

An Inhibitor of the δ PKC Interaction with the d Subunit of F_1F_0 ATP Synthase Reduces Cardiac Troponin I Release from Ischemic Rat Hearts: Utility of a Novel Ammonium Sulfate Precipitation Technique

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Abstract

We have previously reported protection against hypoxic injury by a cell-permeable, mitochondrially-targeted δ PKC-d subunit of F_1F_0 ATPase (d F_1F_0) interaction inhibitor [NH₂-YGRKKRRQRRRMLA TRALSLIGKRAISTVVCAGRKLALKTIDWVSFDYKDDDDK-COOH] in neonatal cardiac myo-cytes. In the present work we demonstrate the partitioning of this peptide to the inner membrane and matrix of mitochondria when it is perfused into isolated rat hearts. We also used ammonium sulfate ((NH₄)₂SO₄) and chloroform/methanol precipitation of heart effluents to demonstrate reduced cardiac troponin I (cTnI) release from ischemic rat hearts perfused with this inhibitor. 50% (NH₄)₂SO₄ saturation of perfusates collected from Langendorff rat heart preparations optimally precipitated cTnI, allowing its detection in Western blots. In hearts receiving 20 min of ischemia followed by 30, or 60 min of reperfusion, the Mean \pm S.E. (n = 5) percentage of maximal cTnI release was 30 \pm 7 and 60 \pm 17, respectively, with additional cTnI release occurring after 150 min of reperfusion. Perfusion of hearts with the δ PKC-d F_1F_0 interaction inhibitor, prior to 20 min of ischemia and 60–150 min of reperfusion, reduced cTnI release by 80%. Additionally, we found that when soybean trypsin inhibitor (SBTI), was added to rat heart effluents, it could also be precipitated using (NH₄)₂SO₄ and detected in western blots. This provided a convenient method for normalizing protein recoveries between groups. Our results support the further development of the δ PKC-d F_1F_0 inhibitor as a potential therapeutic for combating cardiac ischemic injury. In addition, we have developed an improved method for the detection of cTnI release from perfused rat hearts.

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Introduction

Cardiac ischemia/reperfusion (IR) injury is responsible for more annual deaths than any other medical condition. It occurs as a consequence of a heart attack when one or more of the coronary arteries become occluded, typically by atherosclerosis or thrombosis. This leads to regional deprivation of the myocardium to oxygen, nutrients and other factors, and if the blockage is not relieved promptly, cardiac cells experience severe energy deprivation, the loss of ion homeostasis, excessive proteolysis, oxidative stress, and additional damaging events, which culminate in cell death [1,2]. Currently, the most successful strategy for minimizing cardiac IR injury is to restore blood flow and “reperfuse” the tissue. However, considerable injury also occurs during reperfusion [1,2] and improved therapeutics for IR injury are desperately needed.

Following a prolonged IR insult, intracellular proteins are released from cardiac muscle cells and can be detected in serum as biochemical markers of cardiac IR injury [1,3]. At present the preferred protein used for this purpose is cardiac troponin I (cTnI) [4–6]. cTnI is a myofibrillar protein which complexes with

tropomyosin and troponins T and C to minimize Ca⁺⁺ binding to troponin C, and prevent subsequent crossbridge formation between the actin and myosin myofilaments [7]. Hence, it plays a crucial role in the diastolic relaxation. It has only ~50% amino acid homology to skeletal muscle TnI and is found almost exclusively in the heart [3]. The effects of cardiac IR injury on cTnI depend on the duration of the insult. During mild cardiac ischemia proteolysis of cTnI may not lead to its release from cells, and instead contributes to reversible contractile dysfunction known as myocardial stunning [8]. In prolonged ischemia or IR, cTnI is released into serum, mostly as a consequence of cardiac myocyte necrotic cell death [1–3].

Many laboratories have previously focused on mechanisms of cardiac IR injury and protection mediated by the protein kinase C (PKC) family [9–17]. In support of this, previous studies have reported a PKC ϵ -selective activating peptide and a PKC δ translocation inhibitor peptide that induce cardio-protection from IR injury in cardiac myocytes [18], isolated hearts and *in vivo*, in transgenic mice [11,17,18]. Furthermore, a PKC δ -selective translocation inhibitor, administered immediately following a

prolonged ischemic insult, improved the recovery of mouse heart ATP levels during oxygenated reperfusion [18]. We have also previously demonstrated, in neonatal cardiac myocytes, that the ϵ PKC isozyme protects cytochrome oxidase [19–22] during PC and the δ PKC isozyme interacts with the “d” subunit of F_1F_0 ATP synthase (dF_1F_0) to inhibit F_1F_0 function, impair energetics, and reduce survival following prolonged hypoxia [14–16]. Instrumental in this latter work was a peptide developed in our laboratory, which inhibits the δ PKC- dF_1F_0 interaction [15,16].

