Increased Nitration of Sarcoplasmic Reticulum Ca$^{2+}$-ATPase in Human Heart Failure

Andrew J. Lokuta, PhD; Nathan A. Maertz, BS; Sivan Vadakkadath Meethal, PhD; Katherine T. Potter, MS; Timothy J. Kamp, MD, PhD; Héctor H. Valdivia, MD, PhD; Robert A. Haworth, PhD

**Background**—Reduced sarcoplasmic reticulum (SR) Ca$^{2+}$-ATPase (SERCA2a isoform) activity is a major determinant of reduced contractility in heart failure. Ca$^{2+}$-ATPase inactivation can occur through SERCA2a nitration. We therefore investigated the role of SERCA2a nitration in heart failure.

**Methods and Results**—We measured SERCA2a levels and nitrotyrosine levels in tissue from normal and failing human hearts using Western blots. We found that nitrotyrosine levels in idiopathic dilated cardiomyopathic (DCM) hearts were almost double those of control hearts in age-matched groups. Nitrotyrosine was dominantly present in a single protein with the molecular weight of SERCA2a, and immunoprecipitation confirmed that the protein recognized by the nitrotyrosine antibody was SERCA2a. There was a positive correlation between the time to half relaxation and the nitrotyrosine/SERCA2a content ($P<0.01$) in myocytes isolated from control and DCM hearts. In experiments with isolated SR vesicles from porcine hearts, we also showed that the Ca pump is inactivated by peroxynitrite exposure, and inactivation was prevented by protein kinase A pretreatment.

**Conclusions**—We conclude that SERCA2a inactivation by nitration may contribute to Ca pump failure and hence heart failure in DCM.

(Circulation. 2005;111:988-995.)

Key Words: heart failure • sarcoplasmic reticulum • calcium • nitric oxide

Contractile function of the heart depends strongly on the release of Ca from the sarcoplasmic reticulum (SR), and relaxation requires the removal of Ca from the cytosol by SR reuptake or by efflux from the cell. The lack of a positive force-frequency response in failure is related to a failure of the SR to load as frequency increases. Although SR Ca content is often reduced in heart failure, the relative contribution of changes in SR Ca uptake and SR Ca release to this is controversial. Failing human myocardium relaxes more slowly than normal myocardium, and Ca transients decline more slowly. This suggests reduced SR Ca pump activity, although the expression of the SR Ca pump protein SERCA2a may or may not be reduced in heart failure.

Increased nitration of the SERCA2 isoform of the SR Ca pump with aging has been observed in skeletal muscle, associated with decreased pump function. Protein nitration likely results from exposure to peroxynitrite, which is formed by the combination of NO and superoxide. Failing human hearts show increased inducible NO synthase (iNOS) expression, and levels of superoxide are also elevated in an animal model of failure. Additionally, failing human hearts exhibit the end-products of oxidative stress. These findings raise the possibility, investigated here, that SR Ca pump function in human heart failure could be impaired by pump nitration.

**Methods**

**Human Subjects**

Explanted human hearts were obtained through the transplant program of the Department of Surgery of the University of Wisconsin in accord with institutional consent protocols. Control hearts were donor hearts not transplanted because of age or performance. Failing hearts were all diagnosed with idiopathic dilated cardiomyopathy (DCM) without coronary artery disease.

**Preparation of Human Cardiac Homogenates**

Rapidly frozen human ventricular tissue was minced and homogenized in a chilled solution (1 g/5 mL) containing 20 mmol/L Na-PIPES (pH 6.8) and 10% sucrose with a protease inhibitor cocktail (Sigma P2714). The homogenate was centrifuged at 1000 g for 5 minutes at 4°C. The supernatant was quick-frozen and stored at −70°C. Protein was determined by the Bradford method.

