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Myocardial stunning is associated with impaired calcium uptake by sarcoplasmic reticulum

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ABSTRACT

Myocardial stunning (temporary post-ischaemic contractile dysfunction) may be caused by oxidative stress and/or impaired myocyte calcium homeostasis. Regional myocardial stunning was induced in open-chest pigs (segment shortening reduced to 68.3 ± 4.7% of baseline) by repetitive brief circumflex coronary occlusion (I/R). Reduced glutathione was depleted in stunned myocardium (1.34 ± 0.06 vs. 1.77 ± 0.11 nmol/mg, $p = 0.02$ vs. remote myocardium) indicating regional oxidant stress, but no regional differences were observed in protein-bound 3-nitrotyrosine or *S*-nitrosothiol content. Repetitive I/R did not affect myocardial quantities of the sarcolemmal sodium–calcium exchanger, L-type channel, SR calcium ATPase and phospholamban, or the kinetics of ligand binding to L-type channels and SR calcium release channels. However, initial rates of oxalate-supported ^{45}Ca uptake by SR were impaired in stunned myocardium (41.3 ± 13.5 vs. 73.0 ± 15.6 nmol/min/mg protein, $p = 0.03$). The ability of SR calcium ATPase to sequester cytosolic calcium is impaired in stunned myocardium. This is a potential mechanism underlying contractile dysfunction.

Introduction

Brief cardiac ischaemia/reperfusion (I/R) can cause reversible injury leading to myocardial stunning (persistent contractile dysfunction after restoration of normal coronary flow). Oxygen free radicals (ROS, generated largely upon reperfusion) contribute to the pathogenesis of myocardial stunning [1], as may other reactive species. Nitric oxide ($^{\cdot}NO$), for example, is generated upon myocardial reperfusion [2], and reacts rapidly with superoxide ($^{\cdot}O_2^-$) to form the strong oxidant peroxynitrite ($ONOO^-$): $^{\cdot}NO + {}^{\cdot}O_2^- \rightarrow ONOO^-$.

Peroxynitrite and other reactive nitrogen species (RNS) may cause injury by nitration of protein tyrosine residues (producing protein-bound 3-nitrotyrosine, pNT), oxidation of thiols such as glutathione, *S*-nitrosation of thiols (generating nitrosothiols (RSNOs)), and initiation of lipid peroxidation in cellular membranes [3].

However, it is not known whether reperfusion after *brief* ischaemia (leading to myocardial stunning) enhances RNS generation, with published studies reporting either increased myocardial levels of RNS adducts [2,4,5] or no change [6,7]. In the present study, we examine potentially harmful biochemical modifications induced by RNS in a large animal model of brief I/R. We find no evidence that such modifications are more prevalent in stunned myocardium.

The mechanism of contractile dysfunction in myocardial stunning is unknown, but may involve impaired excitation–contraction coupling in cardiac myocytes. Accordingly, reduced L-type calcium current [8], blunted calcium transients [8], impaired ryanodine receptor (RyR) function [9], and inefficient clearance of cytosolic calcium by sarcoplasmic reticulum [9,10] have all been observed in stunned myocardium.

In the present study, we examine the major calcium-handling proteins and find that active calcium uptake by the sarcoplasmic reticulum (SR) calcium ATPase (SERCA-2) is diminished in stunned myocardium.

Materials and methods

Experimental protocol

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 under project license 70/4139. Six large white pigs (35–45 kg) were anaesthetised, ventilated and underwent instrumentation of the heart after left thoracotomy, as previously described [11].

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The completed surgical preparation was allowed to stabilise for 30 min before baseline haemodynamic data were recorded. Repetitive regional ischaemia–reperfusion was induced by ten consecutive 2-min inflations of a hydraulic balloon occluder (placed around the atrio-ventricular circumflex coronary artery), each interspersed with 2-min deflations (total 40 min).