The F_1F_0 -ATP synthase is the terminal complex in oxidative phosphorylation and supplies over 90% of cardiac energy, making it a logical focus of cardiac IR research. It is a 16-subunit enzyme complex associated with the inner mitochondrial membrane (IMM) [23]. During severe heart attacks the IMM gradient, which supplies the energy for F_1F_0 -ATP synthase, is lost [23]. As a consequence, the F_1F_0 -ATP synthase first becomes inhibited and then operates in reverse as an ATPase [1,2,23]. Initially this allows the F_1F_0 complex to pump protons out of the mitochondrial matrix which in-turn, re-establishes some of the lost IMM potential (Ψ_m) [23]. However, if ischemia is not interrupted, F_1F_0 ATPase contributes heavily to the loss of myocardial ATP and consequent cell death [1,2,23]. In addition, the return of aerobic ATP synthesis is impaired following heart attacks, during reperfusion, but the mechanisms for this delay are not completely known. Previous studies have proposed that cardiac preconditioning (PC), a cardio-protective response against IR injury, inhibits F_1F_0 ATPase-mode activity [24,25], which may contribute protection by reducing ATP hydrolysis after ischemic insults. There have also been reports that PC induces an improved recovery of cardiac ATP levels following prolonged IR injury [1,2,6]. The F_1F_0 complex therefore, has the potential to impact thousands of energy-requiring processes that contribute to normal cardiac functioning and IR injury.

In our studies with the δ PKC- dF_1F_0 inhibitor we wanted to complement other methods of quantifying infarct size (e.g. triphenyltetrazolium chloride (TTC) staining techniques) with a confirmatory biochemical marker of myocardial injury, such as cTnI release. This study provides the first demonstration of reduced cTnI release from Langendorff hearts perfused with the δ PKC- dF_1F_0 interaction inhibitor prior to ischemic exposures.

Materials and Methods

Ethics Statement

Extreme care was taken to avoid pain or duress to animals used in this study. Briefly, hearts were isolated from adult male Sprague-Dawley rats (350 g) under complete anesthesia (see below). All experimentation was approved by The Georgia Regents University Institutional Animal Care and Use Committee (approval #2009-156) and was in compliance with The Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Materials

Unless otherwise specified all protease inhibitors and chemicals were from Sigma Chemical Co. Crystalline ammonium sulfate ($(NH_4)_2SO_4$) was from MP Biomedical. Electron microscopy fixatives, resins and other supplies were from Electron Microscopy Sciences (Ft. Washington, PA).

Langendorff Heart Preparations

Anesthesia was conducted by intraperitoneal injection of ketamine HCl (100 mg/kg) and xylazine (10 mg/kg) as previously described [19,22]. A surgical incision to open the chest was made

and heparin (285 units/kg) was administered via injection into the inferior vena cava. Hearts were rapidly excised and rinsed in oxygenated (95% O_2 , 5% CO_2), chilled modified Krebs's buffer (115 mM NaCl, 4 mM KCl, 1.1 mM $MgSO_4 \cdot 7H_2O$, 1.3 mM KH_2PO_4 , 24 mM $NaHCO_3$, 1.0 mM $CaCl_2$, 5.5 mM glucose, 50 μ Unit/ml insulin, 0.1 mM sodium pyruvate, 0.5 mM taurine, pH 7.4) and attached to the aorta to a Langendorff perfusion apparatus. Retrograde-perfusion was conducted with 37°C modified Krebs's buffer at constant pressure (75 mmHg) and a water-inflated, balloon-tipped catheter was inserted into the left ventricle (LV) through the left atrium. LV end diastolic pressure was adjusted to 5 mm Hg and the volume of the balloon was not subsequently changed. LV catheter pressure was detected using a pressure transducer. Cardiac parameters were calculated using AD Instruments PowerLab software. Hearts were paced at 300 bpm using a Grass SD stimulator except during global ischemia when pacing was stopped.

