Nitrosative Stress and Myocardial Sarcoplasmic Endoreticular Calcium Adenosine Triphosphatase Subtype 2a Activity after Lung Resection in Swine

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Background: Chronic, disease-associated oxidative stress induces myocardial peroxynitrite formation that may lead to nitrosative inhibition of the calcium cycling protein sarcoplasmic endoreticular calcium adenosine triphosphatase subtype 2a (SERCA2a). The current study was designed to test the hypothesis that the acute oxidative stress associated with lung resection also induces myocardial nitrosative stress and alters SERCA2a activity.

Methods: Ventricular myocardium from 16 swine was studied; 11 animals had undergone left upper lobectomy (n = 7) or sham thoracotomy (n = 4) 3 days before harvest, and 5 were nonoperated controls. Tissue peroxynitrite was assessed by measurement of 3-nitrotyrosine incorporation into proteins. SERCA2a activity was determined from indo-1 uptake by isolated sarcoplasmic reticular membranes. Expression of SERCA2a and its regulatory protein phospholamban were determined by Western blotting, as was the phospholamban phosphorylation state (when dephosphorylated, phospholamban inhibits SERCA2a). Mechanical significance of changes in SERCA2a activity was assessed from the force–frequency relation of isometric myocardial trabeculae.

Results: Relative to both the control and sham groups, lobectomy animals exhibited a greater than twofold higher myocardial 3-nitrotyrosine incorporation and an approximately 50% lower SERCA2a activity, but no difference in SERCA2a or phospholamban expression or phospholamban phosphorylation. Concomitantly, whereas the trabecular force–frequency relation of control animals was positive, that of lobectomy animals was negative, consistent with impaired calcium cycling.

Conclusions: These data indicate that oxidative/nitrosative stress associated with lung resection influences SERCA2a activity independent of any influence on protein expression or phospholamban phosphorylation. The findings link an acute event with a subcellular process primarily described for chronic illness and suggest a biochemical basis for perioperative changes in myocardial mechanical reserve.

AS a consequence of oxidative stress, superoxide and nitric oxide interact in the heart to produce peroxynitrite that, in turn, can react with cysteine and tyrosine residues within myocardial proteins.1-3 The modification of cysteine (S-nitrosylation or S-glutathiolation) is redox sensitive and reversible,3 whereas tyrosine undergoes nitration by peroxynitrite, a process generally considered to be a permanent modification.3 Accordingly, if tyrosine nitration alters protein function, this effect will persist after the oxidative/nitrosative stress has been relieved.

Previous investigation has established that the cardiac sarcoplasmic endoreticular calcium adenosine triphosphatase subtype 2a (SERCA2a) is inhibited by peroxynitrite exposure and tyrosine nitration.1,4 Because of the key role SERCA2a plays in myocyte calcium cycling, inhibition of SERCA2a function has been speculated to have an adverse impact on myocardial functional reserve.5 Under normal circumstances, myocyte contraction is initiated by the entry of a small amount of “activator” calcium via the L-type voltage-gated calcium channel, which then binds to the ryanodine-sensitive calcium release channel to cause efflux of a large amount of calcium from the sarcoplasmic reticulum (SR). After binding to contractile proteins to produce shortening of the myocyte, calcium is rapidly transferred back into the SR by SERCA2a, where it is sequestered on binding proteins until released again for the next beat. By modulating SR calcium reuptake from the cytosol, SERCA2a plays a prominent role in not only myocyte relaxation but also contraction because the rate of uptake during diastole determines the amount of calcium that can be released to produce the next systole.6 Activity of myocardial SERCA2a is closely regulated by the phosphorylation state of phospholamban, a relatively small pentameric protein located adjacent to SERCA2a in the SR membrane.6 When in the unphosphorylated state, phospholamban exhibits tonic inhibition of SERCA2a, but when phosphorylated at serine 16 (Ser-16) or threonine 17 (Thr-17), this inhibition is lost.6-8 Inhibition of SERCA2a by nitration seems to persist in the presence of phospholamban phosphorylation, possibly contributing to the reduced inotropic reserve (i.e., impaired force–frequency relation) associated with heart failure.1,5,8