Regional myocardial function was assessed by sonomicrometry in both circumflex (ischaemic) and left anterior descending (remote, non-ischaemic) territories. Fractional shortening (FS) was derived from the segment length of myocardium between 2 piezoelectric crystals in end-diastole (SL_D) and end-systole (SL_S): $FS = (SL_D–SL_S)/SL_D$.

Monitoring was continued for a further 30 min, after which lethal propofol overdose was rapidly administered, and the heart immediately excised, sectioned and snap frozen in liquid nitrogen-cooled isopentane.

Glutathione

This technique was adapted from the enzymatic recycling assay of Tietze [12]. About 100 mg samples of myocardium were homogenised in ice-cold buffer, and vortex-mixed to precipitate proteins with either 5% 5-sulphosalicylic acid (SSA, Sigma) for the total glutathione assay, or SSA + 2-vinylpyridine:ethanol (final pH 6–7 [13], followed by incubation at 4 °C for 90 min) for the oxidised glutathione (GSSG) assay. Sample mixtures were centrifuged at 500*g* and 4 °C for 5 min, and the resulting protein-free supernatant withdrawn.

Reagents (see assay reagent mixture, Supplementary material) were mixed with supernatant and placed in a Beckman DU-650 spectrophotometer 1 min after addition of glutathione reductase. Serial measurements of absorbance at 412 nm were made over the next 5 min. Absorbance curves were calibrated against a range of GSH standards (0.25–10 μg/ml).

S-nitrosothiols

Nitric oxide chemiluminesence spectrometry was performed using a Nitric Oxide Analyser (NOA) 280i (Sievers Instruments Inc.). NO derived from contaminant RSNOs and *N*-nitrosamines (RNNOs) was expelled by adding 200 mM $copper_{(II)}$ sulphate into a purge vessel containing glacial acetic acid and potassium iodide, as previously described [14]. Myocardium was homogenised in pre-cooled buffer and mixed with sulphanilamide (2.5% w/v, in 1 M HCl) to render myocardial nitrite inert. Samples were injected for NO analysis twice, with the second injection following decomposition of all sample RSNO by 2.5% $mercury_{(II)}$ chloride.

Protein-bound 3-nitrotyrosine

Frozen myocardium was homogenised in ice-cold saline with chloroform/methanol (2:1) and centrifuged at 2000*g* for 30 min at 4 °C to separate the protein pellet. These pellets were freeze-dried and stable internal standards (50 ng [^{13}C]-nitrotyrosine and 40 μg [2,3,5,6-^{2}H]-tyrosine) added. 3-nitrotyrosine was cleaved from protein by alkaline hydrolysis (4 M sodium hydroxide for 15 h at 120 °C). Following 2 stages of solid-phase extraction, pNT was quantified by gas chromatography–negative ion chemical ionisation mass spectrometry [15]. Results were normalized to tyrosine content to control for between – sample variability in the degree of alkaline hydrolysis.

Western blotting

Frozen myocardium was sonicated in homogenisation buffer. Electrophoresis was performed at 80 V and 21 °C using a 10% acrylamide separating gel in running buffer. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) in transfer buffer at 30 V and 4 °C overnight.

Membranes were incubated with filtered blocking agent for 1 h, with primary antibody/blocking agent for 1 h, with secondary antibody conjugated to horseradish peroxidase for 1 h, and finally left in Tris-buffered saline (TBS) overnight at 4 °C. Membranes were rinsed with bovine serum albumin/Tween/TBS between incubations. Blots were developed in alkaline phosphatase buffer/substrate (ImmunoPure, Pierce), using the protocol provided by the manufacturer. Protein content was calibrated against bovine serum albumin standards using the BioRad Protein Assay Kit.

Radioligand binding studies

Mixed membranes were prepared from frozen myocardium, as described previously [16]. Protein concentrations in all preparations were determined using the Bradford assay [17].

