Injury to the Ca\(^{2+}\) ATPase of the sarcoplasmic reticulum in anesthetized dogs contributes to myocardial reperfusion injury

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Abstract

Objective: Sarcoplasmic reticulum dysfunction may contribute to calcium (Ca\(^{2+}\)) overload during myocardial reperfusion. The aim of this study was to investigate its role in reperfusion injury. Methods: Open chest dogs undergoing 15 min of left anterior descending coronary artery occlusion and 3 h of reperfusion were randomized to intracoronary infusions of 0.9% saline, vehicle, or the Ca\(^{2+}\) channel antagonist, nifedipine 50 \(\mu\)g/min from 2 minutes before to 5 minutes after reperfusion. After each experiment, transmural myocardial biopsies were removed from ischemic/reperfused and nonischemic myocardium in the beating state and analyzed for i) sarcoplasmic reticulum protein content Ca ATPase, phospholamban, and calsequestrin by immunoblotting and ii) Ca\(^{2+}\) uptake by sarcoplasmic reticulum vesicles with and without 300 micromolar ryanodine or the Ca ATPase activator, antiphospholamban 2D12 antibody.

Results: Contractile function did not recover in controls and vehicle-treated dogs after ischemia and reperfusion mean systolic shortening, \(-2 \pm 2\%\), but completely recovered in nifedipine-treated dogs 17 \(\pm\) 2\%, \(p = NS\) vs. baseline, \(p < 0.01\) vs. control. Ventricular fibrillation occurred in 50% of controls and vehicle dogs and 0% of nifedipine-treated dogs (\(p < 0.01\) vs. control). Ryanodine and the 2D12 antibody improved, but did not reverse the low Ca\(^{2+}\) uptake. Protein content was similar in ischemic/reperfused and nonischemic myocardium. In contrast, Ca\(^{2+}\) uptake and the responses to ryanodine and 2D12 antibody were normal in ischemic/reperfused myocardium from nifedipine-treated dogs. Conclusion: Dysfunction of the sarcoplasmic reticulum Ca\(^{2+}\) ATPase pump correlates with reperfusion injury. Reactivation of Ca\(^{2+}\) channels at reperfusion contributed to Ca\(^{2+}\) pump dysfunction. Ca\(^{2+}\) pump injury may be a critical event in myocardial reperfusion injury. © 1997 Elsevier Science B.V.

Keywords: Nifedipine; Calcium channel antagonist; Myocardial ischemia; Calcium, intracellular concentration; Protein analysis; Stunning arrhythmias; Ventricular fibrillation; Reperfusion; Dog, anesthetized

1. Introduction

Myocardial reperfusion injury is probably mediated by oxygen free radicals and transient calcium (Ca\(^{2+}\)) overload [1–7]. Targets of oxyradicals and Ca\(^{2+}\) activated enzymes include the sarcolemma, sarcoplasmic reticulum, and contractile proteins [2,7]. Injury to the sarcoplasmic reticulum may exacerbate Ca\(^{2+}\) overload during early reperfusion and setup the myocardium for reperfusion injury (contraction dysfunction and ventricular fibrillation) [8,9].

The sarcoplasmic reticulum has a major role in myocardial Ca\(^{2+}\) homeostasis and accounts for >90% of the Ca\(^{2+}\) transient in mammals [2,7,8,10,11]. Release is triggered by Ca\(^{2+}\) influx through sarcolemmal Ca\(^{2+}\) channels [11]. Uptake is mediated by a Ca\(^{2+}\) pump (ATPase) regulated by phospholamban [11]. Ca\(^{2+}\) is concentrated in the

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Sarcoplasmic reticulum is injured by transient ischemia and reticulum at the onset of reperfusion may have a major role in this secondary increase in Ca2+. Reactivation of myocardial Ca2+ channels and Ca2+ release by the sarcoplasmic reticulum at the onset of reperfusion may have a major role in this secondary increase [12,13], but prior studies have not investigated how resumption of this normal trig- 

Reperfusion injury correlates with a secondary increase in Ca2+ after reperfusion [10]. The hypotheses tested in this study are: (i) the sarcoplasmic reticulum function from ischemic/reperfused myocardium; (ii) determine protein content by immuno- blotting, and (iii) compare these findings with sarcoplasmic reticulum from ischemic/reperfused myoc- 

The hypotheses tested in this study are: (i) the sarcoplas- mic reticulum function from ischemic/reperfused myocardium from saline and vehicle treated dogs with control nonischemic myoc- 

Our aims were to: (i) compare sarcoplasmic reticulum function in ischemic/reperfused myocardium from saline and vehicle treated dogs with control nonischemic myoc- 

(ii) determine protein content by immuno- blotting, and (iii) compare these findings with sarco- plasmic reticulum function from ischemic/reperfused myoc- 

cardium of dogs treated with intracoronary nifedipine at the onset of reperfusion. Thereby, open chest dogs undergoing 15 min of left anterior descending occlusion and 3 h of reperfusion were randomized to intracoronary saline, vehicle, or nifedipine (50 µg/min from 2 minutes before until 5 minutes after reperfusion). Transmural biopsies were taken from the ischemic/reperfused and nonischemic zones and analyzed for sarcoplasmic reticulum Ca2+ uptake and protein content.