To date, most studies have linked nitrosative inhibition of SERCA2a with the persistent oxidative stress presented by advanced age or chronic disease.9-11 Whether

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short-term oxidative stress, as has been reported to occur during a variety of surgical procedures, can induce myocardial peroxynitrite generation and prolonged nitrosative inhibition of myocardial SERCA2a is unknown. Perioperative oxidative stress has been found to be particularly evident during pulmonary resection, where selective collapse and subsequent hyperoxic re-expansion of the operative lung elicits an immediate, sharp increase in reactive oxygen species that return to baseline over the next 6–8 h. We therefore hypothesized that pulmonary resection with selective lung collapse would be associated with myocardial peroxynitrite generation and postoperative impairment of SERCA2a activity independent of any change in protein expression or phospholamban phosphorylation. To test this hypothesis, myocardial peroxynitrite generation, SERCA2a expression and function, and phospholamban expression and phosphorylation state were compared in swine that had undergone no operation (control), a sham thoracotomy, or left upper lobectomy during selective lung collapse. Finally, in a subset of subjects, the mechanical implication of any nitrosative inhibition of SERCA2a activity was assessed by measurement of the force–frequency relation, an index of SERCA2a-mediated contractile reserve, in isolated myocardial trabeculae.

Materials and Methods

Under protocols approved by the Institutional Animal Care and Use Committee at the Weill Cornell Medical College, New York, New York, 16 swine (30–42 kg in weight) were used for the study. Seven animals underwent removal of the left upper lung lobe during single lung ventilation to facilitate lung collapse, 4 underwent thoracotomy without lung collapse or lobectomy (sham procedure), and the remaining 5 were nonoperated controls. Both the lobectomy and sham groups were studied on the third postoperative day. This time frame was chosen to assure that the acute effects of surgery had dissipated and that all animals had resumed normal activity.

Survival Lobectomy and Sham

After induction of anesthesia with intramuscular tiletamine–zolazepam (4.4 mg/kg) and xylazine (2.2 mg/kg), the trachea was intubated, and the lungs were ventilated with oxygen and isoflurane to maintain end-tidal isoflurane concentration at 1.3% and carbon dioxide at 32–34 mmHg. In addition to the animals that had undergone lobectomy or sham thoracotomy, the nonoperated control animals were prepared in an identical fashion. A femoral arterial catheter was inserted percutaneously for blood pressure monitoring, and a pulmonary artery catheter was inserted via cut-down of the right external jugular vein. A median sternotomy was performed, and the heart was suspended in a pericardial cradle. At the apex, a 5-French micromanometer catheter was inserted via a puncture into the left ventricle for pressure measurement and secured with a purse string suture. In the left ventricular free wall, a pair of 2-mm ultrasonic crystals were inserted into the myocardium approximately 15 mm apart for measurement of left ventricular segment length as a surrogate for volume. Hemodynamic variables were then recorded with left ventricular contractility expressed as peak +dP/dt normalized to left ventricular end-diastolic segment length over the variation in preload induced by acute occlusion of the inferior vena cava. For all animals, stroke volume and cardiac output data were normalized to body surface area and were expressed as stroke volume index and cardiac index, respectively. After in situ data collection, the heart was arrested with iced potassium chloride solution and rapidly removed. Myocardial samples were snap frozen in liquid nitrogen and either used for preparation of SR vesicles (see Force–Frequency Relations in Isometric Trabeculae section below) or stored at −80°C until analysis. In addition, for control and lobectomy animals, free wall tissue was also placed into ice-cold, oxygenated Krebs-Ringer’s solution for isolation of myocardial trabeculae (small muscle strips 1–1.5 mm in diameter, approximately 6 mm in length) and subsequent assessment of contractile reserve (see Force–Frequency Relations in Isometric Trabeculae section below).
Plasma Malondialdehyde and Myocardial Myeloperoxidase as Markers for Oxidative Stress

