Endothelial nitric oxide synthase increases in left atria of dogs with pacing-induced heart failure

FADI H. KHADOUR,1 DARRYL W. O’BRIEN,2 YULING FU,2 PAUL W. ARMSTRONG,2 AND RICHARD SCHULZ1,3
Departments of 1Pharmacology, 2Medicine, and 3Pediatrics, Cardiovascular Research Group, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

Khadour, Fadi H., Darryl W. O’Brien, Yuling Fu, Paul W. Armstrong, and Richard Schulz. Endothelial nitric oxide synthase increases in left atria of dogs with pacing-induced heart failure. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1971–H1978, 1998.—In congestive heart failure (CHF) the alterations in cardiac NO synthase (NOS) isoforms activity and expression are incompletely documented, and the chamber specificity of these changes is unknown. We studied plasma nitrate-nitrite (NOx) levels and in nonpaced control dogs and in dogs paced for 2 or 21 days into CHF. Plasma NOx rose significantly after 7 and 21 days of pacing, whereas creatinine levels remained unchanged. In control dogs Ca2+-dependent NOS activity in left atria was double that of right or left ventricular activity. In paced animals the activity increased only in the atria after 21 but not 2 days of pacing. Levels of endothelial NOS (eNOS) protein were enhanced in the left atria but not ventricles after 21 days of pacing because of a greater quantity of the 150-kDa but not the 135-kDa eNOS. Ca2+-independent NOS activity in left atria was double that of right or left ventricular tissue. The specific upregulation of eNOS in the left atria suggests that NO production may be enhanced in comparison with other tissues, and that NO production may play a role in the pathogenesis of CHF.

Congestive heart failure; atrial hypertrophy; nitrate-nitrite

CONGESTIVE HEART FAILURE (CHF) is a complex syndrome of myocardial dysfunction and diminished cardiac output. Experimental and clinical research indicates that an alteration in cardiac nitric oxide (NO) production may play a role in the pathogenesis of CHF. Three isoforms of nitric oxide synthase (NOS), which catalyze NO biosynthesis from L-arginine have been demonstrated in the heart. There are two constitutively expressed Ca2+-dependent isoforms: endothelial NOS (eNOS), located in the coronary vascular endothelium (1), endocardial endothelium (32), and cardiac myocytes (5, 31), and a neuronal isoform (nNOS) that has been localized in some cardiac neurons (38). An inducible, Ca2+-independent isoform (iNOS) is expressed after exposure of the heart to bacterial endotoxin or proinflammatory cytokines (23, 31). Cardiac constitutive NO production plays physiological roles in regulating coronary blood flow (1) and modulating contractile function (35) by exerting negative inotropic (4, 5, 9) and chronotropic (4, 15) actions. Upregulation of cardiac NO biosynthesis, through iNOS expression, causes a pathophysiological depression in myocardial contractile function (6, 30, 39) and may have a direct cytolytic effect on cardiac myocytes (28).

In a study of heart failure patients, Winlaw et al. (42) observed that plasma levels of nitrate, a stable end product of endogenous NO metabolism, were elevated in these patients compared with healthy individuals. Subsequently, the same investigators reported that the increase in plasma nitrate levels positively correlated with the severity of heart failure (43). However, measurements of cardiac iNOS in human CHF have revealed conflicting results. DeBelder et al. (11, 12) found a Ca2+-independent NOS activity in right ventricular myocardial biopsy specimens of patients with dilated cardiomyopathy and myocarditis but only a Ca2+-dependent NOS activity in those with ischemic and valvular heart diseases. Expression of iNOS mRNA and protein in the ventricles was reported by Haywood et al. (16) in heart failure resulting from ischemic and valvular heart diseases, as well as in dilated cardiomyopathy. In contrast, Thoenes et al. (40) detected iNOS protein in failing hearts from patients in septic shock only. These human studies, however, did not measure the activity of iNOS (16, 40) or examine possible changes in eNOS activity in right ventricular tissues, whereas others reported findings with left ventricular samples. There are no data regarding expression of NOS in the atria of normal or failing hearts; however, this would be of interest because NO has many potential effects on atrial function and structural integrity for reasons given above.

