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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 dephosporylated, 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.**

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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.[5] 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,[12–15] 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 reexpansion of the operative lung elicits an immediate, sharp increase in reactive oxygen species that return to baseline over the next 6–8 h.[15–18] 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,[19] 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, the lungs were ventilated with oxygen and isoflurane, and, for the lobectomy animals, a balloon-tipped catheter was inserted into the left mainstem bronchus under bronchoscopic guidance. When filled with sufficient air, the balloon catheter prevented ventilation of the operative lung during the resection. With animals in right lateral decubitus position and the bronchial-blocking balloon filled to prevent ventilation of the left lung in the lobectomy group, a thoracotomy was performed. For lobectomy animals, anatomic resection of the left upper lobe was performed using vascular and bronchial staplers. For sham animals, no additional surgery was performed in the chest, but the cavity was left open for 40 min, the approximate time period required for lobectomy. For both groups, a 20-French chest tube was then placed and connected to a Heimlich valve. Perioperative analgesia was provided with intrathecal and intravenous morphine in combination with intercostal nerve blockade. Postoperatively, analgesia was maintained with transcutaneous fentanyl and subcutaneous ketoprofen.

Experimental Preparation

Animals were anesthetized as described above (Survival Lobectomy and Sham section), 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[20] 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).

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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.[21] In that inflammation can also elicit local oxidative stress *via* activation of leukocytes and myeloperoxidase is a reflection of this activation,[22] 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.[4,23] 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 100,000*g* 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 12,000*g* 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 μM 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.*[24] 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 Ca^{2+} uptake in enriched SR samples was subsequently measured using the Ca^{2+} fluorescent dye Indo-1 Pentapotassium Salt (Calbiochem, La Jolla, CA). With methods adapted from Tupling *et al.*[24] and O'Brien *et al.*,[25,26] 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 Ca^{2+} bound) and 485 nm (for Ca^{2+} 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 $[1.5\ \mu M]_f$ and $CaCl_2$ $[3.0\ \mu M]_f$ were first added to the cuvette containing uptake buffer (200 mM KCl, 20 mM HEPES, 10 mM NaN_3, 5 μM *N,N,N′,N′*-tetrakis(2-pyridyl-methyl) ethylenediamine, 5 mM oxalate, and 15 mM $MgCl_2$, pH 7.0). The SR sample was then added. After 2 min, adenosine triphosphate (ATP) $[1.5\ mM]_f$ was added to initiate the reaction. When uptake reached plateau, 50 mM EGTA and then 100 mM $CaCl_2$ were added to calibrate indo-1 intensities for use in calculation of Ca^{2+} concentration.[25] 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 mM ryanodine 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-

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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.*[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, $\varepsilon = 1{,}670\ \mathrm{M}^{-1} \cdot \mathrm{cm}^{-1}$. 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_2–5% CO_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% Emax). 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/8SP (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, $P \leq 0.05$ 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,

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Table 1. Hemodynamic Variables among Experimental Groups

	Control	SE	Lobectomy	SE	Sham	SE
HR, beats/min	102	10	91	5	86	8
SI, ml/m^2	26	3	29	2	28	5
CI, $l \cdot min^{-1} \cdot m^{-2}$	2.55	0.24	2.60	0.15	2.39	0.30
MAP, mmHg	83	7	80	5	78	5
MPAP, mmHg	16	1	22	3	16	3
SVRI, $dyn \cdot s \cdot cm^5 \cdot m^{-2}$	2,531	398	2,315	273	2,619	355
PVRI, $dyn \cdot s \cdot cm^5 \cdot m^{-2}$	225	24	400	42	309	36
LVPmax, mmHg	101	8	100	7	97	8
LVEDP, mmHg	9	2	9	1	7	1
LVdP/dt, mmHg/s	953	105	1,054	40	966	79
LVdP/dt/EDSL, $mmHg \cdot s^{-1} \cdot mm^{-1}$	64	7	68	3	66	8

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

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.