For the lobectomy and sham groups, arterial blood samples were drawn into EDTA tubes before opening, and just after closing, the chest during the survival procedure, and again before beginning the organ harvest. For the control animals, blood samples were obtained only at organ harvest. Using the Malondialdehyde Assay Kit (Northwest Life Science Specialties, Vancouver, WA), plasma was added to individual wells and processed according to the manufacturer’s specifications. The resulting malondialdehyde levels reflect peroxidation reactions initiated by oxygen radicals and therefore represent a stable index of oxidative stress. In that inflammation can also elicit local oxidative stress via activation of leukocytes and myeloperoxidase is a reflection of this activation, myocardial samples obtained at sacrifice were analyzed for myeloperoxidase content using a commercial kit (Northwest Life Science Specialties) after tissue homogenization in the recommended lysis buffer.

3-Nitrotyrosine Incorporation as an Indicator of Myocardial Peroxynitrite Production and Nitrosative Stress

Although the profoundly reactive peroxynitrite molecule has an extraordinarily short half-life, the resulting modification of tyrosine residues within proteins (addition of a nitro group to position 3 of the phenyl ring) represents a stable end-product that can be used to index peroxynitrite production. After homogenization in 10 ml/g wet weight of tissue in a buffer consisting of 50 mM Tris, 150 mM NaCl, 0.1 mM EDTA, and 20 mM CHAPS (pH 7.4), the samples were centrifuged at 100,000g for 1 h and the supernatants were removed to fresh tubes. A 100-μl aliquot of the tissue homogenate was then subjected to proteolytic digestion before the extent of protein incorporated 3-nitrotyrosine was quantified. Proteinase K (48U/ml buffer) was added to the tissue homogenate and incubated for 8 h at 55°C. Samples were then allowed to cool to room temperature before addition of ice-cold acetonitrile (3:1 vol/vol), followed by vortexing, cooling on ice for 5 min, and centrifugation at 12,000g at 4°C for 15 min. The resulting supernatants were removed to fresh tubes, dried in a speed vacuum, and resuspended in 400 μl mobile phase containing 90 mM sodium acetate, 35 mM citric acid, 130 mM EDTA, and 460 μM sodium octane sulfonate (pH 4.35). Samples were then processed using an isocratic high-performance liquid chromatography system with multichannel electrochemical CoulArray (ESA Inc., Chelmsford, MA) detection to effectively resolve 3-nitrotyrosine from background species with a 100-mm C-18 column (Microsorb-MV; Varian Inc., Palo Alto, CA) running the mobile phase at a flow rate of 0.75 ml/min and the temperature at 30°C. The optimum potential for detection of 3-nitrotyrosine is +800 mV. For maximum selectivity of the system for 3-nitrotyrosine, two additional electrodes were set either side of the optimum potential for 3-nitrotyrosine, +700 mV and +900 mV, respectively. Further confirmation of the elution of 3-nitrotyrosine was established by addition of 10 mM sodium hydrosulfite to nitrotyrosine. This treatment chemically reduces 3-nitro- to 3-amino-tyrosine, silencing the electrochemical signal.