We hypothesized that ventricular NO production is enhanced, via iNOS expression, during the development of pacing-induced CHF in dogs. This model of heart failure, induced by rapid ventricular pacing at 250 beats/min for 21 days, produces clinical, hemodynamic, and neurohumoral changes similar to those found in humans (2). In normal dogs, dogs subjected to 2 days of pacing, and those to 21 days of pacing (severe CHF) our objectives were to determine 1) NO production via measurement of plasma nitrate-nitrite (NOx), 2) the profile of changes in Ca2+-dependent and -independent NOS activities in the left atria in comparison with those in the left and right ventricles, and 3) the chamber-specific isoform(s) of NOS responsible for these changes.

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MATERIALS AND METHODS

This study was approved and conducted in accordance with the Health Sciences Animal Welfare Committee of the University of Alberta and the Canadian Council of Animal Care. The study group consisted of twenty adult male mongrel dogs weighing 23 ± 1 kg. All dogs were conditioned to the laboratory environment at least 2 days before the beginning of the experiment. Each dog was housed in an individual cage in a room with a temperature of 20°C and lights on from 0700 to 1800. Animals were fed a fixed commercially available dog food once per day in the morning and had free access to tap water. Dogs were randomly divided into three groups: 1) a control group that was not paced (n = 8), 2) dogs paced for 2 days (n = 5), and 3) dogs paced for 21 days into heart failure (n = 7).

Induction of heart failure. Heart failure was induced by right ventricular pacing using a protocol that is known to produce changes in hemodynamic parameters indicative of the development of heart failure (2, 13, 29). In brief, a programmable pulse generator (Medtronic Minex-8341, Medtronic, Mississauga, ON, Canada) was inserted into a subcutaneous cervical pocket and a unipolar pacemaker lead was positioned into the right ventricular apex under general anesthesia with halothane. Each dog was implanted with an externalized chronic indwelling catheter placed in the aortic arch that was used for blood sampling and pressure measurement during hemodynamic studies. Catheter patency was maintained by flushing every other day with saline followed by heparin (1.5 ml, 1,000 IU/ml). Dogs were allowed to recover from surgery for 6–7 days before the beginning of ventricular pacing. For the induction of CHF the pulse generator was programmed to 250 beats/min using a Medtronic 9710A Programmer. Once pacing was initiated daily clinical assessment was performed, including observation of animal behavior, body weight, heart rate, respiratory rate, dyspnea, gingival cyanosis, and ascites.

Echocardiographic and hemodynamic measurements. Hemodynamic and echocardiographic measurements were performed simultaneously at baseline during sinus rhythm just before the initiation of pacing and subsequently within 30 min after cessation of pacing. Dogs paced for 21 days were also evaluated by echocardiography at the end of the first week of pacing. Transthoracic echocardiographic studies were carried out using a Sonos 1000 series echocardiographic imaging system (Hewlett-Packard Medical Products Group, Andover, MA) with a 3.5- or 5-MHz transducer as previously described (13) and were used to determine left ventricular weight and ejection fraction as well as left atrial and ventricular cross-sectional areas. Hemodynamic studies were performed on sedated animals in the right decubitus position. Intravenous morphine boluses of 1–2 mg each (total dose of 4–14 mg) were given as necessary for sedation. A Swan-Ganz balloon-tipped thermodilution catheter (7-F) and a high-fidelity, microtransducer-tipped pigtail Millar catheter (Millar Instruments, Houston, TX) were introduced via the femoral vein (Swan-Ganz) and artery (Millar) to the pulmonary artery and left ventricle, respectively. Right atrial pressure and left ventricular end-diastolic pressure were recorded on a data acquisition system (Datag Instruments, Akron, OH). Cardiac output was determined by the thermodilution technique (COM-2, Baxter-Edwards, Santa Ana, CA), as previously reported (13). After the final hemodynamic and echocardiographic assessments, dogs were anesthetized with pentobarbital (50 mg/kg iv), the chest was opened, and the heart was removed. The left atrial appendages were dissected out and weighed, and tissue samples from the left ventricle (cardiac apex), right ventricular free wall, and left atrium were taken. The endocardium was removed from left atrial samples by scraping the luminal surface with a scalpel blade and from ventricular tissues by removing the inner layer to an approximate depth of 3–4 mm. Myocardial tissues were then rapidly frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