**SDS-PAGE and Western Blots**

Homogenates were not allowed to sit at warm temperatures before dissolution in reducing Laemmli buffer at 37°C. Normal and failing samples were treated identically. Aliquots of homogenate (30 μg protein) were electrophoresed on a 4% to 15% linear gradient polyacrylamide gel, then transferred to a nitrocellulose membrane.
Blots were probed with anti-nitrotyrosine primary antibody (Cayman Chemical), a mouse monoclonal SERCA2a-specific antibody, a rabbit monoclonal calsequestrin primary antibody, and a mouse monoclonal phospholamban primary antibody, all from Affinity Bioreagents. Adherent primary antibody was quantified with the use of secondary antibodies either by peroxidase chemiluminescence or by infrared imaging. For peroxidase chemiluminescence (Figure 1), primary antibody staining was followed by a peroxidase-conjugated anti-mouse IgG secondary antibody (Calbiochem). Chemiluminescence was induced with the use of a SuperSignal detection kit (Pierce) and imaged on a digital camera. The membranes were then stained for total protein with Ponceau S and imaged again. The chemiluminescent signal was normalized to this total protein measure to compensate for any variability in the amount of protein applied in each lane. Simultaneous detection of SERCA2a, calsequestrin, and phospholamban was performed by infrared imaging of fluorescent secondary antibodies. Staining with primary antibodies was followed by AlexaFluor 680 goat anti-mouse IgG secondary antibody (Molecular Probes) and IRDye 800CW goat anti-rabbit IgG secondary antibody (Rockland Immunochemicals). The blots were imaged and quantified with an Odyssey Infrared Imaging System (LI-COR Biosciences). Comparability between gels was obtained with data from 1 sample that was run on every gel.

Immunoprecipitation of SR Ca\(^{2+}\)-ATPase

Homogenates were solubilized in a buffer containing 0.5% CHAPS (1 mg CHAPS/100 µg protein), 10 mmol/L Tris-HCl (pH 7.4), 0.3 mol/L sucrose, with protease inhibitors, for 60 minutes at 4°C. After centrifugation (80,000g for 20 minutes), SERCA2a/2b polyclonal antibody was added to the supernatant and incubated for 3 hours at 4°C. An IgG-agarose slurry was added and rotary mixed overnight at 4°C. The slurry was centrifuged at 4°C for 5 minutes and washed 2 times. Finally, the immunoprecipitated proteins were solubilized in Laemmli sample loading buffer, electrophoresed, and Western blotted as described.

Preparation of SR Microsomes From Swine Left Ventricular Tissue

Animals were treated in compliance with institutional guidelines. Pigs were anesthetized with ketamine (10 mg/kg) by intramuscular injection, a sternotomy was performed, and the heart was exposed. After cardioplegia, SR microsomes were isolated as described.\(^{15}\)

Peroxynitrite Treatment of SR Microsomes

One to 2 µL of 3 to 150 mmol/L peroxynitrite (CalBiochem) in 1 mol/L NaOH was added to 300-µL vesicles (0.4 mg/mL) in reaction buffer (0.3 mol/L sucrose, 50 mmol/L K-MOPS, pH 7.2), and incubated at 34°C for 3 minutes in the dark.

Protein Kinase A Treatment of SR Microsomes

Bovine heart protein kinase A (PKA) catalytic subunit (Sigma) was activated with dithiothreitol, then added (20 µg/mL) to 300-µL vesicles (0.4 mg/mL) in reaction buffer containing ATP (0.5 mmol/L) and okadaic acid (1 µmol/L) and incubated at 34°C for 3 minutes.

\(45\text{Ca}^{2+}\) Translocation Measurements

Treated SR microsomes were added (1:1 vol/vol) to uptake medium for measurement of oxalate-supported Ca uptake as described.\(^{16}\) Briefly, swine SR microsomes were diluted to 0.1 mg/mL in the following: 100 mmol/L KCl, 20 mmol/L imidazole, pH 7.0, 5 mmol/L MgCl\(_2\), 5 mmol/L K-oxalate, 10 mmol/L Na\(_2\)SO\(_4\), 45 µmol/L unlabeled CaCl\(_2\), and 2.28 µmol/L \(^{45}\text{CaCl}_2\) (specific activity 5895.54 cpm/µmol total Ca). Uptakes were initiated by adding 5 mol/L ATP and incubating the reaction for the desired time at 36°C, at which point a 300-µL aliquot was removed and immediately vacuum filtered onto GF/C filters and washed 3 times with 5 mL of ice-cold 100 mmol/L KCl, 5 mmol/L MgCl\(_2\), 0.1 mmol/L EGTA, 20 mol/L sodium azide, pH 7.0. The amount of accumulated \(^{45}\text{Ca}^{2+}\) in the microsomes and thus retained on the filters was then determined by liquid scintillation counting.