SERCA2a-mediated Calcium Uptake by Isolated SR Vesicles

Enriched cardiac SR samples were prepared according to Tupling et al. with modification. Briefly, ventricular tissue in liquid nitrogen was ground with mortar and pestle into a fine powder and diluted 1.5 (wt/vol) in ice-cold buffer containing 5 mM HEPES, 250 mM sucrose, 0.2% sodium azide, and 0.2 mM phenylmethylsulfonyl fluoride (pH 7.5). Samples were homogenized and then centrifuged in ordered steps. Final pellets were resuspended in homogenization buffer plus 50 mM l-histidine, then frozen in liquid nitrogen and stored at −80°C for later analysis. Oxalate-supported Ca²⁺ uptake in enriched SR samples was subsequently measured using the Ca²⁺ fluorescent dye Indo-1 Pentapotassium Salt (Calbiochem, La Jolla, CA). With methods adapted from Tupling et al. and O’Brien et al., measurements were obtained with a luminescence spectrometer (dual emission monochromators) and the FL WinLab software (Perkin Elmer, Beaconsfield, England). The samples were excited at 360 nm with a bandpass of 2.5 nm. The emission monochromators were set at 400 nm (for Ca²⁺ bound) and 485 nm (for Ca²⁺ free) with 3.0-nm band passes. Before beginning, the protein concentration of SR isolates was quantified using a Bradford assay. With temperature maintained at 37°C, indo-1 [1.5 μM] and CaCl₂ [3.0 μM] were first added to the cuvette containing uptake buffer (200 mM KCl, 20 mM HEPES, 10 mM Na₂HPO₄, 5 μM N,N,N’,N’-tetraakis(2-pyridyl-methyl) ethylenediamine, 5 mM oxalate, and 15 mM MgCl₂, pH 7.0). The SR sample was then added. After 2 min, adenosine triphosphate (ATP) [1.5 mM] was added to initiate the reaction. When uptake reached plateau, 50 mM EGTA and then 100 mM CaCl₂ were added to calibrate indo-1 intensities for use in calculation of Ca²⁺ concentration. SERCA2a dependence of the preparation was confirmed by preincubation with 1.25 μM thapsigargin, a SERCA inhibitor, which abolished calcium uptake. In addition, samples were analyzed in the presence of 300 μM cyanide to determine whether SR calcium release via the ryanodine receptor represented a confounding variable.

Incubation of SR Vesicles with Peroxynitrite

To determine whether exposure to peroxynitrite specifically alters calcium uptake, SR vesicles from control hearts were exposed to varying concentrations of per-
oxynitrite for 3 min in the dark before measurement of calcium uptake. In that peroxynitrite concentration within a stock solution can vary widely over time, the actual concentration present was assayed before each experiment using the method of Beckman et al.\textsuperscript{27} Briefly, an aliquot of the peroxynitrite solution was diluted with 1.2 M NaOH, absorbance was measured at 302 nm, and the concentration was calculated using the extinction coefficient, \(e = 1,670 \, \text{m}^{-1} \cdot \text{cm}^{-1}\). As a negative control, an aliquot of peroxynitrite was first prepared in 0.5 M phosphate buffer (pH 7.4). Under these neutral pH conditions, peroxynitrite degrades, rapidly yielding an absorbance that is the same as that of buffer alone. Finally, to determine whether SERCA2a activity is sensitive to varying concentrations of ATP and whether the response is altered by preincubation with peroxynitrite and/or consistent among groups, SR calcium uptake measurements were performed on the same samples using different ATP concentrations to initiate the reaction.

**Western Blotting for SERCA2a**

Myocardial samples were homogenized in ice-cold buffer containing 20 mM Na-HEPES (pH 7.4), 4.0 mM EGTA, 1.0 mM dithiothreitol, and EDTA-free protease inhibitors (Boehringer-Mannheim, Indianapolis, IN) and were then centrifuged, and protein content of the supernatant was determined by Bradford assay. Samples were then subjected to 10% SDS-PAGE, transferred to nitrocellulose, and blocked with 2.5% nonfat dry milk in phosphate buffered saline with Tween 20 (PBS-T) buffer. After washing with PBS-T, blots were incubated with primary antibody for 2 h at room temperature. Primary antibody was diluted in 2.5% nonfat dry milk in PBS-T buffer and included mouse monoclonal antibodies against SERCA2a (Calbiochem), and actin (Oncogene Research Products, La Jolla, CA). Protein bands were analyzed by laser densitometry using actin as an internal standard.

**Western Blotting for the Expression and Phosphorylation of Phospholamban**

After homogenization as noted above and determination of protein content, samples were subjected to 10–20% SDS-PAGE, transferred to nitrocellulose, and blocked with 2.5% nonfat dry milk in PBS-T buffer. After washing with PBS-T, blots were incubated with primary antibodies for total phospholamban as well as phospho-Ser16 phospholamban (P-Ser16 PLB), phospho-Thr17 phospholamban (P-Thr17 PLB), and actin for 2 h at room temperature. After application of secondary antibody (all anti-rabbit immunoglobulin G) and development with enhanced chemiluminescence, protein bands, reflecting both the pentameric and monomeric forms, were analyzed by laser densitometry using actin as an internal standard. P-Ser16 PLB and P-Thr17 PLB were expressed as a fraction of total phospholamban.