Blood sampling and measurements of plasma NOx and creatinine Blood samples were withdrawn from the aorta just before the initiation of pacing and subsequently within 30 min after cessation of pacing. Dogs paced for 21 days were also sampled at the end of the first week. The plasma was separated from blood by centrifugation at 2,000 g for 5 min at 4°C and stored at −80°C until analysis. After thawing, plasma was diluted 1:1 with deionized water. Next, 400 µl of the diluted plasma were deproteinized by centrifugal ultrafiltration (Millipore Ultrafree-MC microcentrifuge tubes UFC3, Bedford, MA). Ultrafiltrates were analyzed for total NOx content by the method of Green et al. (14). The limit of detection was 0.1 µmol/l for both nitrite and nitrate. Renal function was measured by determination of plasma creatinine levels using an automated colorimetric method based on Jaffe’s reaction at the University of Alberta Hospital Laboratory.

Preparation of atrial and ventricular homogenates for determination of NOS activities. The frozen atrial and ventricular myocardial tissues were used for the assay of NOS activities as previously described (19, 30, 31). First, tissues were pulverized in liquid nitrogen, homogenized, and subsequently centrifuged (1,000 g, 5 min, 4°C). Next, the resultant supernatant was utilized for measurement of atrial or ventricular NOS activities by the conversion of L-[14C]arginine to [14C]citrulline. Citrulline production was normalized to the protein concentration of the homogenates, as determined by bichromonic acid assay utilizing BSA as a standard, and was expressed as picomole per minute per milligram protein. The limit of detection was 0.1 pmol·min⁻¹·mg protein⁻¹.

Western blotting and densitometric analysis. Western blot analysis was performed as previously described (19). Sample proteins (50 µg/lane) were size-fractionated by SDS-PAGE (7 and 9%) and transferred to nitrocellulose membranes by wet electroblotting for 170–180 min. In addition to the prestained SDS-PAGE protein standards (Bio-Rad, Hercules, CA), a positive control of recombinant bovine eNOS (Cayman Chemical, Ann Arbor, MI) was utilized. The membranes were blocked at room temperature in 3% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 3 h, incubated with rabbit polyclonal antibody directed against bovine eNOS (Affinity Bioreagents, Golden, CO, prepared in 1% BSA in TBS-T at 1:1,000 dilution) for 1.5 h, and then washed with TBS-T three times (5 min each). This antibody detects canine eNOS but does not cross-react with either iNOS or nNOS. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted at 1:2,000 with 1% BSA in TBS-T for 60 min, washed three times with TBS-T, and finally with PBS alone (5 min each). Immunoreactive proteins were detected by using an enhanced horseradish peroxidase/luminol chemiluminescence reaction kit (ECL, Amersham, Oakville, ON, Canada). Autoradiographs were obtained by exposure to X-ray film (Kodak, X-OMAT) for 1 s, and the density of the bands was quantified by scanning densitometry as previously reported (19) and expressed as a percentage of the positive control.
Table 1. Hemodynamic parameters before and after 2 or 21 days of rapid ventricular pacing in dogs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Preparing (12)</th>
<th>2 days (5)</th>
<th>21 days (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV systolic pressure, mmHg</td>
<td>139 ± 23</td>
<td>123 ± 22</td>
<td>106 ± 11*</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>8 ± 4</td>
<td>13 ± 4</td>
<td>31 ± 9*</td>
</tr>
<tr>
<td>LV dp/dt, mmHg/s</td>
<td>2,778 ± 316</td>
<td>1,755 ± 141*</td>
<td>1,474 ± 253*</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>111 ± 6.2</td>
<td>93.2 ± 4</td>
<td>87.6 ± 2.7*</td>
</tr>
<tr>
<td>Right atrial pressure, mmHg</td>
<td>1.8 ± 0.5</td>
<td>4.9 ± 1.1</td>
<td>7.5 ± 1.4*</td>
</tr>
<tr>
<td>PCWP, mmHg</td>
<td>3.1 ± 0.6</td>
<td>11.1 ± 1.2</td>
<td>19.7 ± 4.7*</td>
</tr>
<tr>
<td>Heart rate (sinus rhythm), bpm</td>
<td>55 ± 1</td>
<td>44 ± 2*</td>
<td>25 ± 2*</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of dogs in parentheses. LV ejection fraction was determined by echocardiography. dp/dt, rate of rise of pressure; PCWP, pulmonary capillary wedge pressure. *P < 0.05 vs. preparing.