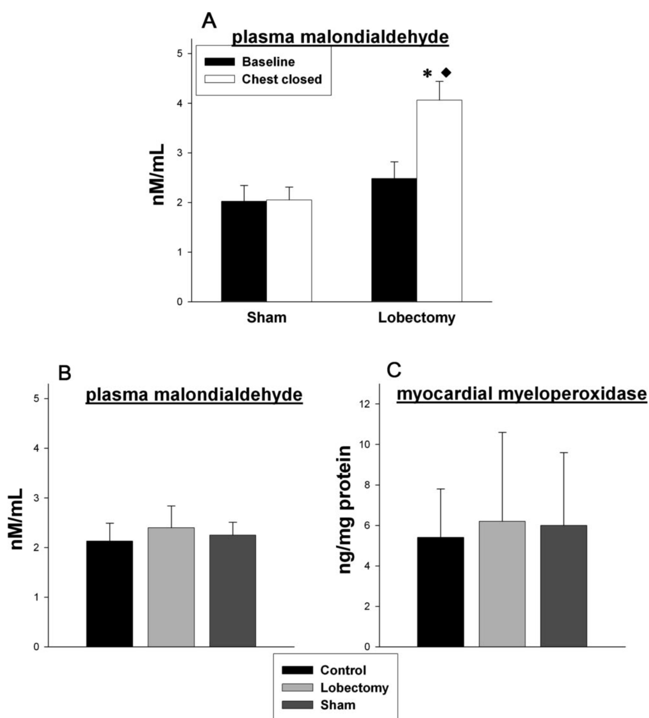

Fig. 1. Comparative levels of plasma malondialdehyde and myocardial myeloperoxidase among groups. (*A*) Malondialdehyde data obtained at the beginning (baseline) and end (chest closed) of a thoracotomy followed by removal of the left upper lung lobe (lobectomy) or no other surgery in the chest (sham). Animals were then allowed to recover before subsequent tissue harvest 3 days later. * Difference from baseline within each group. ♦ Difference between groups at the same time point. (*B* and *C*) Plasma malondialdehyde and myocardial myeloperoxidase values, respectively, from samples obtained at the time of tissue harvest. Depicted are data from control animals that had not previously undergone a surgical procedure as well as those from the sham and lobectomy groups.

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

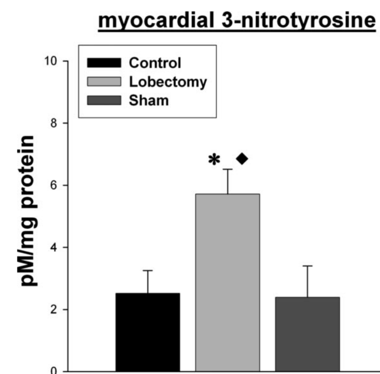

Fig. 2. High-performance liquid chromatography analysis of myocardial 3-nitrotyrosine, a stable product of peroxynitrite generation. * Lobectomy *versus* control difference. ♦ Lobectomy *versus* sham difference.

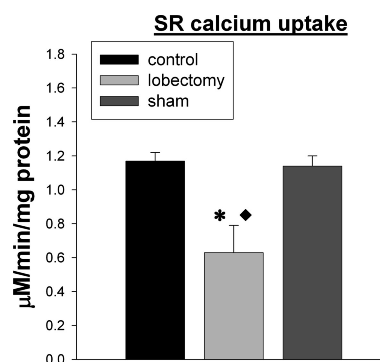

Fig. 3. Compiled data for sarcoplasmic endoreticular calcium adenosine triphosphatase subtype 2a–mediated calcium uptake by vesicles isolated from sarcoplasmic reticulum (SR) of left ventricular myocardium. * Lobectomy *versus* control difference. ♦ Lobectomy *versus* sham difference.

