Contribution of Caveolin Protein Abundance to Augmented Nitric Oxide Signaling in Conscious Dogs With Pacing-Induced Heart Failure

Joshua M. Hare, Robert A. Lofthouse, George J. Juang, Laurence Colman, Kelly M. Ricker, Benjamin Kim, Hideaki Senzaki, Suyi Cao, Richard S. Tunin, David A. Kass

Abstract—Myocardial NO signaling appears elevated in heart failure (HF). Whether this results from increased NO production, induction of the high-output NO synthase (NOS)2 isoform, or changes in NOS regulatory pathways (such as caveolae) remains controversial. We tested the hypothesis that increased abundance of caveolin-3 and/or sarcolemmal caveolae contribute to increased NO signaling in pacing-induced HF. Abundance of caveolin-3 (0.59±0.08 versus 0.29±0.08 arbitrary units, P=0.01) but not caveolin-1 was increased in HF compared with control conditions, assessed by Western blot. Additionally, transmission electron microscopy revealed increased caveolae (2.7±0.4 versus 1.3±0.3 per micrometer myocyte membrane, P<0.005). The association between caveolin-3 and NOS3 at the sarcolemma and T tubules was unchanged in HF compared with control myocytes. The impact of NOS inhibition with L-N\textsuperscript{G}-methylarginine hydrochloride (L-NMMA) on β-adrenergic inotropy was assessed in conscious dogs before and after HF. In control dogs, dobutamine (5 μg·kg\textsuperscript{-1}·min\textsuperscript{-1}) increased +dP/dt by 36±7%, and this was augmented to 66±24% by 20 mg/kg L-NMMA (P=0.04 versus without L-NMMA, n=8) but not affected by 10 mg/kg L-NMMA (34±10%, P=NS; n=8). In HF, dobutamine +dP/dt response was depressed (P<0.001 versus control), and increased concentrations were required to match control inotropic responses (10 to 15 μg·kg\textsuperscript{-1}·min\textsuperscript{-1}, 48±7%). L-NMMA enhanced +dP/dt responses similarly at 10 mg/kg (61±17%, P=0.02; n=4) and 20 mg/kg (54±7%, P=0.04; n=7). Caveolin-3 abundance positively correlated with L-NMMA augmentation of dobutamine inotropic responses in HF (r=0.9, P=0.03; n=4). Thus, in canine pacing-induced HF, expression of caveolin-3 and of sarcolemmal caveolae is increased. This increase is associated with augmented agonist-stimulated NO signaling, likely via a compartmentation effect. (Circ Res. 2000;86:1085-1092.)

Key Words: heart failure • caveolae • signal transduction • compartmentation

The role of NO signaling in the pathophysiology of heart failure (HF) remains controversial. On the one hand, inhibition of NO synthase (NOS) augments β-adrenergic inotropic responses to a greater extent in patients with HF than in normal subjects, suggesting increased myocardial NO activity. On the other hand, HF is associated with diminished endothelium-dependent vasoreactivity (reviewed in Reference 3) and reduced NO regulation of myocardial oxygen consumption. From a biochemical perspective, several studies reporting induction of the high-output NOS2 isoform in cardiomyopathy support the notion that the NO pathway activity is enhanced in HF. Conversely, other studies have shown reduced NO metabolites in coronary sinus blood from human and experimental cardiomyopathy. These seemingly conflicting findings may be reconciled if abnormalities in NOS regulation exist in failing myocardium. NOS3, which is constitutively found in cardiac myocytes in addition to endothelial cells, is regulated by caveolar scaffolding proteins, the caveolins. Caveolin-3 (in myocytes) and caveolin-1 (in endothelial cells) bind NOS3, preventing its subsequent activation by calmodulin. Caveolin interactions have also been reported with NOS1 and NOS2. Stimuli that activate NOS via Ca\textsuperscript{2+} and calmodulin displace NOS from caveolin, thereby relieving its inhibitory effect (reviewed by Michel and Feron). Given the fact that caveolins bring NOS into proximity with sarcolemmal agonists, relative increases in their protein abundance have the potential to augment NO pathway activity in response to agonist (ie, β-adrenergic) stimulation via a compartmentation effect and at the same time to inhibit basal unstimulated activity.