**Force-Frequency Relations in Isometric Trabeculae**

Excised trabeculae were allowed to equilibrate for at least 1 h at low tension (< 50 mg) at 37°C. Aerated Krebs-Henseleit solution gassed with 95% O\textsubscript{2}-5% CO\textsubscript{2} was replaced every 30 min. Electrical field stimulation was continuously applied using a Grass S48 stimulator and a Grass SIU5 stimulus isolation unit (both Astro-Med Inc., Grass Technology Product Group, West Warwick, RI) (1.5 Hz, 90 V, 5 ms). After completion of the initial equilibration period, tissues were slowly stretched to 600 mg and then further progressively stretched to the length of submaximal force generation (approximately 90% E\textsubscript{max}). After an additional reequilibration of 30 min, the stimulation frequency was increased every 2 min at 0.5-Hz increments to a maximum of 3.0 Hz (180 bpm). For each preparation, isometric myocardial force generation was assessed using PowerLab/SP (AD Instruments, Colorado Springs, CO) and referenced to the baseline level at 1.5 Hz.

**Statistical Analysis**

All data are presented as mean ± SE. For hemodynamic and biochemical variables, comparisons between groups were made with one-way analysis of variance. For comparison of different concentrations of ATP on SR calcium uptake in myocardial samples from the same animal, a paired \(t\) test was applied. For isolated myocardial trabeculae data, the contractile force of control and lobectomy tissues was compared by nonpaired \(t\) test. For all analyses, \(P < 0.05\) was considered significant.

**Results**

**In Situ Hemodynamic Response to Lobectomy**

Hemodynamic variables obtained with the chest open and identical end-tidal isoflurane and carbon dioxide levels are shown in table 1. There were no differences in hemodynamic variables between groups, although a trend toward higher mean pulmonary artery pressure and pulmonary vascular resistance was evident in lobectomy animals. Under these “resting” conditions, there were no differences in inotropy among the groups.

**Plasma Malondialdehyde and Myocardial Myeloperoxidase Concentrations**

As shown in figure 1, during the survival lobectomy and sham procedures, malondialdehyde levels were the same before opening the chest, but twice as high in lobectomy animals after chest closure. At the time of organ harvest, however, malondialdehyde levels in the lobectomy group were not different than in either sham or control animals. Similarly, at the time of organ harvest,
there were no differences in myocardial myeloperoxidase content among the groups, indicating that myocardial inflammation and local leukocyte activation was not a source of ongoing oxidative stress within the heart.

Table 1. Hemodynamic Variables among Experimental Groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SE</th>
<th>Lobectomy</th>
<th>SE</th>
<th>Sham</th>
<th>SE</th>
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<tr>
<td>HR, beats/min</td>
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<td>10</td>
<td>91</td>
<td>5</td>
<td>86</td>
<td>8</td>
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<tr>
<td>SI, ml/m²</td>
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<td>3</td>
<td>29</td>
<td>2</td>
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<td>5</td>
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<td>CI, L/min⁻¹·m⁻²</td>
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<td>2.60</td>
<td>0.15</td>
<td>2.39</td>
<td>0.30</td>
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<td>80</td>
<td>5</td>
<td>75</td>
<td>5</td>
</tr>
<tr>
<td>MPAP, mmHg</td>
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<td>1</td>
<td>22</td>
<td>3</td>
<td>16</td>
<td>3</td>
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<td>2,315</td>
<td>273</td>
<td>2,619</td>
<td>355</td>
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<tr>
<td>PVRI, dyn·s·cm⁻⁵·m⁻²</td>
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<td>400</td>
<td>42</td>
<td>309</td>
<td>36</td>
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<td>LVPmax, mmHg</td>
<td>101</td>
<td>8</td>
<td>100</td>
<td>7</td>
<td>97</td>
<td>8</td>
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<td>9</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>7</td>
<td>1</td>
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<td>LVEDP/dt, mmHg/s</td>
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<td>79</td>
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<tr>
<td>LVEDP/dt/EDSL, mmHg·s⁻¹·mm⁻¹</td>
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<td>7</td>
<td>68</td>
<td>3</td>
<td>66</td>
<td>8</td>
</tr>
</tbody>
</table>

Cl = cardiac index; HR = heart rate; LVPmax = peak first derivative of left ventricular pressure divided by end-diastolic segment length; LVEDP = left ventricular end-diastolic pressure; MAP = mean arterial pressure; MPAP = mean pulmonary arterial pressure; PVRI = pulmonary vascular resistance index; SI = stroke volume index; SVRI = systemic vascular resistance index.