Homogenization of Hearts

Rat hearts were isolated as described above. Cardiac ventricles were minced into ~2 mm cubes, followed by homogenization in chilled homogenization buffer (20 mM tris-aminomethane, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 10 μ g/ml each of phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin) for 1 min using a motorized tissue homogenizer. Samples were then placed on ice for 1 min. This procedure was repeated 3 times. Next, samples were centrifuged at 600 \times g to pellet Triton-insoluble proteins. The Triton-extracted supernatant was used as a positive control for cTnI immunoreactivity in western blots and also to optimize cTnI $(NH_4)_2SO_4$ and chloroform/methanol (Chl/MeOH) precipitations.

Perfusion of Hearts with δ PKC- dF_1F_0 Modulatory Peptides and Ischemia/reperfusion (IR) Protocols

Following a 20 min equilibration period, rat hearts were perfused for 1 hr with 50 nM concentrations of the cell-permeable, mitochondrial-targeted δ PKC- dF_1F_0 inhibitor [NH₂-YGRKKRR QRRRMLATRALSLIGKRAISTSV CAGRK-LALKTIDWVSFDYKDDDDK-COOH] peptide or scrambled-sequence (inactive) control peptide [NH₂-YGRKKRRQRRRMLATRALSLIGKRAISTSV CADKIGWAVLRTKSLFDYKDDDDK-COOH] at 37°C in oxygenated, modified Krebs's buffer. Details of peptide syntheses/purification have been provided elsewhere [15,16]. Peptides were not washed out prior to ischemia and we did not supplement reperfusion buffers with peptides.

$(NH_4)_2SO_4$ Precipitation of Proteins in Cardiac Effluents

$(NH_4)_2SO_4$ saturation levels were obtained using the online *EnCor Biotechnology Ammonium Sulfate Calculator Program* (<<http://www.encorbio.com/protocols/AM-SO4.htm>>). Effluents (50 ml) from Langendorff-perfused hearts were collected at the indicated times, frozen in liquid nitrogen, and stored at -80°C until use in $(NH_4)_2SO_4$ precipitations. Thawed effluents were then placed in a 100 ml beaker with a stir bar, and gently stirred in an ice bath. $(NH_4)_2SO_4$ was added slowly (~1.5 g every 3 min), and allowed to dissolve until final concentrations were achieved. Samples were stirred on ice for an additional 1 hr., and then centrifuged (4°C) at 20,000 \times g for 15 min in a Beckman JA-20 fixed-angle rotor. Supernatants were carefully removed, so as not to disrupt the protein pellet at the bottom of the tube, and were discarded. The inside of the centrifuge tubes were blotted dry with Kimwipes, taking care to avoid the bottom 3 cm where the protein pellets

were. Next, proteins were resuspended in 180 μ l of solubilization buffer (SB) (10 mM Tris-HCl, pH 7.4, 2 μ g/ml each of PMSF, leupeptin, and aprotinin) and transferred to a 2 ml microfuge tube, on ice. This step was repeated twice to rinse residual amounts of precipitated protein out of the centrifuge tube. Each 180 μ l wash with SB was placed on ice in a separate 2 ml microfuge tube. Following washes with SB, the large Beckman centrifuge tubes used for the $(\text{NH}_4)_2\text{SO}_4$ precipitation were further washed with 600 μ l of room temperature (RT) methanol, which was then transferred to one of the microfuge tubes that contained the 180 μ l of SB wash prepared in the previous steps. This methanol step was repeated 2 additional times with each 600 μ l methanol volume being placed in one of the remaining microfuge tubes (with 180 μ l of SB wash). During these (SB/methanol) steps, the bottom 3 cm of the large Beckman centrifuge tubes were meticulously washed using a pipettor on all sides, at least 5 times, to recover as much protein as possible. From this point on, all steps were conducted at RT. Samples in the microfuge tubes containing SB (180 μ l) and methanol (600 μ l) were vortexed and then 150 μ l of chloroform was added, followed by vortexing. Next, 450 μ l of deionized water was added to each tube, the tube was vortexed, and then subjected to centrifugation (12,000 rpm) for 5 min in a microfuge. The top phase of the resulting solution was discarded and 450 μ l of methanol was added to each tube, followed by vortexing. A second 12,000 rpm centrifugation was conducted and the supernatant was discarded. The inner walls of the microfuge tube were blotted dry with Kimwipes, being careful not to touch the pellet at the bottom of the tube. At this stage large white pellets were observed which contained substantial amounts of residual $(\text{NH}_4)_2\text{SO}_4$. It was therefore, necessary to re-dissolve the pellets in 150 μ l of SB and then repeat the entire Chl/MeOH precipitation protocol (as described above) one time to rid the sample of excess $(\text{NH}_4)_2\text{SO}_4$. After the second Chl/MeOH extraction, the microfuge tube walls were blotted dry and the resulting pellets were dissolved in 40 μ l of 1:3 diluted Laemmli sample buffer, heated at 85°C for 5 min, and either stored at -20°C until use, or loaded directly on SDS-PAGE gels.