Myocyte Studies

Myocytes were isolated as described by Mattiello et al.\(^{17}\) Myocyte shortening in response to electric field stimulation at 0.5 Hz was measured at 37°C as described previously,\(^{18}\) except that superfusion medium contained 2 mol/L Ca. Time to half relaxation relative to time to peak was measured on 1 to 12 myocytes (7 on average).
isolated from each heart. Mechanical analysis was performed blinded to the SERCA2a and nitrotyrosine content.

**Statistical Analysis**

Data are shown as mean ± SE unless otherwise indicated. Measured parameters were compared with a 2-sample, 2-tailed t test assuming equal variances. Probability values for slopes were determined from an F test.

**Results**

**Human Heart Data**

Patient data for hearts used in this study are given in Tables 1 and 2. Samples from failing hearts approximately age-matched the control hearts.

**Western Analysis of Human Ventricular Homogenates**

Western blots of the human ventricular homogenates probed for SERCA2a showed a single band of molecular weight 100 to 110 kDa (Figure 1A). SERCA2a levels in DCM hearts were unchanged (Figure 1B). SERCA2a levels were also unchanged when measured as SERCA2a/calsequestrin ratio (DCM ratio/control ratio: 1.08 ± 0.08; P = NS).

Similar blots were probed for nitrated proteins with the use of anti-nitrotyrosine antibody, as shown in Figure 1C. The 100- to 110-kDa protein recognized by the antibody was more intensely stained in DCM samples (Figure 1C). Nitrotyrosine level in DCM hearts was significantly increased by 85% (Figure 1D). No other bands were seen on the gels (Figure 1C). We verified that the anti-nitrotyrosine antibody was capable of recognizing other nitrated proteins, if present, from Western blots of ventricular homogenates exposed to peroxynitrite; multiple bands were seen (data not shown).

Artifactual protein denitration is unlikely to play a role in these results. Heat-sensitive extracts of rat lung and spleen can reduce BSA nitration significantly after 10 minutes incubation at 37°C,19 and similar denitrase activity has been found for heart extracts.20 However, tissue was frozen immediately, and homogenates were not allowed to sit at warm temperatures before dissolution in reducing Laemmli buffer at 37°C. In addition, normal and failing samples were treated identically.

To identify the nitrated protein shown in Figure 1, SERCA2 pumps were immunoprecipitated from a control heart homogenate with a SERCA2a/2b polyclonal antibody. Western blots showed that monoclonal antibodies to SERCA2a and nitrotyrosine residues both labeled the immunoprecipitated protein (Figure 2).

To examine whether changes in the abundance of phospholamban (PLN), the main regulatory protein for SERCA2a, or the ratio of PLN/SERCA2a occurred, we performed Western blots simultaneously measuring PLN and SERCA2a. No difference between control and failing samples in PLN content (DCM PLN/control PLN: 1.13 ± 0.32; P = NS) or