Myocardial 3-Nitrotyrosine and Calcium Uptake by Isolated SR Membranes

Despite the lack of biochemical evidence for ongoing systemic and/or myocardial oxidative stress, as shown in figure 2, the myocardial 3-nitrotyrosine content was more than twofold higher in lobectomy animals relative to both control and sham animals, indicating substantial

Fig. 2. High-performance liquid chromatography analysis of myocardial 3-nitrotyrosine, a stable product of peroxynitrite generation. * Lobectomy versus control difference. ♦ Lobectomy versus sham difference.

Fig. 3. Compiled data for sarcoplasmic endoreticular calcium adenosine triphosphatase subtype 2a–mediated calcium uptake by vesicles isolated from sarcoplasmic reticulum (SR) of left ventricular myocardium. * Lobectomy versus control difference. ♦ Lobectomy versus sham difference.
generation of peroxynitrite within the tissue. This increased level of myocardial 3-nitrotyrosine was associated with a nearly 50% lower rate of SERCA2a-mediated calcium uptake in lobectomy animals relative to both control and sham animals (fig. 3). For all preparations, calcium uptake was enhanced to the same degree (approximately 7%) by preincubation with 300 mM ryanodine (data not shown), indicating that decreased uptake in the lobectomy animals was a result of altered SERCA2a activity and not due to increased release of calcium via the ryanodine receptor. To determine whether exogenous peroxynitrite similarly alters SERCA2a function in this preparation, control samples were incubated with peroxynitrite before measurement of calcium uptake. As shown in figure 4, preincubation with 100 μM peroxynitrite reduced SERCA2a-mediated SR calcium uptake by half. However, this effect was partially reversed by increasing the ATP concentration, a finding suggestive of a change in SERCA2a affinity for ATP. Finally, to determine whether SERCA2a activity in lobectomy preparations could also be restored by increased ATP concentrations, SR calcium uptake was repeated in the presence of increasingly greater concentrations of ATP. As shown in figure 4, doubling the ATP concentration increased SR calcium uptake in lobectomy preparations but had no effect in either control or sham myocardium.

**SERCA2a Expression and PLB Phosphorylation**

Decreased SR calcium uptake in lobectomy animals was not associated with a decrease in SERCA2a protein (fig. 5). To evaluate whether changes in phospholamban expression or phosphorylation status (dephosphorylated phospholamban inhibits SERCA2a activity) contributed to the postlobectomy decrease in SR calcium uptake, blotting with both nonspecific antiphospholamban and phosphospecific (Ser16 and Thr17) antibodies was performed. As shown in figure 6, there were no discernible differences in total phospholamban or P-Ser16 PLB and P-Thr17 PLB among the groups.

**Trabecular Force–Frequency Relations after Lobectomy**

As shown in figure 7, trabeculae from control animals exhibited a rate-related increase in contractile force (positive force–frequency relation), indicating a “recruitable reserve” in SERCA2a function. In contrast, those from lobectomy animals exhibited decreased force with increasing stimulation frequency (negative force–frequency relation), suggesting that with increased rate, SERCA2a could not clear calcium from the cytosol fast enough to optimally load the SR for the subsequent systole.
that occur during lobectomy, presumably due to inten-
sification during selective lung collapse elicits myocardial
contraction. Whether acute perioperative oxidative stress
induced by aging and a variety of chronic diseases, super-
oxide and nitric oxide interact within the heart to
produce peroxynitrite that, in turn, nitrates tyrosine res-
ides within many proteins potentially altering func-
tion. Although global significance of nitrosative protein
modifications in the heart remains uncertain, exposure
to peroxynitrite clearly reduces activity of SERCA2a, a
key determinant of calcium cycling within the myo-
cyte. The current study was therefore de-
signed to use SERCA2a activity as a functional endpoint
in testing the general hypothesis that pulmonary resec-
tion during selective lung collapse elicits myocardial
peroxynitrite generation and postoperative impairment
of cardiac protein function. Results of the study support
this hypothesis in that they confirm the presence of
substantial myocardial peroxynitrite generation that is
associated with impaired SERCA2a activity unrelated to
protein expression or phosphorylation state of the
SERCA2a regulatory protein phospholamban.