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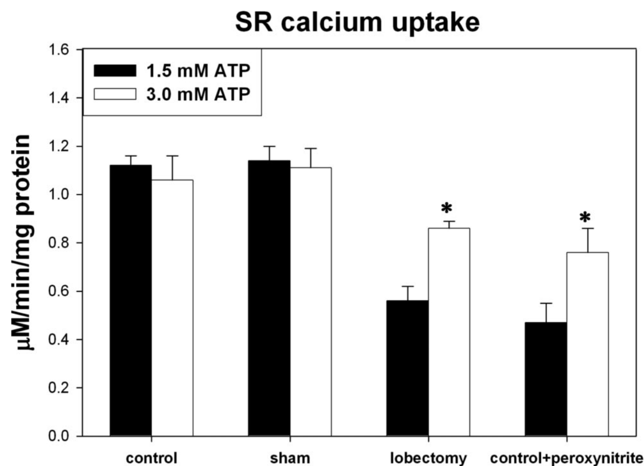

Fig. 4. Effect of doubling adenosine triphosphate (ATP) concentration on sarcoplasmic endoreticular calcium adenosine triphosphatase subtype 2a–mediated uptake of calcium by isolated sarcoplasmic reticulum (SR) vesicles. For control and sham samples, increased ATP had no effect on calcium uptake. In contrast, higher ATP concentration significantly increased SR calcium uptake in samples from lobectomy animals and from control samples pretreated with 100 μM peroxynitrite. * Difference between 1.5 mM and 3.0 mM ATP.

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 beforemeasurement of calcium uptake.[1] 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

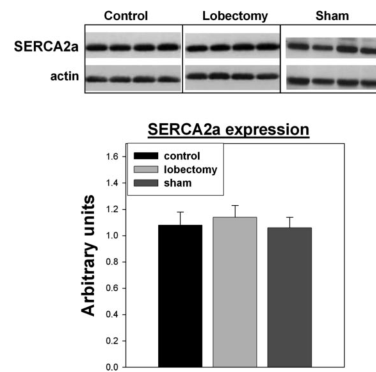

Fig. 5. Representative Western blots and pooled data demonstrating that neither lobectomy nor sham thoracotomy had any effect on sarcoplasmic endoreticular calcium adenosine triphosphatase subtype 2a (SERCA2a) expression relative to nonoperated controls.

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.

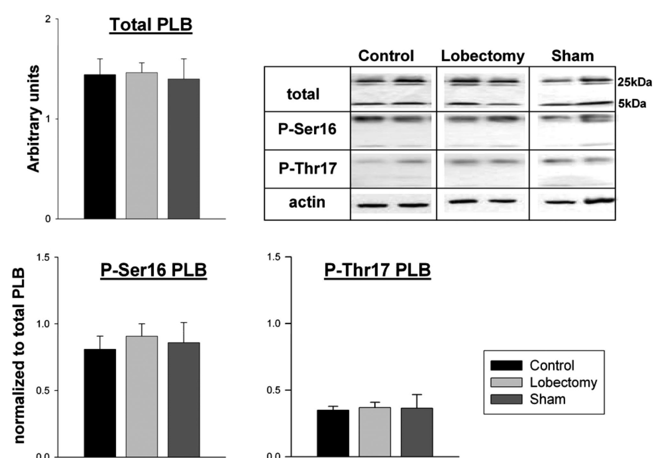

Fig. 6. Representative Western blots and pooled data for total phospholamban (PLB) and phospholamban phosphorylated at serine 16 (P-Ser16) or threonine 17 (P-Thr17). Blots depict both the pentameric (above the 25-kd mark) and smaller monomeric forms (above the 5-kd mark) of phospholamban that were used for analysis.

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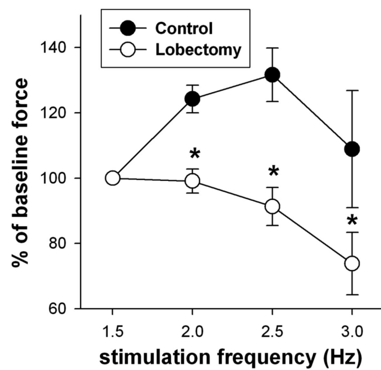