The purpose of the present study was to test the hypothesis that failing myocardium exhibits increased abundance of caveolin-3 and/or caveolae. Experiments were conducted in...
the pacing-induced canine HF model, which exhibits NO-related inhibition of β-adrenergic inotropic responses that is similar to the inhibition found in humans with cardiomyopathy in the absence of increased levels of ventricular NOS.16

Materials and Methods

Chronic Preparation, Hemodynamic Analysis, and Pacing Protocol
Twenty-two mongrel dogs of either sex (20 to 25 kg) were chronically instrumented to measure left ventricular (LV) pressure and dimensions and were paced to HF, as previously described.17 Measurements of preload (end-diastolic dimension [EDD]), afterload (arterial elastance [Ea]), and myocardial contractility (peak +dP/dt, slope of relation between +dP/dt and EDD [+dP/dt-EDD], and ventricular elastance [Ees]) were obtained as previously described17 from conscious animals.

Measurement of Caveolin and NOS Protein Abundance/Enzyme Activity
Western blotting was performed by using protein purified from total LV free wall myocardium as described,18 with use of the following antibodies: monoclonal mouse anti-caveolin-1 and caveolin-3, rabbit polyclonal anti-mouse NOS3, and polyclonal anti-NOS2. Caveolin-3 Western blots were also performed with the use of protein from canine myocytes, isolated by collagenase digestion, as described.19 NOS activity was measured by the conversion of L-[14C]arginine to L-[14C]citrulline by using a modification of the method of Bredt and Snyder20 as described.18

Transmission Electron Microscopy
LV endomyocardial samples obtained from HF and control (CTL) animals (n=2 each) immediately after euthanasia were fixed in 2.5% glutaraldehyde, washed in 0.1 mol/L cacodylate buffer (3 times for 5 minutes), and subjected to postfixation with 1% osmium tetroxide in 0.1 mol/L cacodylate for 1 hour. Formvar-coated copper grids were stained with 2% uranyl acetate for 30 minutes, which was followed by subsequent staining with 0.02% lead citrate for 3 minutes, and imaged by use of a Philips CM 120 transmission electron microscope. Individual myocyte membranes (n=15 or 16 each for CTL and HF groups) were imaged at ×27 500 (27.5 K) and ×74 000 (74 K) magnifications, and caveolae were counted by blinded investigators.

Two-Photon Microscopy
Isolated myocytes from CTL and HF dogs (n=2 each) were attached to coverslips with laminin, fixed in 50% methanol/50% acetone, and incubated first with monoclonal antibodies to caveolin-3 and NOS3 and then with anti-mouse rhodamine (Jackson Immunoresearch) and anti-rabbit Alexa 488 (Molecular Probes). Imaging and colocalization analysis was performed on a Nikon E600FN upright physiological fluorescence microscope with a Bio-Rad MRC-1024/2-P multiphoton imaging system attachment, as described in detail in the online-only Materials and Methods section (see http://www.circresaha.org).

Response to β-Adrenergic Stimulation and NOS Inhibition
The influence of NO was assessed by central venous infusion of the NOS inhibitor L-NMMA (kindly provided as L-NNMMA hydrochloride by Glaxo-Welchol) in 2 concentrations, 10 or 20 mg · kg\(^{-1}\) · h\(^{-1}\) for 1 hour. These infusion rates were selected from clinical trials of L-NMMA for sepsis, representing maximal and half-maximal infusions.21 The concentration-effect relation to the β-adrenergic agonist dobutamine was obtained before and after L-NMMA infusion. Dobutamine was infused via right atrial catheter at 2.5 and 5 μg · kg\(^{-1}\) · min\(^{-1}\) for 5 to 7 minutes until steady-state increases in peak +dP/dt were obtained. In addition to these infusion rates, after the induction of HF, dobutamine was also increased to 10 and 15 μg · kg\(^{-1}\) · min\(^{-1}\). After the dobutamine infusions, normal saline was infused for 15 minutes, and baseline conditions were reestablished. The NOS inhibitor L-NMMA was then infused at either 10 or 20 mg/kg over 1 hour, and hemodynamic measurements were obtained every 10 minutes. After 60 minutes, the L-NMMA was continued, and the dobutamine infusions were repeated.