2. Methods

2.1. General preparation

Mongrel dogs (15–30 kg) were anesthetized with intra- venous sodium pentobarbital (25 mg/kg) and barbitral (200 mg/kg) and ventilated. Saline (0.9%) was infused at 300 ml/hour and body temperature and arterial blood gases maintained at physiological levels. Arterial and left ven- tricular pressures and dP/dt were measured by a high- fidelity, double-tipped pressure transducer catheter (Millar PC 771, Dallas, TX) inserted from the left carotid artery with electronic differentiation. Silastic catheters were inserted in the right femoral artery and vein.

A thoracotomy was performed in the left fifth inter- costal space and the heart suspended in a pericardial cradle. A segment of the left anterior descending coronary artery was isolated proximal to the first major diagonal for an electromagnetic flow probe (Statham SP7515) and a silk ligature. A small distal branch was cannulated retro- gradely with a heparin filled 27 gauge catheter. The tip was advanced into the left anterior descending coronary artery and position confirmed by injection of food color- ing. A catheter was placed in the left atrial appendage for microsphere injection. Dogs were demand atrial paced at 120 bpm via the left atrial appendage. This investigation conforms with the Guide for the Care and Use of Labora- tory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.2. Regional myocardial function

The left anterior descending (ischemic/reperfused zone) and left circumflex coronary distributions (nonischemic zone) were identified by the injection of food coloring and pairs of 5.0 MHz sonomicrometers implanted in the suben- docardium in the circumferential plane in both zones ≥2 cm away from the intracoronary catheter. Segment length was monitored by ultrasonic transit time (Triton Inc., San Diego, CA). End diastole was the onset of the rapid rise in left ventricular pressure and end systole 20 ms before peak negative dP/dt [14]. Systolic shortening (SS) was the change (%) in segment length from end diastole (EDL) to end systole (ESL): SS = ((EDL − ESL)/EDL) × 100. End diastolic segment lengths were normalized baseline [14].

2.3. Regional myocardial blood flow

Myocardial blood flow was measured by radiolabeled microspheres at: (a) 10 min before occlusion; (b) 10 min after occlusion; (c) 30 min after reperfusion; and (d) 3 h after reperfusion [15]. Carbonized microspheres (41Ce, 51Cr, 103Ru, or 85Nb, 15 ± 2 µm, New England Nuclear, Boston, MA) in 10% Dextran and 0.01% Tween 80 were ultrasonicated and vortexed. Twenty µCi were injected in the left atrium. Reference samples were collected from the right femoral artery at 7 ml/min for 130 s starting just before microsphere injection.

After myocardial biopsies, the left anterior descending artery was occluded and India ink injected to delineate the ischemic/reperfused zone. The heart was electrically fib- rillated, removed, and fixed in 10% formalin. Ischemic/reperfused and nonischemic zones were sepa- rated and weighed. Tissue samples (0.8–1.0 g) from the ischemic/reperfused (five) and nonischemic (three) zones were divided into subendo-, mid myo-, and subepi-cardial layers and weighed. Net activity of each isotope in tissue and reference blood samples was measured by a gamma counter (Packard Series 5000, Meriden, CT). Tissue blood flow was calculated by: $Q_m = (Q_r × C_r/C_m) × 100$. $Q_m$ was tissue blood flow (ml/min/100 g). $Q_r$, the withdrawal rate of reference blood sample, $C_r$, the reference sample activity (cpm), and $C_m$ the tissue sample activity (cpm). Blood flows were averaged and dogs excluded for suben- docardial flow > 20 ml/min/100 g during occlusion [16].

2.4. Treatment and ischemia / reperfusion protocols

Dogs were randomized to intracoronary infusions of 0.9% saline (control; 1 ml/min), vehicle (0.9% saline with
4% ethanol and 2% polyethylene glycol 400, or nifedipine (5 mg in 100 ml of 0.9% saline with 4% ethanol and 2% polyethylene glycol 400; 50 µg/min) at the onset of reperfusion. Nifedipine was infused from 2 min before reperfusion until 5 min after reperfusion (total infusion time 7 min) to ensure maximal effects at the onset of reperfusion in the ischemic/reperfused zone [13]. Before and after nifedipine or vehicle, 0.9% saline was infused at 1 ml/min.

After instrumentation and baseline measurements, intracoronary infusion was started and the coronary artery occluded 40 min later. The ligature was released after 15 min and the dogs monitored for 180 min. If ventricular fibrillation occurred, dogs were defibrillated with pads on the lateral left ventricle and the right ventricle with up to 3 shocks (20, 30, and 30 J). A prior study revealed no prolonged effects on function [17].