SDS Polyacrylamide Gel Electrophoresis and Western Blot Analyses

SDS-PAGE (12% acrylamide gels) and electro-elution of proteins onto nitrocellulose paper (NCP) were as previously described [14,16,19,22]. The resulting blots were stained with 0.05% (w/v) Ponceau S stain in 5% acetic acid to visualize proteins, and then probed with anti-cTnI (Santa Cruz (Cat. No sc-52277) or anti-FLAG (Sigma), each at 1:500 dilution) or anti-SBTI (1:30,000, Thermo Scientific) antisera. Detection was by horse radish peroxidase-coupled rabbit anti-mouse 1:1500 (for anti-cTnI and anti-FLAG) or goat anti-rabbit 1:1500 (for anti-SBTI) secondary antisera, and enhanced chemiluminescence (GE Healthcare) [14–16].

Isolation of Crude Mitochondrial Matrix and Inner Membrane Fractions for Western Blot Analyses

The δ PKC-dF₁Fo interaction inhibitor (50 nM) was perfused into Langendorff rat heart preparations for 1 hr at 37°C. Mitochondria were then isolated from hearts using Percoll/Optiprep density gradients [14–16,19,22] and subjected to sub-fractionation as previously described [26]. Briefly, 0.5 mg of isolated mitochondria were incubated 15 min on ice in 500 μ l of hypotonic buffer (5 mM Tris HCl, 1 mM EDTA, pH 7.4), and then centrifuged at 20,000 \times g (10 min, 4°C). The resulting pellet, containing mitoplasts, was resuspended in 500 μ l hypotonic buffer

and sonicated on ice to rupture the inner mitochondrial membrane (IMM). After sonication the fragmented mitoplasts were subjected to centrifugation at 100,000 \times g. The pellet was used as the IMM-enriched fraction and the supernatant contained the mitochondrial matrix protein fraction. The IMM, and matrix fractions were precipitated using chloroform/methanol techniques and then solubilized in Laemmli sample buffer. These samples were heated for 5 min at 85°C and used in western blots.

Electron Microscopy

Fixation, embedding and sectioning of cardiac ventricle or isolated mitochondria and immunogold localization of the FLAG epitope-tagged δ PKC-dF₁Fo interaction inhibitor was performed by The Georgia Regents University Electron Microscopy Core Facility. For these experiments rat hearts were perfused in Langendorff mode for 1 hr, as described above, with 50 nM concentrations of the δ PKC-dF₁Fo interaction inhibitor. Hearts were then quickly removed from the Langendorff apparatus and either homogenized to prepare isolated mitochondria, or perfused with IEM fixative (see below) and 4 thick cross sections of the ventricles were prepared. One or more 2 mm cubes were rapidly excised from one of the sections. Tissue or mitochondria were fixed in 4% formaldehyde, 0.2% glutaraldehyde, 2 mM CaCl₂, 4 mM MgSO₄ and 0.1 mM sodium cacodylate overnight at 10°C. Samples (mitochondrial pellet or cubes from the left ventricle) were then washed in 0.1 M cacodylate buffer, followed by deionized water and were then dehydrated in a graded ethanol series up to 95% and embedded in LR White resin as previously described [27]. Thin sections were cut on a Leica EM UC6 ultramicrotome (Leica Microsystems, Inc. Bannock, IL) and collected on 200 mesh nickel grids. Sections were incubated for 2 hrs at room temperature in a humid chamber with blocking buffer (BSA (5%), normal donkey serum (3%), Tween (0.05%) in Tris-Buffered saline) prior to an overnight incubation at 4°C with M2 anti-FLAG antisera (Sigma) diluted in blocking buffer. Sections were then washed 4 \times at RT with blocking buffer and incubated with anti-mouse 15 nm circular gold particle-labeled secondary antibody for 2 hrs as previously described [28,29]. Grids were washed twice with blocking buffer, followed by deionized water, then post-stained with 2% aqueous uranyl acetate and bismuth subnitrate. Samples were visualized using a JEM 1230 transmission electron microscope (JEOL USA Inc., Peabody, MA) at 110 kV and imaged with an UltraScan4000 CCD camera and First Light Digital Camera Controller (Gatan Inc., Pleasanton, CA).