^{3}H-Ryanodine binding to RyR. Mixed membranes (50 μg protein) were incubated with 10 nM ^{3}[H]-ryanodine in binding buffer at 37 °C for 90 min. Conditions for non-specific binding were similar, but with a 1000-fold excess of cold ryanodine (Sigma). The reaction was halted with excess binding buffer, and mixtures were filtered using a negative pressure manifold through Whatman GF/F glass fibre filters (Fisher Scientific). Filters were then placed in 10 ml scintillant (Ultima Gold MV, Packard Bioscience) and analysed in a Wallac 1219 betarac counter. This procedure was repeated for each sample throughout a range of ^{3}[H]-ryanodine concentrations (0.1, 0.2, 0.5, 1, 2 and 5 nM).

Calcium-dependent ^{3}H-Ry binding. The above experiments were repeated at a constant 10 nM ^{3}[H]-ryanodine and a range of free [Ca^{2+}] (100, 50, 20, 10, 5, 2, 1 and 0.1 μM). Contaminant calcium was buffered using 2 mM *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA). Free [Ca^{2+}] below 50 μM were determined by measuring electromotive force using a calcium-sensitive electrode (Orion Research).

^{3}[H]-PN200-110 binding to L-type channels. Experimental protocol was as for ^{3}[H]-Ry binding studies using the same mixed membrane preparation, with a range of ^{3}[H]-PN200-110 concentrations instead of ^{3}[H]-Ry. Non-specific binding was determined by using a 1000-fold excess of unlabelled nifedipine (Sigma). All solutions containing dihydropyridines were prepared in red-light illumination and covered with foil. All incubations were conducted in darkness.

Active ^{45}calcium uptake by SR

Active calcium transport into SR via SERCA-2 was determined *in vitro* by calculating initial rates of thapsigargin-sensitive ATP-dependant oxalate-supported ^{45}Ca uptake into mixed membranes preparations.

These preparations (40 μg protein each) were incubated at 37 °C for 10 min with uptake buffer plus either dimethyl sulfoxide (DMSO; vehicle, for total uptake) or 200 nM thapsigargin/DMSO (non-specific uptake). Active calcium uptake was initiated with 5 mM ATP, and terminated by adding ice-cold Stop Buffer to each incubate at either 30 s, or 60 s, or 90 s or 120 s after initiation. Incubates were vacuum-filtered onto Whatman GF/F glass fibre filters, placed in scintillant and analysed in a Wallac 1219 betarac counter. Experiments were performed at both 200 nM and 2.5 μM $CaCl_2$. Absolute calcium uptake curves were calibrated against a range of [$^{45}Ca^{2+}$] standards.

Details of reagents, buffers and antibodies used are provided in the Supplementary material.

Statistics

Data are expressed as mean ± SEM. Comparisons between stunned and internal control myocardium were made using Student's paired *t*-test or (where data were not normally distributed) Wilcoxon's matched pairs test. Analysis of trends in haemodynamic data over time was performed using repeated measures one way ANOVA (post hoc test for linear trend employed only if overall ANOVA $p < 0.05$).

Results

Haemodynamic data

Heart rate, arterial blood pressure, peak left ventricular pressure, and LV end-diastolic pressure were unchanged from baseline values during the 30 min after repetitive I/R. dP/dt_{max} was lower than baseline at both 15 and 30 min (1888 ± 195 vs. 1675 ± 144 vs. 1677 ± 125 mmHg/s, respectively; both $p < 0.05$, see Table 1 Supplementary material).

Fractional shortening of remote myocardium did not change significantly from baseline during or after I/R (23.4 ± 2.1% vs. 24.3 ± 1.8%, baseline vs. 30 min, p = ns). I/R of the circumflex territory was associated with repeated cycles of akinesia followed by recovery of contractile function. After I/R, there was a gradual fall in FS (19.1 ± 1.8% vs. 13.2 ± 1.6%, baseline vs. 30 min, $p = 0.05$, see Fig. 1). During this period, circumflex territory segment shortening was significantly lower than that of the remote segment ($p < 0.03$ at 5, 15 and 30 min).