2.5. Biopsy protocol and tissue preparation

Biopsies were taken from ischemic/reperfused and nonischemic myocardial tissue of control, vehicle, and nifedipine-treated dogs. At the end of each experiment, purse string sutures were placed in the ischemic/reperfused and nonischemic zones. Eleven mm transmural core biopsies were removed in the beating state in random order, flash frozen in liquid nitrogen and stored at −70°C until analysis.

2.6. Isolation of sarcoplasmic reticulum vesicles

Each pair of ischemic/reperfused and nonischemic biopsy samples (0.5–1.0 g) were homogenized at 4°C in a Polytron PT-10-35 (Brinkmann Instruments) for 90 s in 10 ml (v/w) of 10 mM NaHCO₃ [18]. Sarcoplasmic reticulum vesicles were isolated by the method of Jones and Cala [19]. Homogenates were centrifuged at 14,000 g max at 4°C for 20 min to remove large membrane particles and organelles. The supernatant was then centrifuged at 45,000 g max at 4°C for 30 min to yield membrane vesicles enriched in sarcoplasmic reticulum. The vesicles were washed once with 0.6 M KCl, 30 mM histidine (pH 7.0) to extract loosely bound proteins. The vesicles were then resuspended in 0.25 M sucrose and 30 mM histidine (pH 7.0) and stored frozen at −40°C. Protein content was measured by the Lowry method [20].

2.7. 45Ca²⁺ uptake studies

Ca²⁺ uptake and the interaction of the Ca²⁺ pump (ATPase) with phospholamban were studied as previously described [21,22]. Studies with the monoclonal antiphospholamban 2D12 antibody were performed at low ionized Ca²⁺ (0.05 µM) because the antibody only stimulates Ca²⁺ transport at low concentrations [21]. ATP dependent 45Ca²⁺ uptake studies at low ionized Ca²⁺ were performed at pH 7.0 and 37°C in 1 ml of uptake medium containing (millimolar): 50 MOPS, 3 MgCl₂, 100 KCl, 10 potassium oxalate, 3 Na₂ATP, 5 NaN₃, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), and 0.2 CaCl₂ with tracer ⁴⁵Ca²⁺ [21]. Reactions were started by adding sarcoplasmic reticulum vesicles to the uptake medium after preincubation in the presence and absence of the 2D12 antibody [21,22]. At the selected time points in the studies, 100 µl samples were removed for Ca²⁺ uptake analysis [19,22].

The role of the Ca²⁺ release channel was studied by determining ryanodine effects on Ca²⁺ uptake at high ionized Ca²⁺ (1.0 µM) to maximize its stimulation of Ca²⁺ uptake [23]. Studies were done in an uptake medium containing (millimolar): 50 histidine (pH 7.0), 3 MgCl₂, 100 KCl, 5 potassium oxalate, and 0.05 CaCl₂ with tracer ⁴⁵Ca²⁺ [21]. Sarcoplasmic reticulum vesicles were preincubated in uptake medium for 10 min at 37°C with or without 300 µM ryanodine, prior to initiation of reactions by adding 3 mM ATP. At the same time points in the studies, 1600 µl samples were removed for Ca²⁺ uptake analysis [19,22].

The suction filtration method for measuring Ca²⁺ uptake has been reported by this laboratory [19,22]. The 100 and 1600 µl samples from the low (± 2D12) and high ionized Ca²⁺ (+ryanodine) uptake solutions, respectively, were analyzed for protein content by a modified Lowry assay and ionized Ca²⁺ by spectrophotometry with 25 µM 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid as the Ca²⁺ indicator [19,22]. Aliquots were suction filtered on a manifold through a filter (0.45 µm) and washed with 100 mM KCl, 20 mM imidazole (pH 7.0), 1 mM EGTA, and 5 mM MgCl₂. Radioactivity (cpm) of the filtered and filtrate fractions was counted and ⁴⁵Ca²⁺ uptake determined (nmol of Ca²⁺/mg of protein) by the following formula:

\[
\text{cpm (vesicles) − blank} \times \frac{\text{nmol Ca}^{2+}/50 \text{ or } 800 \mu l}{\text{total (cpm)}} = \text{nmol Ca}^{2+}/50 \text{ or } 800 \mu l/\text{mg protein}/50 \text{ or } 800 \mu l
\]

2.8. Immunoblotting of sarcoplasmic reticulum proteins

The aliquot of the sarcoplasmic reticulum vesicle fraction was thawed and 300 µl added to 600 µl of 20% sodium dodecyl sulfate (SDS) [18]. The mixture was incubated at 25°C for 20 min and ultracentrifuged for 5 min at 100,000 rpm (Beckman TL-100.2 rotor). Supernatants were collected and protein content determined by the Lowry method [20].

