated by eNOS located in endothelial cells, the predominant distribution of DDAH-1 and DDAH-2 in vascular endothelial cells could act to modulate the local ADMA concentration and regulate NO production by removing ADMA inhibition of eNOS. The decreased DDAH-2 protein content and activity that we observed in the failing heart could result in the accumulation of ADMA in coronary endothelial cells, thereby inhibiting endothelial NO production and contributing to endothelial dysfunction. The increased PCR product despite no change in protein expression in the failing heart suggests that the change in DDAH-1 was mediated at the transcriptional level or that the rate of DDAH-1 protein degradation was increased in the failing heart. We found that PRMT-1 was unchanged in the failing heart, so that increased ADMA production is unlikely to account for the elevation of plasma ADMA in heart failure.

The decreases of DDAH activity and DDAH-2 protein in the failing heart may be related to increased cytokines and/or inducible NOS expression in CHF. A recent report (18) demonstrated that cytokine stimulation caused decreased DDAH activity and increased DDAH-2 nitrosylation/degradation in cultured endothelial cells that were associated with increased...
inducible NOS expression. In contrast, another study (17) reported that IL-1-β stimulation increased DDAH activity and DDAH-1 expression in smooth muscle cells. These differing results may be related to the use of different cell types, different cytokines, and/or the concentration of cytokine used. Using microarray technology, we recently (7) found that the unloading of the failing human heart with an LV assist device resulted in the upregulation of the DDAH-1 gene (DDAH-1 protein was also increased) that was negatively correlated with the expression of several cytokines including tumor necrosis factor. In contrast to the increased expression of the DDAH-1 gene, expression of the DDAH-2 gene was unchanged after LV assist device support, demonstrating that DDAH-1 and DDAH-2 can respond differently to reductions of LV loading. A recent study demonstrated that s-nitrosylation markedly inhibited DDAH activity in vitro (18) and that oxidative stress exacerbated the decrease of DDAH activity (20). We therefore speculate that increased oxidative stress and DDAH s-nitrosylation may have contributed to the decreased DDAH activity in the failing heart. The increased PCR product in the failing heart, indicating increased gene expression of DDAH-1, in the presence of unchanged DDAH-1 protein suggests that both protein synthesis and protein degradation of DDAH-1 may be increased in the failing heart.

Limitations. Although the present data as well as several previous studies have demonstrated that plasma ADMA concentrations are increased in heart failure (9, 25), no studies have directly measured endothelial cell ADMA concentrations. Even though we found that DDAH-2 protein and DDAH activity were decreased in the failing heart, whereas plasma ADMA was increased, the critical question is whether the ADMA in endothelial cells of coronary resistance vessels is also increased in vivo. We did not examine the kinetics of ADMA inhibition of eNOS activity in vivo, although we did demonstrate that ADMA can depress acetylcholine-induced coronary vasodilation. Several investigators have reported associations between elevated plasma ADMA levels and endothelial dysfunction in the peripheral circulation of patients with CHF (12, 16). Thus ADMA levels are elevated in patients with CHF, and short-term administration of L-arginine has been reported to partially reverse endothelial dysfunction in the forearm vascular bed of patients with heart failure (12, 26). Future studies will be needed to determine whether coronary endothelial dysfunction in heart failure or as produced by ADMA can also be reversed by high-dose arginine.

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REFERENCES