Materials. All other reagents were purchased from Sigma except L-[U-14C]arginine monohydrochloride (Amersham) and N6-monomethyl-L-arginine acetate (Alexis, San Diego, CA).

Statistical analyses. Data are presented as means ± SE of samples from n separate experimental animals. One-way analysis of variance followed by the Tukey post hoc test was used to evaluate differences between the groups. Data analysis was done using a statistical software package (SigmaStat, version 2.0, Jandel Scientific, San Rafael, CA). Differences were considered significant at P < 0.05.

RESULTS

CHF induced by rapid ventricular pacing in dogs. Dogs paced for 2 days showed only an increase in heart rate (sinus rhythm) and a decrease in left ventricular rate of rise in pressure (dp/dt) and ejection fraction (Table 1). In contrast, dogs paced for 21 days showed an increase in left ventricular end-diastolic pressure, right atrial pressure, pulmonary capillary wedge pressure, and heart rate with a decrease in left ventricular systolic pressure and dp/dt, mean arterial pressure, cardiac output, and ejection fraction consistent with the induction of CHF (2, 13, 29). The body weight of the dogs did not significantly change from the prepacing state of 21.0 ± 1.7 kg (n = 12) to 22.7 ± 1.1 kg (n = 5) after 2 days and 21.4 ± 1.6 kg (n = 7) after 21 days of pacing. As shown in Table 2, left atrial appendage weight doubled after 21 days of pacing in comparison to control and 2-day values. However, left ventricular mass and total heart weight remained unchanged between control and paced dogs. Accordingly, the ratio of left atrial appendage weight to heart weight increased significantly after 21 days of pacing compared with controls and with dogs paced for 2 days. A significant dilatation of the left atrium occurred as early as 2 days of pacing and increased as pacing continued until 21 days, as evidenced from the data of left atrial cross-sectional area (Table 2). Left ventricular cross-sectional area was significantly elevated first after 7 days and remained at this level after 21 days of pacing.

Plasma NOx and creatinine levels. Figure 1 shows the changes in plasma NOx and creatinine levels with ventricular pacing in dogs. Before pacing was started, the mean plasma NOx level was 19 ± 1 µmol/l (n = 12). After 2 days of pacing it was unchanged from control and then it more than doubled after 7 days of pacing (Fig. 1A). After 21 days of pacing plasma NOx remained significantly higher than prepacing and 2-day values. These changes in plasma NOx levels occurred in the absence of any significant alteration in plasma creatinine levels (Fig. 1B).

Atrial and ventricular NOS activities. The changes in Ca2+-dependent NOS activity in different chambers of the heart, in control and paced dogs, are shown in Fig. 2. Left atrial tissues from control dogs had Ca2+-dependent NOS activity of about 0.4 pmol·min⁻¹·mg protein⁻¹. This was significantly higher than the activity in both left and right ventricular tissues from the same animals. Moreover, the left atrial activity significantly increased in dogs paced for 21 days but not in those paced for 2 days only. In marked contrast to these findings, no significant changes in left or right ventricular Ca2+-dependent NOS activities were detected in dogs paced for 2 or 21 days. Thus after 21 days of pacing the enzyme activity in the left atria was ~3.5-fold higher than that in either left or right ventricle. Ca2+-independent NOS activity was not detectable in any of these tissues (data not shown).

Table 2. Changes in left atrial and ventricular weights and cross-sectional area before and after 2, 7, or 21 days of rapid ventricular pacing in dogs