SDS-polyacrylamide gel electrophoresis (PAGE) was performed with an 8% gel using a modified method of Porzio and Pearson [24]. Five and 15 µg of protein from each supernatant was electrophoresed per lane. For immunoblotting, protein samples were transferred to 0.2-µm pore size nitrocellulose membranes (Schleicher and Schuell, Keene, NH).
Table 1

**Hemodynamics**

<table>
<thead>
<tr>
<th>Controls, N = 8</th>
<th>Vehicle, N = 5</th>
<th>Nifedipine, N = 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate (bpm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>128 ± 5</td>
<td>124 ± 5</td>
</tr>
<tr>
<td>0</td>
<td>126 ± 4</td>
<td>125 ± 6</td>
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<tr>
<td>15 min OCC</td>
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</tr>
<tr>
<td>5 min REP</td>
<td>124 ± 5</td>
<td>122 ± 5</td>
</tr>
<tr>
<td>30 min REP</td>
<td>126 ± 6</td>
<td>123 ± 5</td>
</tr>
<tr>
<td>1 h REP</td>
<td>129 ± 6</td>
<td>124 ± 5</td>
</tr>
<tr>
<td>3 h REP</td>
<td>130 ± 6</td>
<td>123 ± 5</td>
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<table>
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<tr>
<th><strong>MAP (mmHg)</strong></th>
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<tbody>
<tr>
<td>BL</td>
<td>96 ± 5</td>
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<tr>
<td>0</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>15 min OCC</td>
<td>99 ± 6</td>
</tr>
<tr>
<td>5 min REP</td>
<td>97 ± 5</td>
</tr>
<tr>
<td>30 min REP</td>
<td>106 ± 6</td>
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<tr>
<td>1 h REP</td>
<td>102 ± 7</td>
</tr>
<tr>
<td>3 h REP</td>
<td>106 ± 9</td>
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<table>
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<tr>
<th><strong>Normalized EDL</strong></th>
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<td>10 ± 0</td>
</tr>
<tr>
<td>0</td>
<td>10 ± 0.1</td>
</tr>
<tr>
<td>15 min OCC</td>
<td>11.5 ± 0.2</td>
</tr>
<tr>
<td>5 min REP</td>
<td>10.9 ± 0.3</td>
</tr>
<tr>
<td>30 min REP</td>
<td>11.2 ± 0.2</td>
</tr>
<tr>
<td>1 h REP</td>
<td>11.0 ± 0.4</td>
</tr>
<tr>
<td>3 h REP</td>
<td>10.6 ± 0.4</td>
</tr>
</tbody>
</table>

Mean ± SEM, standard error of mean; BL, baseline values after instrumentation; OCC, occlusion; REP, reperfusion; MAP, mean arterial pressure; Pos dp/dt, peak positive derivative of LV pressure; Normalized EDL, end diastolic segment length in the ischemic/reperfused zone normalized to baseline.

Table 2

**Regional myocardial blood flow (ml/min/100 g)**

<table>
<thead>
<tr>
<th>Preocclusion 10 min OCC</th>
<th>30 min REP</th>
<th>3 h REP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonischemic zone — subepicardium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (N = 8)</td>
<td>103 ± 13</td>
<td>140 ± 28</td>
</tr>
<tr>
<td>Vehicle (N = 5)</td>
<td>92 ± 9</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>Nifedipine (N = 8)</td>
<td>108 ± 6</td>
<td>114 ± 7</td>
</tr>
</tbody>
</table>

Subendocardium

| Controls (N = 8)         | 99 ± 13   | 119 ± 17|
| Vehicle (N = 5)          | 86 ± 13   | 83 ± 7  |
| Nifedipine (N = 8)       | 96 ± 3    | 98 ± 5  |

Ischemic / reperfused zone — subepicardium

| Controls (N = 8)         | 94 ± 13   | 10 ± 5  |
| Vehicle (N = 5)          | 87 ± 12   | 12 ± 6  |
| Nifedipine (N = 8)       | 100 ± 4   | 6 ± 2b  |

Subendocardium

| Controls (N = 8)         | 89 ± 22   | 2 ± 1b  |
| Vehicle (N = 5)          | 82 ± 13   | 5 ± 3b  |
| Nifedipine (N = 8)       | 84 ± 3    | 1 ± 1b  |

Endo / epi flow ratio

| Controls (N = 8)         | 0.97 ± 0.09 | 0.20 ± 0.10 | 0.71 ± 0.04 | 0.79 ± 0.07 |
| Vehicle (N = 5)          | 0.95 ± 0.04 | 0.40 ± 0.08b| 0.76 ± 0.04 | 0.94 ± 0.04 |
| Nifedipine (N = 8)       | 0.86 ± 0.08 | 0.16 ± 0.10b| 0.99 ± 0.05b| 0.94 ± 0.07 |

Mean ± SEM, standard error of mean; OCC, occlusion; REP, reperfusion; Endo, endocardial; Epi, epicardial.