Markers of reactive oxygen and nitrogen species

Reduced glutathione (GSH) was depleted in stunned myocardium (1.77 ± 0.11 vs. 1.34 ± 0.06 nmol/mg, remote vs. stunned, $p = 0.02$). Oxidised glutathione (GSSG) concentrations were similar in both territories (91.9 ± 15.7 vs. 85.3 ± 7.3 pmol/mg, p = ns).

Myocardial *S*-nitrosothiol content was similar in both territories when normalized to myocardial mass (394 ± 66 vs. 343 ± 30 pmol/g, remote vs. stunned, p = ns) or to protein content (310 ± 70 vs. 293 ± 58 pmol/mg, p = ns).

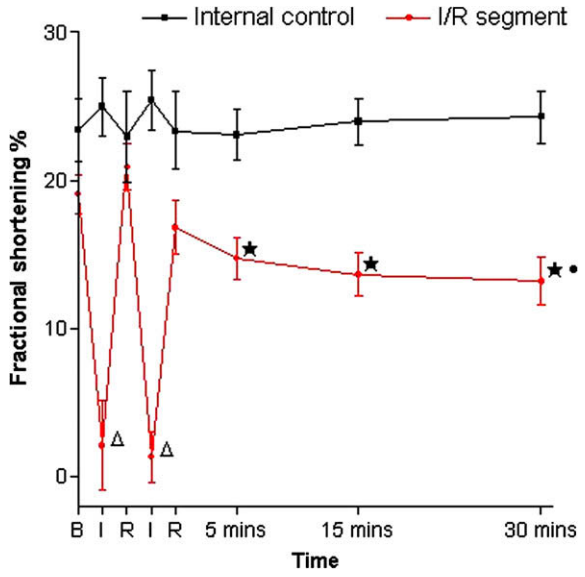

Fig. 1. Regional contractile function (expressed as fractional shortening) at baseline (B), during the first and final cycles of ischaemia (I) and reperfusion (R), and during recovery. Data are mean ± SEM. Δ$p < 0.01$ vs. baseline; •$p = 0.05$ vs. baseline; ★$p < 0.03$ vs. internal control.

Similarly, no regional difference was found in myocardial protein-bound 3-nitrotyrosine concentration (22.6 ± 3.6 vs. 29.9 ± 8.5 pg/μg tyrosine, remote vs. stunned, p = ns). Western blotting was performed using anti-nitrotyrosine primary antibody to help resolve whether nitration of *specific* proteins is altered by I/R. A typical blot is shown in Fig. A (Supplementary material). Analysis of selected band optical densities (Fig. B, Supplementary material) supports the visual impression that no nitrated protein is significantly more abundant in stunned myocardium.

Studies of calcium-handling proteins

Optical densities (arbitrary units, normalized to myosin content) of western blot bands representing calcium-handling proteins in pig heart are presented in Fig. 2. The quantities of the L-type calcium channel, the sarcolemmal sodium–calcium exchanger, the SR calcium ATPase or phospholamban (total and serine-16 phosphorylated) were similar in both stunned and remote myocardium.

Radioligand binding curves for the L-type channel and the ryanodine receptor are presented in Fig. 2. The kinetics of ligand binding (as defined by maximal binding B_{max}, and dissociation constant K_d) were similar in remote and stunned myocardium for both the L-type channel (B_{max} 63.7 ± 6.4 vs. 71.7 ± 15.0 fmol/mg protein, K_d 118.6 ± 20.4 vs. 171.9 ± 31.6 pM, both p = ns) and the ryanodine receptor (B_{max} 246 ± 11 vs. 264 ± 13 fmol/mg protein, K_d 0.801 ± 0.12 vs. 0.953 ± 0.16 nM, both p = ns). I/R did not alter the sensitivity of RyR opening to extraluminal [Ca^{2+}] (B_{max} 238 ± 19 vs. 260 ± 21 fmol/mg protein; EC_{50} 3.68 (2.36–5.75) vs. 3.89 (2.50–6.06) μM, mean (95%CI); remote vs. stunned, both p = ns).