Statistical Analyses

Differences between two groups were assessed using unpaired Student's t-test, and multiple group comparisons were evaluated using one-way ANOVA with Bonferroni's post hoc test. A p value \leq 0.05 was considered significant.

Results

Chloroform/methanol (Chl/MeOH) Precipitation Improves Detection of Low Levels of Cardiac Troponin I in Western Blots

As indicated in Methods, it was necessary to use Chl/MeOH precipitation of proteins following $(\text{NH}_4)_2\text{SO}_4$ protocols to reduce the amount of residual $(\text{NH}_4)_2\text{SO}_4$ in our samples prior to loading them on SDS-PAGE gels for Western blots. We anticipated that the amount of cTnI released from our Langendorff heart preparations might be at the lower limit of detection and that there could be poor recovery of low amounts of cTnI following the Chl/MeOH extractions. We therefore, first evaluated the

efficiency of Chl/MeOH precipitation of cTnI from rat heart homogenates (Figure 1). In these analyses, 10–50 μg of rat heart ventricle protein was subjected to either Chl/MeOH extraction prior to solubilization in Laemmli sample buffer (Figure 1, left side) or was directly solubilized in Laemmli sample buffer without Chl/MeOH extraction (Figure 1, right side). Samples were then subjected to SDS-PAGE and western blot analysis using anti-cTnI antisera and enhanced chemiluminescence detection methods. In these analyses we detected a single cTnI species of ~ 25 kDa, which is similar to the previously published molecular mass (26 kDa) for the rat holo-cTnI protein [30]. Of most importance, we observed no loss of cTnI detection when samples were precipitated with Chl/MeOH just prior to SDS-PAGE. In fact, with 10 μg of cardiac ventricle protein there was a $64 \pm 14\%$ improvement (Mean \pm S.E., $n = 4$, $p < 0.02$) in cTnI detection (Figure 1).

Utility of Exogenously Added Soybean Trypsin Inhibitor (SBTI) for Normalization of Protein Recoveries in $(\text{NH}_4)_2\text{SO}_4$ cTnI Precipitations

In Figure 2, 50 ml of chilled Krebs's buffer was supplemented with protease inhibitors (SBTI, leupeptin, aprotinin, PMSF), and 200 μg of rat heart ventricle homogenate (used as a source of cTnI). Proteins were then concentrated using $(\text{NH}_4)_2\text{SO}_4$ precipitation and residual amounts of $(\text{NH}_4)_2\text{SO}_4$ were removed using Chl/MeOH techniques. Maximal precipitation of total cardiac proteins occurred using solutions that were $\geq 80\%$ saturated with $(\text{NH}_4)_2\text{SO}_4$ (Figure 2A). Of interest, the major protein detected by Ponceau S staining in our nitrocellulose paper (NCP) blots was a protein of ~ 20 kDa (See arrow, Figure 2A). This protein was not present in the rat heart homogenate (Figures 3 and 4, lane 1) loaded on gels as a positive control for cTnI immunoreactivity. This suggested to us that the unidentified ~ 20 kDa protein was not likely coming from the cardiac ventricle protein in our samples. Since we did not add SBTI to the rat ventricle homogenate, but it was added to the 50 ml Krebs's buffer samples used for cTnI precipitation we hypothesized that the ~ 20 kDa protein might be SBTI. SBTI is not found naturally in rat hearts, and we added a known amount of it to each $(\text{NH}_4)_2\text{SO}_4$ precipitated sample. We reasoned that SBTI might therefore, be