<table>
<thead>
<tr>
<th>Weight</th>
<th>Preparing (12)</th>
<th>2 days (5)</th>
<th>7 days (7)</th>
<th>21 days (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left atrial appendage, g</td>
<td>1.8 ± 0.2 (8)</td>
<td>1.9 ± 0.2</td>
<td>112 ± 5</td>
<td>101 ± 6</td>
</tr>
<tr>
<td>Left ventricle, g</td>
<td>96 ± 4</td>
<td>112 ± 5</td>
<td>101 ± 6</td>
<td>104 ± 1</td>
</tr>
<tr>
<td>Heart, g</td>
<td>177 ± 14 (8)</td>
<td>180 ± 7</td>
<td>179 ± 13</td>
<td>179 ± 13</td>
</tr>
<tr>
<td>Left atrial appendage-heart, g</td>
<td>0.009 ± 0.003</td>
<td>0.010 ± 0.002</td>
<td>0.020 ± 0.005†</td>
<td></td>
</tr>
<tr>
<td>Cross-sectional area, cm²</td>
<td>0.8 ± 0.3</td>
<td>10.6 ± 0.4*</td>
<td>12.7 ± 0.6*†</td>
<td>15.2 ± 0.6*†</td>
</tr>
<tr>
<td>Left atrium, g</td>
<td>14.4 ± 0.6</td>
<td>14.7 ± 0.8</td>
<td>19.2 ± 0.9*†</td>
<td>21.4 ± 1.4*†</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of dogs in parentheses. Left ventricle weight and left atrial and ventricular cross-sectional areas were determined by echocardiography. Other weights were obtained by weighing tissues after the dog was killed. Left atrial appendage and total heart weights under “Preparing” were obtained from the nonpaced control group (n = 8). *P < 0.05 vs. preparing or control. †P < 0.05 vs. 2 days.
Western blot and densitometric analysis. Figure 3 shows representative blots of immunoreactive eNOS proteins from the heart chambers of control, 2-, and 21-day paced dogs as resolved by 7% SDS-PAGE. In left atrial tissues, three distinct bands can be seen in the region corresponding to the known molecular mass of eNOS. On the basis of eNOS studies in rat cardiac myocytes (7), the upper and middle bands from the left atrial tissues represent the 150- and 135-kDa eNOS proteins, respectively. At this time we do not know the identity of the lowest band (estimated molecular mass of 120–125 kDa). These blots demonstrate a marked enhancement of expression of the 150-kDa eNOS protein in the left atrium concomitant with the development of heart failure after 21 days of pacing. In left and right ventricular tissues, only two bands were seen corresponding to the 150- and 135-kDa eNOS proteins. The density of these two bands did not change significantly with pacing. Triplet atrial and doublet ventricular eNOS bands were also detected in a similar fashion using 9% SDS-PAGE in the current study (data not shown). As depicted in Fig. 4, densitometric analyses of all hearts subjected to Western blotting show enhanced expression of the 150-kDa eNOS band at 21 days of pacing in left atrial tissues. This was associated with a significant increase in total eNOS expression, whereas no significant changes were noted in the 135-kDa band. The ratio of 150/135-kDa band densities, approximately 1/6–1/7 in control atrial tissues, increased to about 1/1 in atria from dogs paced for 21 days into heart failure. The density of the 150-kDa as well as that of 135-kDa eNOS bands did not change significantly in either left or right ventricular tissues. Consequently, the density ratio between these two bands in ventricular tissues after pacing was also unchanged in comparison to its value in the control group (Fig. 4).

**DISCUSSION**

This study is the first to demonstrate the enhancement of left atrial, but not ventricular, Ca\(^{2+}\)-dependent NOS activity with the induction of CHF by rapid ventricular pacing in dogs. The changes in enzyme activity were accompanied by an enhanced total eNOS protein expression, specifically the 150-kDa but not the 135-kDa eNOS.

The observed hemodynamic changes in dogs paced for 21 days document the occurrence of severe heart failure.
failure in these animals as previously reported in this model (2, 13, 22, 29). On the other hand, dogs paced for 2 days had some hemodynamic changes, in the absence of clinical signs of failure, suggesting the development of an early ventricular dysfunction. A unique finding in this canine model of heart failure is the development of atrial but not ventricular hypertrophy (22, 33), which is possibly caused by contraction of the atria against closed atrioventricular valves due to asynchronous electrical activation of the cardiac chambers (22, 33).