**Table 1. Patient Characteristics for Failing (DCM) Hearts**

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Sex</th>
<th>Comorbidities</th>
<th>EF, %</th>
<th>Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>M</td>
<td>CRI</td>
<td>18</td>
<td>Dopamine, dobutamine, milrinone, heparin, captopril, digoxin, furosemide, KCl, famotidine</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td></td>
<td>32</td>
<td>Milrinone, digoxin, furosemide, carvedilol, lisinopril, warfarin, bupropion HCl</td>
</tr>
<tr>
<td>58</td>
<td>M</td>
<td></td>
<td>15</td>
<td>Benazepril, furosemide, digoxin, amiodarone, warfarin, KCl</td>
</tr>
<tr>
<td>44</td>
<td>M</td>
<td>Type 2 DM, AF, CRI</td>
<td>25</td>
<td>Dobutamine, milrinone, heparin, digoxin, bumetanide, spironolactone, levothyroxine, KCl, allopurinol</td>
</tr>
<tr>
<td>46</td>
<td>M</td>
<td></td>
<td>20</td>
<td>Carvedilol, enalapril, losartan, digoxin, furosemide, KCl, warfarin, atorvastatin</td>
</tr>
<tr>
<td>50</td>
<td>M</td>
<td>Type 2 DM, CAD, alcoholism</td>
<td>15</td>
<td>Dobutamine, metoprolol, lisinopril, digoxin, furosemide, KCl, warfarin, insulin</td>
</tr>
<tr>
<td>56</td>
<td>M</td>
<td></td>
<td>20</td>
<td>Dobutamine, dopamine, milrinone, hydralazine, isosorbide dinitrate, alendronate, amiodarone, erythropoietin, folic acid</td>
</tr>
</tbody>
</table>

Mean 45
SD 13.5

**Table 2. Patient Characteristics for Control (Nonfailing) Donor Hearts**

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Sex</th>
<th>Comorbidities</th>
<th>Cause of Death</th>
<th>Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>M</td>
<td>HTN, type 2 DM, CAD, AF</td>
<td>ICH, burn injury</td>
<td>Dopamine, propranolol, digoxin, phenylephrine, hydrochlorothiazide, warfarin</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td></td>
<td>Head trauma</td>
<td>Dopamine</td>
</tr>
<tr>
<td>61</td>
<td>M</td>
<td>HTN</td>
<td>SAH</td>
<td>Dopamine, lisinopril</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td></td>
<td>Head trauma</td>
<td>Dopamine</td>
</tr>
<tr>
<td>53</td>
<td>F</td>
<td></td>
<td>ICH</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>47</td>
<td>M</td>
<td></td>
<td>SAH</td>
<td>Norepinephrine</td>
</tr>
</tbody>
</table>

Mean 42.7
SD 18.5

HTN indicates hypertension; ICH, intracranial hemorrhage; and SAH, subarachnoid hemorrhage. Other abbreviations as in Table 1.
PLN/SERCA2a ratio (DCM ratio/control ratio: 1.11±0.12; $P=\text{NS}$) was observed.

**Functional Consequences of SERCA Nitration in Isolated Myocytes**

Myocyte shortening in response to electric field stimulation was $6.13\pm0.60\%$ for control cells (n=6 preparations) and $5.55\pm0.67\%$ for DCM cells (n=5 preparations; $P=0.53$; $P=\text{NS}$). Although these values were only slightly different, there was a difference in the time to half relaxation, calculated relative to the time of peak contraction: for control myocytes, this time was $152\pm31$ ms, and for DCM myocytes it was $245\pm43$ ms. Although this difference did not reach statistical significance as a comparison between preparation average values ($P=0.106$), it did when all individual measures for control myocytes were compared against all individual measures for DCM myocytes (n=87 cells in total; $P=0.025$, in a 2-sample $t$ test without averaging over heart).

To determine whether there was a relationship between the levels of nitrotyrosine and the functional properties of the myocytes, we plotted the time to half relaxation against nitrotyrosine content (Figure 3A) and against SERCA2a content (Figure 3B). Correlations between these quantities did not reach statistical significance (Figure 3A, 3B), perhaps because of the small sample size. However, closer examination suggested that the combination of SERCA2a nitration and SERCA2a level, acting oppositely, together determined the time to half relaxation. This was tested by plotting the time to half relaxation against the ratio of nitrotyrosine to SERCA content (Figure 3C), and a positive correlation with high significance was then observed (Figure 3C; $P<0.01$). There was no correlation between PLN or PLN/SERCA2a ratio and relaxation (data not shown).