Values for Ca\(^{2+}\) ATPase (SERCA2), phospholamban, and calsequestrin were compared by the Student’s t test for paired samples. Repeated-measures and multi (three to six)-way analysis of variance (ANOVA) with the Bonferroni t-test were used to identify changes in continuous data within and between groups, respectively. χ² analysis was used to evaluate the effects of animal exclusion. Protein content and maximal (33 min after initiation of Ca\(^{2+}\) uptake) ryanodine and antiphospholamban antibody induced Ca\(^{2+}\) uptake of ischemic/reperfused myocardium were normalized by dividing the values by the respective values of each pair of specimens of nonischemic myocardium. Linear regression analysis was used to determine whether normalized changes in protein content of the sarcoplasmic reticulum from ischemic/reperfused myocardium correlated with normalized changes in maximal ryanodine or antiphospholamban antibody induced Ca\(^{2+}\) uptake. A two tailed p < 0.05 was considered significant.

Schuell by electrophoresis at 3.0 amp for 90 min in 50 mM NaHPO\(_4\) buffer (pH 7.5) and stained with amido black [25]. The nitrocellulose membranes were cut into horizontal strips corresponding to the mobility regions of Ca\(^{2+}\) ATPase (SERCA2), low and high molecular weights of phospholamban, and calsequestrin. Immunoblotting was performed using a 1:500 dilution of ascites fluid containing monoclonal antibody 2A7-A1, which is specific for the cardiac isoform of SERCA2; a 1:500 dilution of asites fluid containing monoclonal antibody 2D12, which reacts with phospholamban; and polyclonal rabbit antibody to canine cardiac calsequestrin, affinity purified by incubation with canine cardiac calsequestrin bound to nitrocellulose membranes followed by acid extraction [18]. The transfer was blocked with 2% bovine serum albumin. Thereafter, the strips were incubated with the appropriate antibodies followed by incubation with \(^{125}\)I-labeled protein A (New England Nuclear). Protein bands identified by autoradiography were excised and bound radioactivity quantified by gamma counting. Background counts (< 10% of the total counts for each band) were subtracted from all measurements.
3. Results

3.1. Animal data

Thirty-two dogs were randomized to the three experimental groups. Ventricular fibrillation occurred in 12 dogs (32%): 50% (8/16) of controls, 50% of vehicle (4/8) and 0% (0/8, \( p < 0.05 \) vs. control/vehicle) of nifedipine dogs. Only 2 (17%) dogs were successfully defibrillated. One control was excluded due to high collateral blood flow during coronary occlusion. The control, vehicle, and nifedipine groups consisted of eight, five, and eight dogs, respectively.

3.2. Systemic hemodynamics

Hemodynamic data are summarized in Table 1. Hemodynamics in control and vehicle dogs were similar throughout the experimental protocol. Nifedipine infusion only reduced mean arterial pressure during drug infusion.

Heart rate and peak positive dP/dt were unaffected and remained similar to the other groups throughout the protocol. Mean arterial pressure was similar to controls and vehicle dogs before and during coronary occlusion, lower during the first 5 minutes of reperfusion, but similar thereafter. Normalized end diastolic segment length increased during occlusion and recovered by 1–2 h after reperfusion, similar to controls and vehicle dogs.

Fig. 2. \( \text{Ca}^{2+} \) uptake by sarcoplasmic reticulum vesicles from controls (\( N = 8 \)) with and without preincubation with the antiphospholamban (2D12) antibody or 300 \( \mu \text{M} \) ryanodine (Ryn). \( \text{Ca}^{2+} \) uptake studies were performed at (A) low (0.05 \( \mu \text{M} \)) and (B) high ionized \( \text{Ca}^{2+} \) (1.0 \( \mu \text{M} \)), respectively. Values are mean \( \pm \) SEM. (A) Shows that preincubation of nonischemic myocardium (NZ) with 2D12 increased \( \text{Ca}^{2+} \) uptake by 5-6 fold. In contrast, \( \text{Ca}^{2+} \) uptake in ischemic/reperfused myocardium (IZ) was much less. Uptake responded to 2D12, but remained much less. (B) Shows that Ryn doubled \( \text{Ca}^{2+} \) uptake in nonischemic myocardium (NZ). Again, \( \text{Ca}^{2+} \) uptake in ischemic/reperfused myocardium was much less than nonischemic. Uptake responded to Ryn, but remained much less. \( ^{*} p < 0.05 \) vs. NZ.

3.3. Myocardial blood flow

Table 2 summarizes myocardial blood flow in ischemic/reperfused and nonischemic zones. Blood flow before occlusion was similar in controls, vehicle and nifedipine dogs. Collateral blood flow to the ischemic/reperfused zone including the subendocardium was similar in all groups during occlusion. After reperfusion, blood flow to the ischemic/reperfused zone in controls and vehicle dogs was reduced at 30 min and returned to normal at 3 h. Ischemic/reperfused zone blood flow in nifedipine dogs was significantly reduced compared to controls and vehicle dogs.
dogs was similar to baseline at 30 min (p < 0.05 vs. controls) and 3 h after reperfusion. Nonischemic zone blood flow was similar in all groups at all timepoints.