The concept of lung resection eliciting a short-term
systemic oxidative stress is well described. Previous in-
vestigators have reported exhaled peroxide along with
large increases in plasma markers for oxidative stress
that occur during lobectomy, presumably due to inten-
tional lung collapse followed by hyperoxic reexpan-
sion. Recent data have characterized this response
(quantified as plasma malondialdehyde) as peaking
shortly after reexpansion of the remaining operative
lung and then declining to a level below baseline over
the next 8 h. Although previous studies have
attempted to link the magnitude of this short-term increase
in plasma malondialdehyde to complications after lobec-
tomy, there are no clinical data indicating a specific
physiologic effect of the acute oxidative stress associated
with lung resection. We previously reported experimental
data demonstrating that after lobectomy during inten-
tional lung collapse, tetrahydrobiopterin, a critical cofac-
tor for nitric oxide synthase, becomes oxidized to
dihydrobiopterin within both the remaining operative
lung and the nonoperated contralateral lung. Function-
ally, this oxidation of tetrahydrobiopterin uncouples
components of the nitric oxide synthase complex, caus-
ing the enzyme to produce superoxide instead of nitric
oxide. The end result is not only endothelial dysfunction
(inability to produce nitric oxide), but also the possibil-
ity of further local oxidative stress via a “feed forward”
mechanism, i.e., ongoing superoxide formation leads to
more oxidation of tetrahydrobiopterin.

For the current study, we chose to focus downstream
on myocardial 3-nitrotyrosine as a marker for peroxyni-
trite generation within the heart in response to oxidative
stress. Consistent with clinical studies, our data indicate
an acute increase in malondialdehyde after lung resec-
tion, but not sham thoracotomy, that is no longer
present by the third postoperative day. However, de-
spite normalization of plasma malondialdehyde levels,
72 h after lung resection, myocardial 3-nitrotyrosine
incorporation was more than twofold higher than that
measured in both nonoperated controls and animals that
had undergone sham thoracotomy. These data strongly
suggest that the lung resection procedure elicited suffi-
cient myocardial oxidative stress to trigger substantial
peroxynitrite generation within the heart. It is unclear,
however, whether this response reflects events that oc-
curred intraoperatively or ongoing postoperative pro-
cesses. Nonetheless, myocardial levels of myeloperox-
idase were found to be the same in control, lobectomy,
and sham animals, suggesting that ongoing myocardial
inflammation after lobectomy was not a major compo-
nent of the response.

Previous studies have indicated that cardiac SERCA2a
activity is reduced by aging and pathologic conditions
such as dilated myopathy and diabetes. While recent
data have raised the prospect that SERCA2a nitra-
tion may be a contributing factor diminished SERCA2a activity has largely been attributed to reduced
protein expression and/or a shift in the expression or
phosphorylation state of the SERCA2a regulatory protein
phospholamban. For example, it has been speculated
that because unphosphorylated phospholamban elicits

Fig. 7. The relation between the contractile force of isometric
myocardial trabeculae (expressed as percent of baseline value)
and stimulation rate (expressed in Hz). In trabeculae from con-
trol swine, increased stimulation frequency initially augments
contractile force (a positive force–frequency relation). In con-
trast, trabeculae obtained after lobectomy exhibit a decline in
contractile force with increasing stimulation frequency (a neg-
ative force–frequency relation). Trabeculae were not obtained
from sham animals. * Difference relative to control.