**Figure 2.** The major nitrotyrosine-containing protein is SERCA2a. Immunoprecipitation of control heart homogenate with SERCA2a/2b polyclonal antibody was performed as described in Methods. Only 1 major band was observed with either SERCA2a or nitrotyrosine antibody, and it appears to be the same protein.

**Figure 3.** Effect of SERCA2a level and SERCA2a nitration on the time to half relaxation in isolated myocytes. A, SERCA2a nitration; B, SERCA2a level; C, SERCA2a nitration/SERCA2a level. Circles indicate control hearts; squares, DCM hearts. Each point represents the average time to half relaxation, relative to time to peak, of measurements on all myocytes isolated from 1 heart. Cells were not successfully isolated from 2 of the DCM hearts. Abscissa units are protein band intensity normalized for total protein applied (see Methods).

**Functional Consequences of SERCA Phosphorylation and Nitration in Isolated Cardiac SR**

Peroxynitrite exposure was implicated in the progressive age-associated nitration of SERCA2a in rat skeletal muscle SR, accompanied by a decreased ATPase activity of the Ca$^{2+}$-

---

Exposure of skeletal muscle microsomes to peroxynitrite caused an intermediate degree of inhibition of Ca uptake, presumably because these microsomes contain a mixture of SERCA2a and SERCA1 isoforms, and the SERCA1 isoform does not become nitrated. We therefore investigated the functional consequences of peroxynitrite exposure in heart using porcine cardiac SR microsomes, which contain only the SERCA2a isoform. We observed a complete inhibition of thapsigargin-sensitive Ca uptake with peroxynitrite (Figure 4A, 4C), with IC₅₀ ≈ 150 μmol/L (Figure 4B).

As expected, the catalytic subunit of PKA was able to stimulate ⁴⁵Ca²⁺ translocation into SR microsomes (Figure 4C). Unexpectedly, preincubation of microsomes with PKA prevented the inhibitory effect of exogenous 300 μmol/L peroxynitrite on ⁴⁵Ca²⁺ translocation, whereas PKA had no effect after peroxynitrite (Figure 4C). PKA was less effective at preventing inhibition by 1 mmol/L peroxynitrite (Figure 4C).

**Discussion**

The profound impact of SERCA2a function on the development of heart failure has been established in transgenic studies in which enhancement of SERCA2a activity resulted in the prevention of failure. We have observed an increased level of SERCA2a nitration in DCM hearts, and the time to 50% relaxation of myocytes isolated from these hearts correlated positively with the ratio of SERCA2a nitration to SERCA2a level, whereas each alone showed trends but no significant correlation (Figure 4). This suggests that both SERCA2a level and SERCA2a nitration determine relaxation time through their impact on the rate of SR Ca reuptake. The increased time to half relaxation in failing myocytes is accompanied by an increased time to half decrease of the Ca transient in human heart failure, and SERCA2a dysfunction is generally accepted as contributing to diastolic as well as systolic dysfunction in heart failure. Inactivation of SERCA2a function through nitration could therefore play an important role in the development of heart failure. Additional work is clearly needed to further elucidate the mechanism of the functional impact of SERCA2a nitration on cardiac function.

Others have found either no change or a modest reduction in SERCA2a protein levels in DCM hearts. In the studies in which SERCA2a levels were unchanged, reductions in Ca²⁺-ATPase activity were noted, even though levels of PLN also were unchanged. PLN levels are commonly found to not change in human heart failure, and our PLN measurements also agree with this. Because in our study SERCA2a levels were similar between control and DCM hearts, the longer average time to half relaxation of the DCM myocytes could not have been the result of different levels of SERCA2a and could have been entirely the result of the impact of nitration on Ca pump function.