Initial rates of thapsigargin-sensitive oxalate-supported $^{45}Ca^{2+}$ uptake by SR (see Fig. 3) were similar in remote and stunned myocardium at 2.5 μM free Ca^{2+} (1.86 ± 0.52 vs. 1.71 ± 0.20 pmol/s/μg protein, remote vs. stunned, p = ns), but were significantly lower in stunned myocardium at 200 nM free Ca^{2+} (1.21 ± 0.26 vs. 0.71 ± 0.19 pmol/s/μg protein, $p = 0.03$). In both segments, ^{45}Ca uptake increased with increasing free [Ca] (200 nM vs. 2.5 μM, $p = 0.03$ in both remote and stunned myocardium).

Discussion

Our main findings are that in stunned myocardium:

- GSH is depleted, indicating regional oxidant stress.
- The 'footprints' of reactive nitrogen species (*S*-nitrosothiols and protein-bound 3-nitrotyrosine) are no more abundant than in remote tissue.
- The quantities of the major calcium-handling proteins remain unaltered.
- The kinetics of ligand–receptor binding for both the L-type channel and ryanodine receptor are unchanged.
- The ability of SR calcium ATPase to sequester cytosolic calcium is impaired at physiological pCa.

Depletion of GSH in stunned myocardium is a non-specific marker of oxidant stress arising from I/R. Both GSH depletion and unchanged GSSG levels have also been reported in stunned rat myocardium [18], but no such data have been published for large animals (except when full recovery from stunning has already occurred [19]). GSH consumption cannot be taken as evidence of specific RNS-induced injury (for example, $ONOO^- + GSH \rightarrow$ *S*-nitrosoglutathione), since ROS can also initiate *S*-glutathionylation of proteins [20]. Indeed, we observed no increase in total RSNO content of stunned myocardium, suggesting that GSH consumption was largely due to ROS.