Data Analysis
Data are presented as mean±SEM and were analyzed by t test, multiple linear regression, or ANOVA, as appropriate.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Hemodynamic Response to Rapid Pacing
Resting hemodynamics were obtained in 16 CTL dogs before and in 9 dogs after the induction of HF with rapid ventricular pacing (210 to 240 bpm). As shown in Table 1, pacing produced the hemodynamic features of dilated cardiomyopathy and congestive HF with elevated preload (end-diastolic dimension and pressure), elevated afterload (Ea), and diminished contractility (Ees, +dP/dt, and dP/dt-EDD).

Caveolin-1 and -3 Abundance
Concentrations of the muscle-specific isoform, caveolin-3, were increased in HF versus CTL myocardium (0.59±0.08 versus 0.29±0.08 arbitrary units, P=0.01; n=10 or 11 each; data from 2 separate blots; Figure 1). These findings were confirmed in an additional blot using protein from isolated canine myocytes (0.63±0.33 versus 0.27±0.25 arbitrary units for HF versus CTL, respectively; n=3 each). In contrast, the endothelial cell–specific isoform, caveolin-1, was unchanged in HF compared with CTL myocardium (Figure 1).

Caveolar Abundance
Transmission electron microscopy was performed to quantify caveolar abundance. Caveolae, defined as 50- to 100-nm membrane invaginations, were 2-fold more abundant (2.7±0.4 versus 1.3±0.3 per micrometer plasmalemmal membrane, P<0.005) in HF compared with CTL myocytes (Figure 2).

Two-Photon Imaging
To determine whether caveolin-3 and NOS3 had similar colocalization in myocytes, 2-photon imaging was performed

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<tr>
<th>TABLE 1. Resting Hemodynamic Conditions</th>
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<tr>
<td>Peak +dP/dt, mm Hg/s</td>
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<td>Ees, mm Hg/mm</td>
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<td>+dP/dt-EDD, mm Hg · s(^{-1}) · mm(^{-1})</td>
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<td>LVEDP, mm Hg</td>
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<tr>
<td>Ea, mm Hg/mm</td>
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<td>EDD, mm</td>
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Values are mean±SEM. LVESP indicates LV end-systolic pressure; LVEDP, LV end-diastolic pressure.

*P<0.05 vs CTL.
with the use of myocytes costained with caveolin-3 and NOS3 antibodies (Figure 3). Caveolin-3 staining localized to the plasmalemmal membrane as well as to T-tubular structures in both CTL and HF myocytes. NOS3, on the hand, exhibited a diffuse staining pattern throughout the cell. The staining patterns colocalized only at the sarcolemma and T tubules.

Response to NOS Inhibition in Normal Dogs

Dobutamine produced a positive inotropic effect reflected by increases in all indices of myocardial contractility (Table 2). In the CTL group, the peak infusion of dobutamine (5 μg · kg⁻¹ · min⁻¹) increased peak +dP/dt by 869±148 mm Hg/s (36±7%). This was suppressed in the HF group, with +dP/dt increasing by 162±64 mm Hg/s or 9±2% at 5 μg · kg⁻¹ · min⁻¹ (P<0.001 versus CTL group). To achieve increases in +dP/dt similar to those in the CTL group, dobutamine infusions were increased to rates of 10 to 15 μg · kg⁻¹ · min⁻¹, so as to increase peak +dP/dt by 828±26 mm Hg/s (48±7%).

NOS inhibition was tested at 2 concentrations of L-NMMA. In the CTL group, the lower infusion (10 mg · kg⁻¹ · h⁻¹) did not affect the inotropic response to dobutamine, whereas twice the concentration (20 mg · kg⁻¹ · h⁻¹) augmented the +dP/dt response to a 1735±244 mm Hg/s increase (66±24%, P<0.001; Table 2). This effect was also evident in the load-insensitive indices of contractility, Ees and dP/dt-EDD (Table 2). In contrast to the CTL condition, after the induction of HF, L-NMMA significantly augmented β-adrenergic contractility at both infusion rates (Table 2). Moreover, the effect was the same for the 10 and 20 mg · kg⁻¹ · h⁻¹ infusion rates, assessed by 3-way ANOVA.