3.4. Regional myocardial function

Systolic shortening in the ischemic/reperfused and nonischemic zones is plotted in Fig. 1A and 1B, respectively. Systolic shortening before occlusion was similar in all groups. Coronary occlusion caused similar degrees of systolic lengthening. After reperfusion, systolic shortening remained depressed in controls and vehicle dogs, but completely recovered in nifedipine-treated dogs within the first hour. Nonischemic zone systolic shortening was similar in all groups at baseline and similarly increased during coronary occlusion and recovered after reperfusion.

3.5. $^{45}\text{Ca}^{2+}$ uptake studies

The yield of sarcoplasmic reticulum membrane protein was similar in nonischemic and ischemic/reperfused myocardium from control, vehicle, and nifedipine-treated dogs. The yields were 1.57 ± 0.24 mg/g, 2.19 ± 0.25 mg/g, 2.10 ± 0.45 mg/g, and 2.02 ± 0.61 mg/g, respectively.

Fig. 2A and 2B plot $\text{Ca}^{2+}$ uptake at low ionized $\text{Ca}^{2+}$ by sarcoplasmic reticulum vesicles from vehicle treated dogs (N = 5) with and without preincubation with the antiphospholamban (2D12) antibody or 300 μM ryanodine (Ryn). $\text{Ca}^{2+}$ uptake studies were performed at (A) low (0.05 μM) and (B) high ionized $\text{Ca}^{2+}$ (1.0 μM). Values are mean ± SEM. (A) Shows that $\text{Ca}^{2+}$ uptake at low $\text{Ca}^{2+}$ in ischemic/reperfused myocardium (IZ) was similar to nonischemic myocardium (NZ) and responded normally to 2D12. (B) Shows that similar findings at high $\text{Ca}^{2+}$ in ischemic/reperfused myocardium and a normal response to Ryn. p < 0.05 vs. NZ.

Fig. 3A and 3B plot $\text{Ca}^{2+}$ uptake by sarcoplasmic reticulum vesicles from nonischemic and ischemic/reperfused myocardium of controls measured at low ionized $\text{Ca}^{2+}$ with and without the 2D12 antiphospholamban antibody and at high ionized $\text{Ca}^{2+}$ with and without 300 μM ryanodine, respectively. The antibody increased $\text{Ca}^{2+}$ uptake of nonischemic myocardium at low ionized $\text{Ca}^{2+}$ by 5–6 fold (Fig. 3A). Normalized $\text{Ca}^{2+}$ uptake in ischemic/reperfused myocardium was markedly reduced (11 ± 6%, p < 0.01). Antibody caused a 4 fold increase in uptake, but normalized uptake remained depressed (22 ± 5%, p < 0.01). Ryanodine doubled $\text{Ca}^{2+}$ uptake of nonischemic myocardium at high ionized $\text{Ca}^{2+}$ (Fig. 3B). Again, normalized $\text{Ca}^{2+}$ uptake in ischemic/reperfused myocardium was depressed (27 ± 9%, p < 0.01). Ryanodine doubled uptake, but normalized uptake remained depressed (27 ± 6%, p < 0.01).

Fig. 3A and 3B plot $\text{Ca}^{2+}$ uptake by vesicles from nonischemic and ischemic/reperfused myocardium of vehicle dogs measured at low ionized $\text{Ca}^{2+}$ with and without the 2D12 antibody and at high ionized $\text{Ca}^{2+}$ with and without ryanodine, respectively. Findings were similar to controls. Normalized $\text{Ca}^{2+}$ uptake in ischemic/reperfused myocardium was depressed at low ionized $\text{Ca}^{2+}$ (p < 0.01).
Fig. 5. Composite autoradiograph of the immunoblot of paired specimens of ischemic/reperfused (IZ) and nonischemic (NZ) myocardium. Lanes 1-10 are paired immunoblots of ischemic/reperfused and nonischemic myocardium from control dogs using 15 μg of enriched sarcoplasmic reticulum fraction. Lanes 11-14 are the immunoblots from ischemic/reperfused and nonischemic myocardium from vehicle dogs. The top portion represents bound antibody to Ca\(^{2+}\) ATPase 2A7-A1, the middle to calsequestrin (CSQ), and the bottom to the high (H) and low (L) molecular weight forms of phospholamban. Migration of molecular weight standards (kd) is indicated at the right.

with and without the 2D12 antibody (10 ± 5% and 18 ± 4%, respectively) and at high ionized Ca\(^{2+}\) (p < 0.01) with and without ryanodine (28 ± 7% and 33 ± 8%, respectively).