Fig. 7. The relation between the contractile force of isometric myocardial trabeculae (expressed as percent of baseline value) and stimulation rate (expressed in Hz). In trabeculae from control swine, increased stimulation frequency initially augments contractile force (a positive force–frequency relation).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

Discussion

As a consequence of the myocardial oxidative stress induced by aging and a variety of chronic diseases, superoxide and nitric oxide interact within the heart to produce peroxynitrite that, in turn, nitrates tyrosine residues within many proteins potentially altering function.[3,23] Whether acute perioperative oxidative stress such as that elicited by pulmonary resection during intentional lung collapse can elicit similar nitrosative effects on the myocardium is unknown. However, given the profound reactivity of peroxynitrite and the fact that tyrosine nitration is generally considered a permanent protein modification,[3] the possibility exists that if a discreet 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,[28] exposure to peroxynitrite clearly reduces activity of SERCA2a, a key determinant of calcium cycling within the myocyte.[1,2,4,9,10,23] The current study was therefore designed to use SERCA2a activity as a functional endpoint in testing the general hypothesis that pulmonary resection 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 investigators have reported exhaled peroxide along with large increases in plasma markers for oxidative stress that occur during lobectomy, presumably due to intentional lung collapse followed by hyperoxic reexpansion.[15–18] 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.[16,17] Although previous studies have attempted to link the magnitude of this short-term increase in plasma malondialdehyde to complications after lobectomy,[16] 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 intentional lung collapse, tetrahydrobiopterin, a critical cofactor for nitric oxide synthase, becomes oxidized to dihydrobiopterin within both the remaining operative lung and the nonoperated contralateral lung.[29] Functionally, this oxidation of tetrahydrobiopterin uncouples components of the nitric oxide synthase complex, causing 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 possibility 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 peroxynitrite generation within the heart in response to oxidative stress. Consistent with clinical studies, our data indicate an acute increase in malondialdehyde after lung resection, but not sham thoracotomy, that is no longer present by the third postoperative day. However, despite 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 sufficient myocardial oxidative stress to trigger substantial peroxynitrite generation within the heart. It is unclear, however, whether this response reflects events that occurred intraoperatively or ongoing postoperative processes. Nonetheless, myocardial levels of myeloperoxidase were found to be the same in control, lobectomy, and sham animals, suggesting that ongoing myocardial inflammation after lobectomy was not a major component of the response.

Previous studies have indicated that cardiac SERCA2a activity is reduced by aging and pathologic conditions such as dilated myopathy and diabetes.[1,10,11,30] While recent data have raised the prospect that SERCA2a nitration may be a contributing factor,[1,23] 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

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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.[30] 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 stress[3] or as a result of some persistent, ill-defined aspect of anesthesia and/or surgery. Unfortunately, trabeculae were not harvested from the sham animals, so it remains unknown whether the force–frequency relation of trabeculae isolated from this group would be positive or negative. Whatever the underlying mechanism behind diminished rate-related contractile reserve in the lobectomy animals, our observations are consistent with a diminished myocardial capacity to compensate for postoperative stress[19,32] and suggest that although resting cardiac function was not influenced by lung resection, the response to stimuli such as fever, exercise, or supraventricular tachycardia may well have been altered.

Results of the study must be interpreted in the context of certain limitations. First, although both myocardial peroxynitrite generation and SERCA2a inhibition are evident on the third postoperative day, the data do not provide evidence for concomitant onset or duration, or establish a temporal relation between stimulus and maximal response. Second, the study does not define the precise functional role of myocardial peroxynitrite formation. Although there are considerable, compelling data in support of SERCA2a modification and inhibition by peroxynitrite, whether interruption of peroxynitrite generation by pretreatment with an antioxidant and/or blockade of nitric oxide synthesis will alter the observed effects on both SERCA2a activity and the myocardial force–frequency relation remains to be determined. Finally, the study was not designed to determine the independent effects of anesthesia, relative hyperoxia, surgical trauma, and single-lung ventilation on myocardial peroxynitrite generation and/or SERCA2a inhibition. Nonetheless, the finding that 3-nitrotyrosine and SERCA2a activity data from the sham group were virtually identical to those from nonoperated controls, and distinctly different from the lobectomy animals, suggests that anesthesia, hyperoxia, and thoracotomy alone did not have any effect. Although these data must be considered in light of the relatively small sample size, analysis of variance of 3-nitrotyrosine and SERCA2a activity data demonstrated power values of 0.8 and 1.0, respectively. 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