To assess whether caveolin-3 protein abundance was correlated with the effect of L-NMMA to augment dobutamine-stimulated +dP/dt, we quantified caveolin-3 abundance in 4 dogs with HF. Caveolin-3 abundance positively correlated with the L-NMMA effect (r=0.9, P=0.03; Figure 4).

In contrast to β-adrenergic effects, inhibition of NOS affected resting myocardial contractility in CTL but not HF dogs, producing concentration-dependent reductions in myocardial contractility. No effect was observed at 10 mg · kg⁻¹ · h⁻¹, but a progressive decline in +dP/dt occurred with the 20 mg · kg⁻¹ · h⁻¹ infusion, reaching a plateau of −19±4%.

Figure 1. Protein abundance of caveolin-3 (A and B) and caveolin-1 (C and D) in canine myocardium before and after the induction of HF. A, Representative Western blot using monoclonal anti–caveolin-3 antibody (18 kDa). B, Bar graph summarizing caveolin-3 optical density from 2 Western blots. C, Representative Western blot using monoclonal anti–caveolin-1 antibody (22 kDa). D, Bar graph summarizing caveolin-1 optical density from 2 Western blots. +C indicates positive control; BAE, bovine aortic endothelial cell. *P=0.01 vs control.
After induction of HF, neither infusion of L-NMMA affected resting contractility. These data suggest that although NO exerts an increased contribution to β-adrenergic inotropic responses in heart failure, its effect on resting contractility is reduced.

**Effect of NOS Inhibition on Loading Conditions**

As anticipated, L-NMMA produced potent dose-dependent increases in arterial afterload (Ea) in the CTL group, consistent with increased vascular tone from inhibition of vascular endothelial NOS. Ea increased by 40±11% and 70±15% in response to the 10 and 20 mg/kg doses, respectively. In the HF group, pressor responses were markedly attenuated, and Ea rose by 22±5% and 15±7% for the 10 and 20 mg · kg⁻¹ · h⁻¹ infusion rates, respectively (P=0.05 for each versus baseline, P=NS for comparison between the 2 dose responses). Neither infusion rate of L-NMMA changed EDD (preload) in the CTL or HF groups.

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**Figure 2.** A and B, Electron microscopic visualization of plasma-membrane caveolae in membranes of myocytes from dogs before (A) and after (B) pacing-induced HF. Shown are ×27 500 (left) and ×74 000 (right) magnifications of the same membrane. C, Average data from 14 to 16 myocytes from 2 dogs for each group. *P<0.005 vs control.
NOS Enzyme Activity and Protein Abundance
LV myocardium from pacing-induced cardiomyopathic dogs has previously been shown to have unchanged levels of NOS activity. In the present study, we confirmed this observation: After the induction of HF, Ca$^{2+}$-dependent NOS activity was unchanged from the CTL value (4.1 ± 0.6 versus 3.9 ± 0.6 pmol/mg protein in CTL versus HF groups, respectively; n = 11 or 12 each), and Ca$^{2+}$-independent (NOS2) activity was not detected in either case. Western blotting using a NOS3 antibody revealed similar protein abundance in HF and CTL myocardium, and NOS2 was not detected in either CTL or HF tissue by Western blot (data not shown).

Discussion
The major new finding of the present study is that myocardium from dogs with pacing-induced cardiomyopathy exhibits an increase in both caveolin-3 protein abundance and membrane caveolae. Cardiomyopathic dogs had augmentation of $\beta$-adrenergic inotropic responses to NOS inhibition that was similar to that in humans with idiopathic dilated cardiomyopathy, and the abundance of caveolin-3 was positively correlated with augmentation of inotropic responses elicited by NOS inhibition. In contrast, L-NMMA reduced basal contractility in CTL but not HF dogs, suggesting decreased basal NO activity in HF. Despite increased abundance, caveolin-3 had similar subcellular localization and colocalization with NOS3 in HF compared with CTL myocytes. These findings suggest a novel mechanism by which agonist-stimulated NOS activity is increased in the failing heart and have implications for other signaling pathways regulated by caveolae.