Fig. 4A and 4B plot Ca\(^{2+}\) uptake by vesicles from nonischemic and ischemic/reperfused myocardium of nifedipine-treated dogs at low ionized Ca\(^{2+}\) with and without 2D12 antibody and at high ionized Ca\(^{2+}\) with and without ryanodine, respectively. Normalized Ca\(^{2+}\) uptake in ischemic/reperfused myocardium was similar to nonischemic myocardium at low ionized Ca\(^{2+}\) with and without the 2D12 antibody (105 ± 8% and 94 ± 12%, respectively) and at high ionized Ca\(^{2+}\) with and without ryanodine (84 ± 15% and 88 ± 10%, respectively). Absolute Ca\(^{2+}\) uptake in ischemic/reperfused myocardium of these dogs at low ionized Ca\(^{2+}\) with and without 2D12 antibody was similar (p = NS) to control and vehicle nonischemic myocardium and higher (p < 0.01) than control and vehicle ischemic/reperfused myocardium. Absolute Ca\(^{2+}\) uptake in nonischemic myocardium of these nifedipine-treated dogs at high ionized Ca\(^{2+}\) with ryanodine was mildly depressed compared to control dogs (see Fig. 2B and 4B). This effect was attributed to the vehicle, as a similar depression in Ca\(^{2+}\) uptake was observed using sarcoplasmic reticulum vesicles from nonischemic myocardium of vehicle-dogs.

3.6. Immunoblotting of sarcoplasmic reticulum proteins

Figs. 5–7 demonstrate that the reduced Ca\(^{2+}\) uptake by the sarcoplasmic reticulum in dysfunctional myocardium after ischemia and reperfusion did not result from alter-
Oxygen free radicals and transient Ca\(^{2+}\) overload probably mediate myocardial reperfusion injury (contractile dysfunction and ventricular fibrillation) [1–7]. Isolated heart studies have consistently demonstrated a transient, secondary increase in intracellular Ca\(^{2+}\) just after the onset of reperfusion [10]. Pretreatment with activators and inhibitors of Ca\(^{2+}\) release and inhibitors of the Ca\(^{2+}\) ATPase by sarcoplasmic reticulum have been shown to reduce injury in isolated hearts by depleting Ca\(^{2+}\) stores and inhibition of Ca\(^{2+}\) release [12,26,27]. Antagonists of Ca\(^{2+}\) influx also reduce injury in vivo and in vitro, but again were most effective with pretreatment [1–3,9,28–35].

Our laboratory has observed that intracoronary nifedipine prevents reperfusion injury [13], but has not investigated how resumption of this normal trigger for contraction could mediate injury. The sarcoplasmic reticulum Ca\(^{2+}\) ATPase is susceptible to injury by oxyradicals and Ca\(^{2+}\) activated proteases [2,7,25,36]. Since it is the major mediator of Ca\(^{2+}\) homeostasis [11], dysfunction could set up the myocardium for induced protease or oxyradical induced injury.

Most studies regarding sarcoplasmic reticulum function after ischemia and reperfusion have been done in isolated myocytes and Langendorff perfused hearts. Increased Ca\(^{2+}\) release channel open probability and transport capacity correlate with infarction rather than reversible injury [7,37]. Impaired Ca\(^{2+}\) pump function has also been found in myocardial infarction [7,38–40], but reports of function with reperfusion after transient (<20 min) ischemia in isolated hearts have been inconsistent due to the mild dysfunction (>80% of baseline) [7,41–45]. The only previous study in open chest dogs used repetitive 5 min occlusions and reperfusion to induce dysfunction (~10% of baseline) [46]. Ca\(^{2+}\) pump activity was reduced by only 15–20%, but the injury differs in this model [1–3]. Intracellular Ca\(^{2+}\) does not secondarily increase and reperfusion ventricular fibrillation is uncommon [1–7,10,47].

Several other important aspects of sarcoplasmic reticulum function also have not been investigated in reversible regional reperfusion injury. Prior studies have not: (i) directly compared Ca\(^{2+}\) pump, release channel, and phospholamban function; (ii) quantitated sarcoplasmic reticulum protein content; or (iii) evaluated sarcoplasmic reticulum function in ischemic/reperfused myocardium from animals treated with inhibitors of Ca\(^{2+}\) influx.

To investigate these questions, this study documented...
function of Ca²⁺ release channels, phospholamban, and the Ca²⁺ pump of the sarcoplasmic reticulum in nonischemic myocardium and ischemic/reperfused myocardium from control, vehicle, and intracoronary nifedipine-treated dogs. Protein content was measured by immunoblotting. Injury was more severe than that reported from repetitive short-term occlusion and reperfusion [46]. Control and vehicle dogs demonstrated a high rate of reperfusion ventricular fibrillation and severe dysfunction. Nifedipine prevented both aspects of reperfusion injury.

Likewise, abnormalities in Ca²⁺ uptake were more severe than reported after repetitive ischemia and reperfusion [46]. Ca²⁺ release channel and phospholamban function were preserved, but the Ca²⁺ pump was impaired. Ca²⁺ uptake at low and high ionized Ca²⁺ was only 11 and 27% of control myocardium, respectively. Both the 2D12 antibody and ryanodine caused the same relative increase in uptake in nonischemic and ischemic/reperfused myocardium, but absolute Ca²⁺ uptake remained severely depressed in ischemic/reperfused myocardium from control and vehicle dogs.