Aging increases nitrotyrosine formation from peroxynitrite in the SR Ca pump of skeletal muscle. We also observed borderline correlation between age and nitrotyrosine content (P = 0.0504). The increased nitrotyrosine content of tissue from DCM hearts could not be caused by age-related factors, however, because our control group was age-matched with the DCM group. An increase in SERCA2a nitration with age
could therefore contribute significantly to the susceptibility of the elderly to heart failure.26

A role for NO in the pathophysiology of heart failure has long been suspected.27 Many studies have shown an increased expression of iNOS in human dilated cardiomyopathy.28–30 Neuronal NO synthase could also play a role. This isoform is localized to the SR31 and is upregulated and translocated to the sarcolemma after myocardial infarction in senescent rats32 and in the failing human heart.33 Location is a significant factor in NOS pathophysiology, as is shown from the cardio-myopathy resulting from mislocalization of eNOS.34

iNOS activity alone is not sufficient to promote failure because hearts overexpressing iNOS can tolerate high levels of iNOS activity without hemodynamic consequences.35 There is evidence that NO actually benefits the failing heart by increasing preload recruitable stroke work, which is especially important in failing hearts because of reduced inotropic reserve.36 Thus, an increase in NOS could be viewed as an adaptation that allows a failing heart to work better than it otherwise would, by improving diastolic function.

At the same time, iNOS could promote systolic dysfunction. iNOS protein expression was negatively correlated with left ventricular ejection fraction in DCM hearts,11 and this was even more evident in patients with HIV-associated cardiomyopathy.11 NO can become toxic in combination with superoxide, with which it avidly combines to form peroxynitrite.37 There is evidence that superoxide levels are increased in failing hearts: superoxide generation was increased in dogs with heart failure induced by ventricular pacing,12 and failing human (DCM) hearts exhibit the end-products of oxidative stress.13 Nitration of SERCA2a could therefore occur in failing hearts via peroxynitrite.

SERCA2a nitration could contribute to systolic as well as diastolic dysfunction in heart failure, as impaired SR function is generally accepted to contribute to both.2 Its effect on diastolic dysfunction was evident in isolated myocytes from their prolonged relaxation (Figure 3). Its impact on systolic function was not, however, evident in isolated myocytes because the percent shortening of myocytes from failing hearts was only slightly different from control. This is probably because our measurements of percent shortening were made at the relatively low stimulation frequency of 0.5 Hz. At low frequencies of stimulation, failing cardiac trabeculae can generate as much force as normal trabeculae, but they show a negative force-frequency relation, whereas normal trabeculae show a positive force-frequency relation.23,38 Moreover, V\text{max} of SR Ca\textsuperscript{2+} -ATPase activity correlates with the frequency-dependent change in force of contraction.23 This suggests that the impact of SERCA2a activity on systolic function could be most important at higher heart rates.

Thus, NO may exert an acute beneficial effect on diastolic function in the failing heart but in combination with superoxide may cause SERCA2a nitration, promoting systolic dysfunction and further chamber remodeling. If increased NOS is indeed an adaptation to failure, this dual action of NO could contribute to the downward spiral characteristic of the disease. Both of these aspects can be seen in the recent study by Vanderheyden et al.39 These authors found that iNOS-positive DCM hearts had larger left ventricular volumes and depressed cardiac function, as measured by ejection fraction, compared with iNOS-negative DCM hearts, but their higher diastolic distensibility allowed them to better utilize the Starling relationship to maintain function.39

Evidence already exists that SERCA2a ATPase activity is inhibited by nitration. Treatment of skeletal muscle SR vesicles with peroxynitrite induced nitration of Tyr\textsuperscript{294} and Tyr\textsuperscript{295} in SERCA2a, associated with a parallel loss of Ca\textsuperscript{2+} -ATPase activity, whereas SERCA1a did not become nitrated.8 In heart myocytes, where SERCA2a is the only isoform present, SERCA2a nitration would therefore have an even more profound impact on SR function. Our results therefore suggest that exposure to peroxynitrite could impair the Ca\textsuperscript{2+} -ATPase and hence Ca pump function in DCM hearts.