Effect of NO on Myocardial Contractility
The results of the present study regarding myocardial contractile responses are in agreement with several earlier studies performed in vitro and in vivo showing that inhibition of NOS leads to an enhancement of $\beta$-adrenergic inotropic responses. This observation has particular relevance to situations such as sepsis, aging, and HF, which are associated with $\beta$-adrenergic chronotropic and inotropic downregulation.

![Figure 3. Two-photon imaging of myocytes from CTL group (top row) and HF group (bottom row) stained with caveolin-3 (left column, green) and NOS3 (middle column, red). Caveolin-3 localized to the sarcolemma and T tubules in both CTL and HF myocytes, whereas NOS3 localized more diffusely throughout the cell. Shown in the right column in yellow is the colocalization between caveolin-3 and NOS-3. As depicted, the colocalization occurred at the sarcolemma and T tubules.](http://circres.ahajournals.org/)

### Table 2. Inotropic Response to Dobutamine (Percentage of Increase)

<table>
<thead>
<tr>
<th></th>
<th>Dobutamine Effect</th>
<th>+ L-NMMA 10 mg · kg$^{-1}$ · min$^{-1}$</th>
<th>+ L-NMMA 20 mg · kg$^{-1}$ · min$^{-1}$</th>
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<td>CTL</td>
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<tr>
<td>Peak +dP/dt</td>
<td>36±7</td>
<td>34±10</td>
<td>66±24†</td>
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<tr>
<td>Ees</td>
<td>25±8</td>
<td>27±20</td>
<td>144±55*</td>
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<tr>
<td>+dP/dt-EDD</td>
<td>47±13</td>
<td>49±19</td>
<td>132±34†</td>
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<tr>
<td>HF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak +dP/dt</td>
<td>48±7</td>
<td>61±17*</td>
<td>54±7*</td>
</tr>
<tr>
<td>Ees</td>
<td>22±6</td>
<td>50±16*</td>
<td>35±15*</td>
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<tr>
<td>+dP/dt-EDD</td>
<td>32±10</td>
<td>93±13†</td>
<td>58±22†</td>
</tr>
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</table>

Values are mean±SEM. Dobutamine was 5 μg · kg$^{-1}$ · min$^{-1}$ for CTL and 10 to 15 μg · kg$^{-1}$ · min$^{-1}$ for HF.

*P<0.05 and †P<0.01 vs dobutamine.
and in which NO has been implicated as contributing to these changes. With regard to HF, we have previously shown that L-NMMA enhances adrenergic contractility to a greater degree in patients with idiopathic dilated cardiomyopathy than in control subjects with normal LV function.1

The mechanism(s) contributing to enhanced inotropic responses with NOS inhibition in HF remains controversial. On the one hand, it has been suggested that induction of high-output NOS2,6,26 in failing myocardium leads to excess NO production. This view has received support from studies reporting the appearance of circulating cytokines capable of stimulating NOS2 induction in HF.27 On the other hand, NOS2 induction is not observed in genetic28 and pacing-induced16 HF models, and coronary sinus NO metabolites are decreased with HF in experimental models and in humans.7 One possible explanation that reconciles these seemingly conflicting findings may be differential NO signaling under resting versus stimulated conditions. The NO metabolite studies were performed in the absence β-adrenergic29 activation or heart-rate increases,30 which stimulate NO production.

Our observations regarding the effect of L-NMMA on adrenergic contractility suggest differential NO responses at baseline and during β-adrenergic stimulation. L-NMMA suppressed resting myocardial contractility in CTL but not HF animals. The loss of NO pathway activity in HF animals is consistent with an increased inhibitory factor, such as caveolin. Divergent NO influences on contractility (inhibiting resting contractility but augmenting stimulated contractile responses) have been previously observed.18,31 NO signaling exerts its effects on β-adrenergic inotropy most likely via cAMP production, which antagonizes the effects of cGMP.32–34 Different cGMP-independent NO-signaling pathways may also contribute to NO influences on resting myocardial performance. Notably, NO activates the L-type calcium channel and the ryanodine receptor via thiol nitrosylation reactions. Further support for the role of NO in Ca2+ cycling is provided by the observation that NOS1 localizes to cardiac sarcoplasmic reticulum.37