Pacing-induced heart failure caused an elevation in plasma NOx levels in dogs as early as 7 days after pacing and remained so until pacing was terminated. This occurred in the absence of any measurable renal impairment as the creatinine levels were within the normal range (70–150 mmol/l) for this parameter in all three groups. This is in accordance with our previous report of increased plasma NOx levels with age in a rat genetic model of heart failure, in which no changes in plasma creatinine were also observed (19). Therefore, enhanced levels of NOx are not a consequence of renal dysfunction. In contrast, Bernstein et al. (8) reported in dogs paced at 210 beats/min in the first 3 wk then at 240 beats/min for another week into heart failure, no changes in plasma NOx within the first 3 wk of pacing, whereas it then rose in the fourth week in direct relation to the increase in creatinine levels. They suggested that the rise in plasma NOx was due to a decline in renal function and not an increase in NO production. This controversy may be related to differences in the pacing protocols and the severity of pacing-induced heart failure. Because plasma NOx does not discriminate as to the tissue or organ source of the increased NO production, the changes in plasma NOx levels cannot be attributed simply to enhanced atrial NOS activity. We cannot substantiate the source of increased plasma NOx early in the development of heart failure (7 days) because myocardial NOS activity was not measured at this time. The contribution of NO from other sources such as the peripheral vasculature cannot be totally excluded, although a reduction in eNOS gene expression was reported in the aortic endothelium of dogs paced via the left ventricle for 1 mo into severe CHF (37).

This report is the first to show that normal left atria have a significantly greater Ca2+-dependent NOS activity than that in either left or right ventricles. We have also seen significantly greater Ca2+-dependent NOS activity in the atria compared with ventricles in normal rat hearts (Khador and Schulz, unpublished observations). Furthermore, atrial but not ventricular Ca2+-dependent NOS activity increased with pacing for 21 days and the development of heart failure. This activity measures in principle that from both eNOS and nNOS isoforms. The latter, however, has a limited distribution within nerve fibers in the heart (38) and has not been...
reported to contribute significantly to the cardiac Ca$^{2+}$-dependent NOS activity. Enhanced eNOS activity may be an effect of pacing itself because isolated adult rat ventricular myocytes show increased NOS activity, as measured by nitrite accumulation in culture media, with increasing pacing frequency (18) or it may be caused by increased stretching (3) due to dilatation of the atria. On the other hand, the increased atrial eNOS activity in association with atrial, but not ventricular, hypertrophy suggests that the alterations in eNOS are more directly related to the changes in cardiac structure (hypertrophy) induced by the etiologic factor(s) of heart failure rather than the failure state per se. This is supported by our data in a genetic model of heart failure in rats where left ventricular hypertrophy was accompanied by upregulation of eNOS in the same chamber (19). Upregulation of NO production in these settings may be considered a counterbalancing mechanism to the contractions of the hypertrophied atria or ventricles (19) because NO is known to have negative inotropic effects in the normal heart (4, 5, 9).

Although our data are in accordance with other reports showing an enhanced endothelium-dependent relaxation response and NO production in the coronary arteries of dogs with pacing-induced CHF (20, 25), they contrast with the evidence for a diminished coronary and peripheral vascular NO generation reported by other investigators (37, 41). This controversy may be related to the severity of CHF in any particular study as determined by the rate or the duration of pacing. Moreover, these investigators utilized isolated coronary vascular rings (20, 25) or studied the whole coronary circulation in situ (41) compared with our findings in the left atrial tissues. On the other hand, these data may suggest a differential regulation of NO generation in the coronary and peripheral vascular tree from that in atrial tissue in CHF induced by ventricular pacing. Because the endocardium was removed from all heart samples, we can exclude its contribution to atrial NOS activity.

Previous studies in humans addressed the hypothesis that NO elaborated by the induction of Ca$^{2+}$-independent NOS (iNOS) is a potential factor associated with the development of CHF. They reported that the expression of iNOS mRNA and/or protein takes place in ventricular but not atrial tissues and in patients with a variety of causes of failure (11, 12, 16, 40). Contrary to our expectation, iNOS activity was not detectable in atria and ventricles from control or paced dogs in this study. This is in accordance with a recent report from this laboratory whereby iNOS activity was also not detected in ventricles from heart failure-prone rats (19). These findings cannot be explained by the insensitivity of our NOS assay because we were able to detect Ca$^{2+}$-independent NOS activity in hearts from rats treated with bacterial endotoxin (19). In comparison with human studies, the lack of iNOS expression in this study or the rat genetic model of heart failure (19) may be related to species variations or because of differences in the etiologies of heart failure. Whether the upregulation of eNOS in dogs with heart failure is cardioprotective through vasodilatory, anti-aggregatory, and negative chronotropic actions of NO or detrimental in a manner usually ascribed to the expression of iNOS in the heart whereby enhanced NO production, in addition to its negative inotropic effects (4–6, 9, 30, 39), may induce myocyte death through cytolysis (28) or apoptosis (24) is unknown.