The reduced Ca²⁺ uptake of ischemic/reperfused myocardium resulted from Ca²⁺ pump dysfunction. Sarcoplasmic reticulum protein yield and content in ischemic/reperfused myocardium were similar to that of nonischemic myocardium. Ca²⁺ uptake was severely reduced irrespective of protein content. The data are comparable to a dog study reporting preserved protein content after prolonged ischemia [48].

In contrast, sarcoplasmic reticulum function was preserved in ischemic/reperfused myocardium of nifedipine-treated dogs. Ca²⁺ uptake at low and high Ca²⁺ responded normally to the 2D12 antibody and ryanodine. These data imply that reactivation of myocardial Ca²⁺ channels at the onset of reperfusion contributes to injury to the sarcoplasmic reticulum Ca²⁺ pump. The impaired Ca²⁺ uptake then sets up the myocardium for injury by escalating diastolic Ca²⁺ during early reperfusion. The preserved content of sarcoplasmic reticulum proteins and the absence of degradative products suggest free radicals rather than proteases probably mediated the injury [1–9].

4.1. Limitations

The role of impaired Ca²⁺ pump activity in reperfusion injury was derived indirectly. Myocardial Ca²⁺ transients were not measured because techniques have not been adapted in vivo [2]. Multiple studies have demonstrated the specificity of nifedipine against Ca²⁺ channels [34] and that the protective effects result from inhibition of myocardial Ca²⁺ channels [13,18]. Nifedipine at concentrations up to 50 μM has no effects on Na⁺/Ca²⁺ exchange [34]. Furthermore, several studies have demonstrated that the myocardial manifestation of inhibition of Na⁺/Ca²⁺ exchange is positive inotropy [18,49]. Dose response studies from this laboratory demonstrate that the dose of nifedipine infused in this study was very negatively inotropic [13].

The method of isolation of sarcoplasmic reticulum vesicles may alter Ca²⁺ uptake data from ischemic/reperfused myocardium, but the methods utilized in this study produce vesicles representative of the whole cell homogenate [48,50]. The isolation technique produces a subtraction of sarcoplasmic reticulum vesicles without significant contamination by sarcolemma [19]. The effects of tissue deterioration were minimized by taking myocardial biopsies in the beating state and freezing the specimens in liquid nitrogen. The isolation was completed in <2 h at 4°C [19].

The function of the calcium release channels and phospholamban was derived indirectly, but prior work has validated the use of ryanodine and the 2D12 antibody to investigate their respective function and Ca²⁺ pump activity in sarcoplasmic reticulum vesicles [21–23]. Ryanodine at 300 μM stimulates Ca²⁺ uptake by completely inhibiting Ca²⁺ release at a pH of 7.2. The monoclonal 2D12 antibody has the same effect as phosphorylation of phospholamban, removing its inhibitory effect on the Ca²⁺ pump [21,22]. Ryanodine maximally stimulates Ca²⁺ uptake at high ionized Ca²⁺ without altering Ca²⁺ pump activity [23], whereas the 2D12 antibody maximally stimulates Ca²⁺ uptake at low ionized Ca²⁺ by increasing Ca²⁺ pump activity [22].

Previous studies have also validated the immunoblotting technique with monoclonal or polyclonal antibodies for protein content [18]. These antibodies are specific for the canine isoforms of calsequestrin, phospholamban, and Ca²⁺ ATPase [18]. Sarcoplasmic reticulum Ca²⁺ release channel content (i.e., ryanodine binding) was not measured because it has previously been shown to be normal or only mildly reduced, rather than increased [7,37].

Only the strips of the Western blots corresponding to the mobility regions of Ca²⁺ ATPase (SERCA2), low and high molecular weights of phospholamban, and calsequestrin were immunoblotted with monoclonal antibody 2A7-A1, monoclonal antibody 2D12, and polyclonal antibody calsequestrin, respectively. Thus, the protocol may not have detected degradation products of SERCA2 or calsequestrin smaller than the region encompassed by the respective strips.

4.2. Conclusions and clinical implications

Ca²⁺ uptake by the sarcoplasmic reticulum was markedly depressed in this model of severe reperfusion injury. The mechanism was depressed Ca²⁺ pump function rather than alterations in protein content or function of Ca²⁺ release channels and phospholamban. Resumption of Ca²⁺ influx at the onset of reperfusion contributed to injury to the Ca²⁺ pump. Thus, injury to the Ca²⁺ pump of the sarcoplasmic reticulum at the onset of reperfusion may be a critical step that accelerates the secondary increase in intracellular Ca²⁺ when Ca²⁺ influx resumes. Inhibition of myocardial Ca²⁺ channels at the onset of
reperfusion may be an optimal target for the minimizing reperfusion injury by delaying resumption of Ca²⁺ influx and preventing injury to the Ca²⁺ pump of the sarcoplasmic reticulum.

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References

[38] Dhalla NS, Panagia V, Singal PK, Makino N, Dixon IM, Eyolfson DA. Alterations in heart membrane calcium transport during the