1. Lokuta AJ, Maertz NA, Meethal SV, Potter KT, Kamp TJ, Valdivia HH, Haworth RA: Increased nitration of sarcoplasmic reticulum Ca2+-ATPase in human heart failure. Circulation 2005; 111:988–95

2. Gutierrez-Martin Y, Martin-Romero FJ, Inesta-Vaquera FA, Gutierrez-Merino C, Henao F: Modulation of sarcoplasmic reticulum Ca(2+)-ATPase by chronic and acute exposure to peroxynitrite. Eur J Biochem 2004; 271:2647–57

3. Pacher P, Schulz R, Liaudet L, Szabo C: Nitrosative stress and pharmacological modulation of heart failure. Trends Pharmacol Sci 2005; 26:302–10

4. Knyushko TV, Sharov VS, Williams TD, Schoneich C, Bigelow DJ: 3-Nitrotyrosine modification of SERCA2a in the aging heart: A distinct signature of the cellular redox environment. Biochemistry 2005; 44: 13071–81

5. Gianni D, Chan J, Gwathmey JK, del Monte F, Hajjar RJ: SERCA2a in heart failure: Role and therapeutic prospects. J Bioenerg Biomembr 2005; 37:375–80

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6. Pantano S, Carafoli E: The role of phosphorylation on the structure and dynamics of phospholamban: A model from molecular simulations. Proteins 2007; 66:930–40

7. Waggoner JR, Kranias EG: Role of phospholamban in the pathogenesis of heart failure. Heart Fail Clin 2005; 1:207–18

8. Mattiazzi A, Mundina-Weilenmann C, Guoxiang C, Vittone L, Kranias E: Role of phospholamban phosphorylation on Thr17 in cardiac physiological and pathological conditions. Cardiovasc Res 2005; 68:366–75

9. Viner RI, Ferrington DA, Williams TD, Bigelow DJ, Schoneich C: Protein modification during biological aging: Selective tyrosine nitration of the SERCA2a isoform of the sarcoplasmic reticulum Ca2+-ATPase in skeletal muscle. Biochem J 1999; 340:657–69

10. Xu S, Ying J, Jiang B, Guo W, Adachi T, Sharov V, Lazar H, Menzoian J, Knyushko TV, Bigelow D, Schoneich C, Cohen RA: Detection of sequence-specific tyrosine nitration of manganese SOD and SERCA in cardiovascular disease and aging. Am J Physiol Heart Circ Physiol 2006; 290:H2220–7

11. Belke DD, Dillmann WH: Altered cardiac calcium handling in diabetes. Curr Hypertens Rep 2004; 6:424–9

12. Berg K, Haaverstad R, Astudillo R, Bjorngaard M, Skarra S, Wiseth R, Basu S, Jynge P: Oxidative stress during coronary artery bypass operations: Importance of surgical trauma and drug treatment. Scand Cardiovasc J 2006; 40:291–7

13. Bailey DM, Raman S, McEneny J, Young IS, Parham KL, Hullin DA, Davies B, McKeeman G, McCord JM, Lewis MH: Vitamin C prophylaxis promotes oxidative lipid damage during surgical ischemia-reperfusion. Free Radic Biol Med 2006; 40:591–600

14. Glantzounis GK, Tsimaris I, Tselepis AD, Thomas C, Galaris DA, Tsimoyiannis EC: Alterations in plasma oxidative stress markers after laparoscopic operations of the upper and lower abdomen. Angiology 2005; 56:459–65

15. Cheng YJ, Chan KC, Chien CT, Sun WZ, Lin CJ: Oxidative stress during 1-lung ventilation. J Thorac Cardiovasc Surg 2006; 132:513–8