We have detected in the atria by Western blot analysis using a polyclonal eNOS antibody the presence of three bands corresponding to the known molecular weight of eNOS, whereas only two bands were found in the ventricles. Previous work demonstrated doublet bands corresponding to the 150- and 135-kDa proteins in adult rat ventricular myocytes (7) and in COS-7 cells that had been transfected with wild-type eNOS or myristoylation-deficient eNOS mutant cDNAs (10) using 7% SDS-PAGE. Unlike these studies (7, 10), in our hands multiple atrial and ventricular bands in dogs were also detected by 9% SDS-PAGE. At the present time, we do not know the nature of the third (lowest) band detected in the atrial samples, but it is unlikely that it represents a degradation product because atrial and ventricular tissues were harvested from dogs at the same time, processed, and analyzed simultaneously. It may be related to another posttranslational modification of eNOS in the atria, for example glycosylation that may not take place in the ventricles.

eNOS is synthesized as a 150-kDa protein (7), which undergoes posttranslational modification before it is targeted to sarcolemmal caveolae in a process that involves myristoylation (10, 34, 36) and palmitoylation (21, 36) as a 135-kDa protein. Our data indicate that pacing-induced heart failure increases the absolute abundance of 150-kDa eNOS as well as its expression relative to that of the 135-kDa. Because total eNOS and 150-kDa expression were enhanced with no changes in the expression of the 135-kDa protein, it is possible that these changes are related to increased eNOS mRNA synthesis in association with insufficient intracellular mechanisms for the processing and trafficking of eNOS to the plasmalemmal caveolae. Interestingly, these alterations in the expression of 150-kDa eNOS were accompanied by increased enzyme activity, despite findings suggesting that posttranslational processing and targeting of eNOS into the plasmalemmal caveolae are necessary for its optimal activity (10, 21, 34, 36). In a study of cultured adult rat cardiomyocytes, Belhassen et al. (7) reported that even a short-term elevation in intracellular cAMP inhibits the posttranslational processing and the translocation of 150-kDa eNOS. However, either basal cAMP production or that stimulated by forskolin or isoproterenol is reduced in the ventricles of dogs paced into CHF (17, 27). If this is also the case for cAMP levels in atrial myocytes from CHF dogs, then the increase in the atrial 150-kDa eNOS seen in this study cannot be explained by the same mechanism.

Several limitations of this study warrant further discussion. This method of pacing-induced CHF in dogs may not model various types of human heart failure that develop as a result of ischemic or valvular heart
diseases or inflammatory cardiomyopathies but it does mimic ventricular dysfunction and cardiomegaly seen in patients with chronic uncontrolled tachycardia (26). Moreover, there are clear differences between dogs and humans with respect to age and the rate of development of CHF in addition to the possible species variations. We did not investigate right atrial tissues in this study as they were used in a different study and tissue sampling was limited to one site in each of the three chambers studied. There are, however, no previous data to support the possibility of differences in the activity and/or expression of NOS between and/or within heart chambers. In addition, we do not know the cellular source of enhanced eNOS expression in the left atria; whether this occurs in coronary endothelial cells and/or cardiac myocytes is unknown and would require immunohistochemical localization. Despite these limitations, our data stress the need to carefully examine the role of eNOS in the setting of human heart failure and hypertrophy, which has been overlooked. It may be of particular interest to investigate this in patients with mitral stenosis which causes left atrial but not left ventricular hypertrophy.

We have shown that increased left atrial, but not ventricular, Ca\(^2\)-dependent NOS activity along with enhanced expression of a 150-kDa eNOS occurs in vivo with rapid ventricular pacing-induced heart failure in dogs. Further investigation is necessary to reveal the consequences of these alterations in eNOS activity and expression in regard to the possible cardioprotective or detrimental roles of NO in the myocardium.

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Address for reprint requests: R. Schulz, Depts. of Pediatrics and Pharmacology, 4–62 Heritage Medical Research Center, Univ. of Alberta, Edmonton, Alberta, Canada T6G 2S2.

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