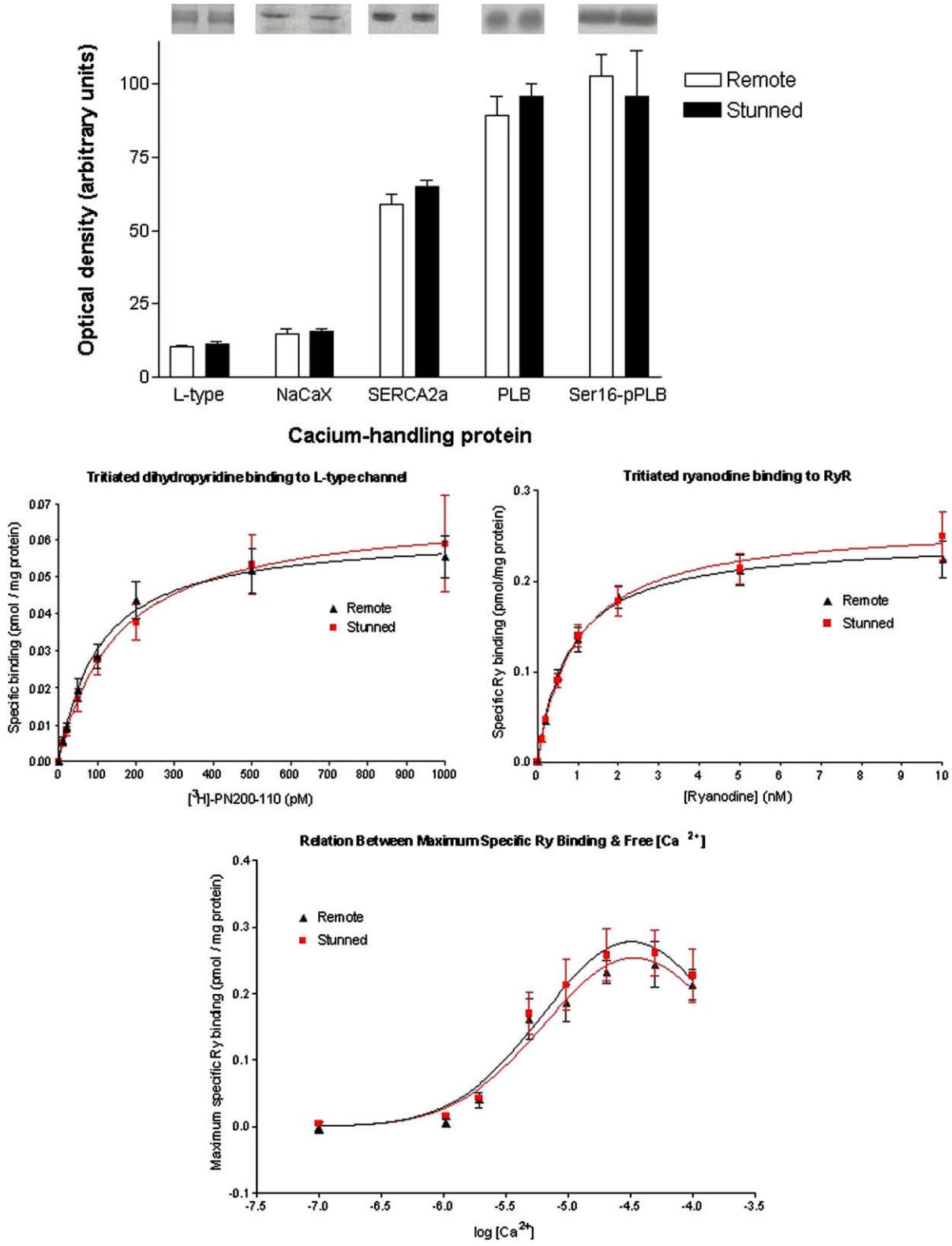

Fig. 2. Data from Western blots and radioligand binding studies of calcium-handling proteins performed on stunned and remote (internal control) pig myocardium. Data are mean ± SEM.

No published data exist on RSNO content in stunned large animal hearts. The present findings do not exclude the possibility that excess RSNO generation is a very brief and reversible reperfusion-associated event that may not be detectable in our pig hearts (from which samples were extracted after 30 min reperfusion *in vivo*). The biological stability of different RSNOs is highly variable [21], and a substantial amount may have been degraded before the opportunity arose to measure it. Furthermore, levels of specific RSNO groups may have increased in a pattern that correlates with myocardial stunning. Data from two different quantitative

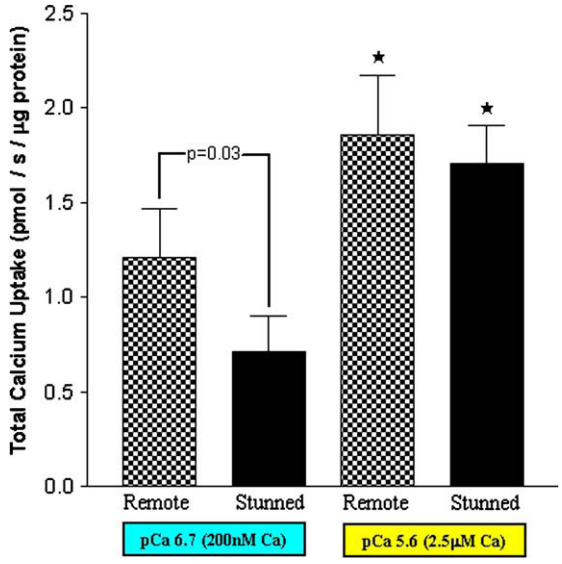

Fig. 3. Initial rates of thapsigargin-sensitive ATP-dependant oxalate-supported ^{45}Ca uptake into mixed membrane preparations from stunned and remote (internal control) pig myocardium. Data are mean ± SEM. ★$p = 0.03$ vs. 200 nM.

methods (mass spectrometry and immunoblotting) concur that I/R did not alter total myocardial pNT content. Furthermore, Western blotting with anti-nitrotyrosine antibody did not expose any specific proteins with enhanced nitration. Taken together, these data indicate that no biochemical 'footprints' of RNS-induced injury remain during the period of contractile dysfunction, suggesting RNS are not central to the pathogenesis of stunning.