16. Misthos P, Katsaragakis S, Theodorou D, Milingos N, Skottis I: The degree of oxidative stress is associated with major adverse effects after lung resection: A prospective study. Eur J Cardiothorac Surg 2006; 29:591–5

17. Misthos P, Katsaragakis S, Milingos N, Kakaris S, Sepsas E, Athanassiadi K, Theodorou D, Skottis I: Postresectional pulmonary oxidative stress in lung cancer patients: The role of one-lung ventilation. Eur J Cardiothorac Surg 2005; 27: 379–82

18. Lases EC, Duurkens VA, Gerritsen WB, Haas FJ: Oxidative stress after lung resection therapy: A pilot study. Chest 2000; 117:999–1003

19. Munch G, Bolck B, Brixius K, Reuter H, Mehlhorn U, Bloch W, Schwinger RH: SERCA2a activity correlates with the force-frequency relationship in human myocardium. Am J Physiol Heart Circ Physiol 2000; 278:H1924–32

20. Doty JM, Saggi BH, Sugerman HJ, Blocher CR, Pin R, Fakhry I, Gehr TW, Sica DA: Effect of increased renal venous pressure on renal function. J Trauma 1999; 47:1000–3

21. Flemming Nielsen F, Bo Borg Mikkelsen BB, Jesper Bo Nielsen JB, Helle Raun Andersen HR, Grandjean P: Plasma malondialdehyde as biomarker for oxidative stress: Reference interval and effects of life-style factors. Clin Chem 1997; 43:1209–14

22. Finkel T: Oxidant signals and oxidative stress. Curr Opin Cell Biol 2003; 15:247–54

23. Viappiani S, Schulz R: Detection of specific nitrotyrosine-modified proteins as a marker of oxidative stress in cardiovascular disease. Am J Physiol Heart Circ Physiol 2006; 290:H2167–8

24. Tupling R, Green H: Silver ions induce Ca2+ release from the SR *in vitro* by acting on the Ca2+ release channel and the Ca2+ pump. J Appl Physiol 2002; 92:1603–10

25. O'Brien PJ: Calcium sequestration by isolated sarcoplasmic reticulum: Real-time monitoring using ratiometric dual-emission spectrofluorometry and the fluorescent calcium-binding dye indo-I. Mol Cell Biochem 1990; 94:113–9

26. O'Brien PJ, Shen H, Weiler J, Mirsalami M, Julian R: Myocardial Ca-sequestration failure and compensatory increase in Ca-ATPase with congestive cardiomyopathy: Kinetic characterization by a homogenate microassay using real-time ratiometric indo-I spectrofluorometry. Mol Cell Biochem 1991; 102: 1–12

27. Beckman JS, Chen J, Ischiropoulos H, Crow JP: Oxidative chemistry of peroxynitrite. Methods Enzymol 1994; 233:229–37

28. Kanski J, Behring A, Pelling J, Schoneich C: Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: Effects of biological aging. Am J Physiol Heart Circ Physiol 2005; 288:H371–81

29. Heerdt PM, Lane PB, Crabtree MJ, Park BJ: Systemic oxidative stress associated with lung resection during single lung ventilation. Eur J Cardiothorac Surg 2006; 30:568–9

30. Neumann J: Altered phosphatase activity in heart failure, influence on Ca2+ movement. Basic Res Cardiol 2002; 97 (suppl 1):I91–5

31. Muller FU, Lewin G, Matus M, Neumann J, Riemann B, Wistuba J, Schutz G, Schmitz W: Impaired cardiac contraction and relaxation and decreased expression of sarcoplasmic Ca2+ ATPase in mice lacking the CREM gene. FASEB J 2003; 17:103–5

32. Heerdt PM, Holmes JW, Cai B, Barbone A, Madigan JD, Reiken S, Lee DL, Oz MC, Marks AR, Burkhoff D: Chronic unloading by left ventricular assist device reverses contractile dysfunction and alters gene expression in end-stage heart failure. Circulation 2000; 102:2713–9

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