**Role of Caveolins in NO Signaling**

Caveolins are scaffolding proteins found in caveolae, which are plasmalemmal microdomains that participate in signal transduction by means of colocalizing membrane receptors with signal transduction effectors.38–40 Because caveolin inhibits NOS activity by preventing calmodulin activation, it may exert dual regulation of NOS; inhibition of basal activity yet augmentation of agonist-stimulated actions. In this regard, Feron et al41 have shown that agonist-stimulated NOS signaling is absent in isolated cardiac myocytes transfected with myristoylation-deficient NOS3 that is unable to interact with caveolin, but reconstituted by transfection of NOS3 able to bind caveolin-3. As discussed above, our physiological observations with L-NMMA are consistent with the paradigm that NO pathway activity in HF is reduced basally but augmented during agonist stimulation.

Given findings previously reported and confirmed in the present study that NOS isoforms or activity are unchanged in failing canine ventricular myocardium, we explored the hypothesis that an endogenous regulator of NOS action might be altered in HF. Western analysis revealed increased caveolin-3 protein abundance, and electron microscopy showed increased numbers of myocyte sarcolemmal caveolae. Confocal imaging identified caveolin-3 localized to the sarcolemma and T tubules and colocalized with NOS3 at these sites in HF myocytes. Moreover, caveolin-3 abundance correlated with the augmentation of dobutamine contractility due to NOS inhibition in HF dogs.

Alterations in caveolin and/or caveolae are only recently being implicated in pathophysiology. For example, caveolin-3 gene mutations and increased protein abundance are observed in patients with muscular dystrophy. Myocardial caveolin abundance is reduced by the infusion of isoproterenol in mice,12 possibly via cAMP production, as demonstrated in rat myocytes.44 Thus, the HF-associated reduction in myocardial cAMP production may contribute to increased caveolin abundance in this condition.

These observations are limited because we could not assess functional consequences of acute perturbation of caveolin-3 abundance or activity. There are no described pharmacological means to do so, and current evaluations of caveolin function have required in vitro manipulation, such as the application of antisense RNA or caveolin peptide fragments.45 Accordingly, it is important to consider that increased NO pathway activity in HF may result from changes in pathways unrelated to caveolin.46 For example, factors such as oxidative stress or altered phosphodiesterase activity may modulate NO signaling.33,46,47 Moreover, increased caveolin-3 abundance may play roles in HF that are unrelated to NOS and that may influence both myocardial structure and function.48–50 In this regard, there are reports of both receptor translocation50 and away from caveolae during agonist stimulation. The implications of these pathways for cardiac function in HF await future study.

**Figure 4.** Impact of caveolin-3 abundance on augmentation of dobutamine-stimulated inotropic responses by L-NMMA. Chronically instrumented dogs were studied after the induction of HF by rapid pacing for 4 weeks. The inotropic response to dobutamine was measured by Western blot from full-thickness LV myocardial samples obtained at euthanasia.
The present observations clarify an earlier controversy regarding the physiological impact of NOS inhibition on myocardial contractility in normal LV function. At the lower concentration tested (10 mg · kg⁻¹ · h⁻¹), L-NMMA augmented β-adrenergic inotropy in HF but not CT1 animals, in a manner similar to that found in our earlier human observations. Only at higher L-NMMA concentrations (20 mg · kg⁻¹ · h⁻¹) was the inotropic response augmented in CT1 animals. Thus, it is likely that earlier observations not showing augmentation of β-adrenergic inotropy in subjects with normal LV function, including our own studies, were limited by either inadequate concentration or time of administration of a NOS inhibitor.

In the present study, we have demonstrated that dogs with pacing-induced HF exhibit increased sensitivity to the augmentation of β-adrenergic inotropic responses by NOS inhibition. These functional changes occurred in the absence of changes in NOS isoform activity or abundance. Failing myocardium and myocytes exhibited increased abundance of caveolin-3 and caveolae. Thus, increases in the concentration of these caveolar scaffolding proteins that compartmentalize NOS with stimulatory agonist signals suggest a novel mechanism by which the NO pathway activity may be increased in HF.

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References


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