Discussion

As a consequence of the myocardial oxidative stress
produced by aging and a variety of chronic diseases, su-
peroxide and nitric oxide interact within the heart to
produce peroxynitrite that, in turn, nitrates tyrosine res-
ides within many proteins potentially altering func-
tion. Whether acute perioperative oxidative stress
such as that elicited by pulmonary resection during in-
tentional lung collapse can elicit similar nitrosative ef-
fects on the myocardium is unknown. However, given
the profound reactivity of peroxynitrite and the fact
that tyrosine nitrination is generally considered a permanent
protein modification, the possibility exists that if a dis-
creet oxidative challenge is of sufficient magnitude, a
persistent effect on vulnerable aspects of myocardial
function may occur.

Although global significance of nitrosative protein
modifications in the heart remains uncertain, exposure
to peroxynitrite clearly reduces activity of SERCA2a, a
key determinant of calcium cycling within the myo-
cyte. The current study was therefore de-
signed to use SERCA2a activity as a functional endpoint
in testing the general hypothesis that pulmonary resec-
tion during selective lung collapse elicits myocardial
peroxynitrite generation and postoperative impairment
of cardiac protein function. Results of the study support
this hypothesis in that they confirm the presence of
substantial myocardial peroxynitrite generation that is
associated with impaired SERCA2a activity unrelated to
protein expression or phosphorylation state of the
SERCA2a regulatory protein phospholamban.

The concept of lung resection eliciting a short-term
systemic oxidative stress is well described. Previous in-
vestigators have reported exhaled peroxide along with
large increases in plasma markers for oxidative stress
that occur during lobectomy, presumably due to inten-
tonic inhibition of SERCA2a, any change in SERCA2a expression must be interpreted in light of simultaneous changes in phospholamban, i.e., the SERCA2a/phospholamban ratio has functional significance.31 Alternatively, because phosphorylation of phospholamban removes inhibitory effects of the protein on SERCA2a function, alterations in protein phosphatase activity may need to be considered because this effect can modulate SERCA2a activity by affecting the rate at which phospholamban is dephosphorylated.32 In the current study, neither a shift in the SERCA2a/phospholamban ratio or a change in phosphatase activity seemed to be factors because we did not observe a difference among groups in regard to expression of SERCA2a or phospholamban, or phospholamban phosphorylation at either Ser16 and Thr17. Accordingly, although our data do not specifically demonstrate a direct SERCA2a modification by peroxynitrite, this prospect is supported by the marked decline in SERCA2a activity independent of any changes in the expression of SERCA2a or phospholamban, or phospholamban phosphorylation. Furthermore, the finding that SERCA2a activity in lobectomy animals and control samples treated with peroxynitrite, but not untreated controls, can be enhanced by increased ATP suggests a change in ATP affinity, a finding seemingly consistent with reported steric effects of SERCA2a modification by peroxynitrite.4

In that rate-related changes in myocardial inotropy are regarded in some ways as a bioassay for SERCA2a,19,32 the observation that myocardial trabeculae isolated from control animals exhibited augmented contractile force when stimulated with increasing frequency (positive force–frequency relation) whereas those harvested from lobectomy animals exhibited decreased contractile force (negative force–frequency relation) is consistent with impaired postoperative SERCA2a activity and calcium cycling. Nonetheless, there were no in situ differences in ventricular inotropy. Although seemingly at odds with the isolated tissue responses, these observations are, in fact, in accord with previous data demonstrating that decreased SERCA2a activity has a greater impact upon inotropic reserve than resting function in myocardial trabeculae.32 It is possible, however, that postoperative changes in mechanical reserve could have been modulated, at least in part, by other reported effects of oxidative/nitrosative stress3 or as a result of some persistent, established 3-nitrotyrosine incorporation and lower SERCA2a activity. Accordingly, the conclusion that higher myocardial 3-nitrotyrosine incorporation and lower SERCA2a activity were primarily the result of the lung resection procedure seems valid.

In summary, results of the study indicate that lung resection is associated with significant left ventricular nitrosative stress and impaired myocardial calcium cycling. The data support the probability that acute oxidative/nitrosative stress can alter SERCA2a activity independent of any influence on protein expression or phospholamban phosphorylation and suggest a biochemical basis for perioperative changes in myocardial mechanical reserve.

References


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