These are the first data showing that repetitive brief I/R does not affect the quantities of sarcolemmal sodium–calcium exchanger or L-type channel as measured by Western blotting, although a previous radioligand binding study of the L-type channel also supports this [22]. Our finding that SERCA-2 protein quantity is also unchanged concurs with results from other *in vivo* studies of regional myocardial stunning [8,23]. De-phosphorylation of PLB at the serine-16 site enhances its inhibition of active calcium uptake through SERCA-2, and has been proposed as a mechanism of myocardial stunning. It has been observed in regionally stunned hearts from conscious pigs [8] and dogs [23], but not in the present study.

PLB phosphorylation is cAMP-dependent, and partly driven by β-adrenoceptor stimulation [24]. Acute surgical trauma and general anaesthesia inherent to an open-chest animal model increase cardiac β-adrenoceptor stimulation. This could artifactually mask underlying de-phosphorylation that may otherwise be apparent in a conscious animal. Nevertheless, PLB de-phosphorylation cannot be the mechanism of stunning in the present model, since pPLB levels were comparable in both territories but stunning was observed in post-ischaemic myocardium alone.

Our finding of unaltered ligand–receptor interaction at the L-type channel in stunned myocardium concurs with previously published data [22]. We noted a similar pattern for ryanodine binding to the RyR, in contradiction with another study [9] that reported reduced B_{max}. However, contractile dysfunction was much more severe and persistent than we observed (fractional shortening reduced to 20% of baseline compared with 69% in the present study), suggesting greater oxidant stress and possibly structural change to the RyR accounting for impaired ryanodine binding.

There are, to our knowledge, no published data examining the sensitivity of RyR opening to activating calcium in stunned myocardium. It is pathologically high in chronic heart failure leading to depletion of SR calcium stores [25], and is a plausible mechanism of contractile failure. However, our findings suggest that this mechanism does not operate in the acute setting of myocardial stunning.

SR calcium uptake following I/R has been reported as being either impaired [26] or near normal [27] in isolated rat hearts, and *temporarily* impaired (recovering with extended reperfusion) in isolated rabbit hearts [28] and *in vivo* dog hearts [10,29,30]. It may be that ischaemia impairs SERCA-2 function, with recovery of function occurring to a variable degree during reperfusion. As with myocardial stunning, the extent of recovery may depend upon the initial ischaemic burden and/or the duration of reperfusion.

Two studies have reported oxalate-supported calcium uptake rates by cardiac SR in anaesthetised pigs after I/R [9], Lamers et al. reporting a 17% *increase* in calcium uptake after I/R [31] and Valdivia et al. a 37% reduction [9]. Our data show that at physiological [Ca^{2+}], calcium uptake by SERCA-2 in stunned myocardium is 41% lower than in control tissue. As expected, calcium uptake rises with increasing free [Ca^{2+}], but stunned tissue displayed a greater rise in uptake rate so that no regional difference in calcium uptake was observed at 2.5 µM Ca^{2+}. The cause of this is not clear. Loss of SR protein during sample preparation from stunned myocardium should not have occurred because whole mixed membranes were used rather than purified heavy SR, and no difference between stunned and control myocardium was seen at high free [Ca^{2+}]. Repetitive I/R may have induced structural "damage" in the SERCA-2 protein or its regulators (such as PLB) that somehow alters its sensitivity curve to free [Ca^{2+}]. It is noteworthy that enhanced transcription of SERCA-2 and PLB messenger RNA has been observed in stunned pig myocardium, possibly reflecting a repair mechanism for damaged protein [32].

Impaired SR calcium uptake could underlie myocardial stunning by increasing diastolic free [Ca^{2+}], reducing SR calcium load, and thereby reducing the magnitude of the calcium transient in cardiac myocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.115.

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