The complete inhibition of Ca uptake by cardiac microsomes induced by peroxynitrite treatment (Figure 4) provides the mechanism by which increased nitrotyrosine/SERCA2a correlates with increased time to half relaxation (Figure 3C), because SERCA2a dysfunction contributes to diastolic dysfunction in heart failure.2 Moreover, it is unlikely that the effect of peroxynitrite on cardiac SR Ca uptake was the result of nonspecific destruction of the SR vesicles because it could be prevented by PKA pretreatment (Figure 4C). PKA pretreatment could prevent the effect of peroxynitrite by inducing dissociation of PLN from SERCA2a, resulting in conformational changes in SERCA2a that block tyrosine nitration. Tyr\textsuperscript{294} and Tyr\textsuperscript{295} are situated at the luminal end of transmembrane helix M4,8 adjacent to transmembrane helix M6,40 which interacts directly with PLN.41 A single point mutation in M4 at Val\textsuperscript{300} can shift the pump affinity for Ca, and this shift is abolished by PLN.41 The PKA effect on SERCA2a nitration could therefore well be related to PLN dissociation. This could have consequences for the development of heart failure in animal models in which the interaction between PLN and SERCA2a is enhanced, which show an increased susceptibility to failure.42 If this interaction puts SERCA2a into a conformation in which it is susceptible to inactivation by nitration, this could be a mechanism whereby failure is promoted. Likewise, β-adrenergic activation would dissociate PLN and inhibit nitration.

The level of nitrotyrosine measured in our control hearts could well be greater than that of true normal hearts because the hearts came from donors who may have experienced shock, which can induce iNOS.43 iNOS expression has indeed been detected in donor hearts.44

It is striking that the only protein observed to be nitrated to any extent in Western blots of our heart homogenates was SERCA2a. In a rat model of heart failure by coronary artery ligation, an increase in the level of nitration of the myofibrillar creatine kinase (40 kDa) was observed in Western blots of isolated myofibrils.45 This protein could also be nitrated by exposure of isolated myofibrils to peroxynitrite.45 We also found that other unidentified proteins in the heart homogenates could be nitrated by exogenous peroxynitrite, but SERCA2a was still the dominant protein nitrated (data not shown). Other effects of peroxynitrite on heart have been observed that may not be related to tyrosine nitration.
Per oxygenitrite treatment of cultured myocytes resulted in arrest in diastole, associated with an increase in intracellular Ca and a decrease in sulfhydryl content but no nitrosylation formation.46 Perfusion of rat hearts with peroxynitrite resulted in greatly depressed myofilament responsiveness to Ca, which was cGMP mediated.47 Increased iNOS and xanthine oxidase expression in isolated rat hearts was found to decrease cardiac function in the absence of an impact on cardiac energetics or oxygen consumption, resulting in reduced efficiency.48 These acute effects of peroxynitrite could be primarily related to myofilament desensitization. The effects of peroxynitrite on SR Ca uptake, on the other hand, could be cumulative because of very slow reversal and hence be manifest more with chronic exposure.

In conclusion, we have found an increased nitration of the SERCA2a Ca pump in hearts from DCM patients, associated with impaired relaxation. These results suggest that SR Ca pump function in DCM hearts could be inhibited by SERCA2a nitration and that this could contribute to the failure state. This mechanism also could contribute to the association of heart failure with aging and with increased PLN/SERCA2a ratio.

Acknowledgments

This study was supported by grants HL61534, HL47053, and HL61537. We thank David Redon for technical help and Alejandro Munoz del-Rio for statistical advice. We gratefully acknowledge the kind cooperation of Dr Robert Love and colleagues in the Division of Cardiothoracic Surgery and Dr Anthony D’Alessandro and colleagues in the University of Wisconsin Organ Procurement Services.

References


Increased Nitration of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase in Human Heart Failure
Andrew J. Lokuta, Nathan A. Maertz, Sivan Vadakkadath Meethal, Katherine T. Potter, Timothy J. Kamp, Héctor H. Valdivia and Robert A. Haworth

*Circulation.* 2005;111:988-995; originally published online February 14, 2005; doi: 10.1161/01.CIR.0000156461.81529.D7
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/111/8/988

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/