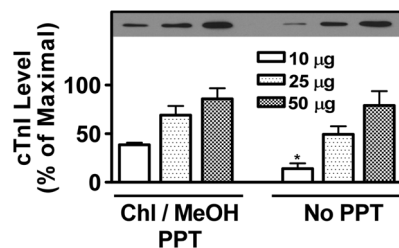


Figure 1. Chloroform methanol precipitation of proteins improves cardiac troponin I (cTnI) detection in Western blots. Cardiac left ventricular tissue from adult rats was homogenized as described in Methods. Homogenate protein (10–50 μg) was (left side) or was not (right side) subjected to chloroform/methanol precipitation prior to its resolution on SDS-PAGE. Western blot analyses with anti-cardiac troponin I (cTnI) antisera were then conducted. A typical autoradiograph is shown (top). Histograms represent Mean \pm S.E. densitometry values, expressed as the percentage of maximal densitometry, from 4 independent experiments. Asterisk shown in the figure indicates statistically significant difference ($p < 0.01$) between 10 μg protein groups for chloroform/methanol precipitated (left) vs. not chloroform/methanol precipitated (right) proteins. doi:10.1371/journal.pone.0070580.g001

useful as a standard to normalize the recovery of protein from the centrifugation steps in each $(\text{NH}_4)_2\text{SO}_4$ precipitation. We next probed these blots with SBTI antisera in western blot analyses (Figure 2B). We consistently observed strong SBTI immunoreactivity in the same blot region as the Ponceau S stained ~ 20 kDa protein. In our experiments SBTI did not precipitate with $(\text{NH}_4)_2\text{SO}_4$ saturation levels at or below 30% saturation levels (Figure 2C–D). We found that $32 \pm 8\%$ (X \pm S.E., $n = 6$) of SBTI maximal immunoreactivity was detectable in 50% $(\text{NH}_4)_2\text{SO}_4$ saturated solutions ($p < 0.002$ vs. 10% $(\text{NH}_4)_2\text{SO}_4$), but maximal precipitation of SBTI ($98 \pm 2\%$ ($p < 0.0001$ vs. 10% $(\text{NH}_4)_2\text{SO}_4$), and $89 \pm 7\%$ ($p < 0.0001$ vs. 10% $(\text{NH}_4)_2\text{SO}_4$), (Mean \pm S.E., $n = 6$) occurred with samples that were 80 or 90% saturated with $(\text{NH}_4)_2\text{SO}_4$ (Figure 2A and B). These results indicated that the combined $(\text{NH}_4)_2\text{SO}_4$ and Chl/MeOH techniques we utilized, precipitated SBTI, allowing its reproducible detection in western blots.

Refinement of Protein Precipitation Techniques for Cardiac Troponin I (cTnI) Detection

We next conducted experiments in which 50 ml of Krebs's buffer was supplemented with 200 μg of rat cardiac ventricular homogenate to determine the optimal $(\text{NH}_4)_2\text{SO}_4$ solution saturation level for the precipitation of cTnI (Figure 2C). These analyses detected a single ~ 25 kDa protein, which was maximally precipitated when solutions were $\geq 50\%$ saturated with $(\text{NH}_4)_2\text{SO}_4$ (Figures 2C–D). When compared to the 10% $(\text{NH}_4)_2\text{SO}_4$ group we observed statistically significant increases in the cTnI precipitated in the 50% ($p < 0.0001$), 80% ($p < 0.003$), and 90% ($p < 0.04$) $(\text{NH}_4)_2\text{SO}_4$ groups. This is consistent with previous studies which used $(\text{NH}_4)_2\text{SO}_4$ in the 30–60% saturation range to precipitate all troponin proteins from bovine heart or rabbit back and thigh muscles [31,32]. However, these previous studies were not dealing with the low levels of cTnI found in our experiments, so we felt it was necessary to establish optimal conditions for our analyses. With 80% $(\text{NH}_4)_2\text{SO}_4$ saturation, reproducible levels of cTnI were also detected, but they were 71% ($n = 6$, $p < 0.04$) of the cTnI that was precipitated with 50% $(\text{NH}_4)_2\text{SO}_4$. With 90% $(\text{NH}_4)_2\text{SO}_4$ solutions we observed 52% of the 50% $(\text{NH}_4)_2\text{SO}_4$ saturation level (Figure 2C–D). Recall that maximal SBTI precipitation was observed using $(\text{NH}_4)_2\text{SO}_4$ saturation levels $\geq 80\%$. However, close analysis of the mean \pm S.E. data revealed that, even though SBTI precipitation was not maximal using 50% saturated $(\text{NH}_4)_2\text{SO}_4$ solutions, a reproducible amount of SBTI was precipitated using either 50% or 80% saturated $(\text{NH}_4)_2\text{SO}_4$ solutions. Also, solutions that were 50% saturated with $(\text{NH}_4)_2\text{SO}_4$ contributed less residual $(\text{NH}_4)_2\text{SO}_4$ in SDS-PAGE analyses.

Commercially Available SBTI is Free of Cardiac Troponin I (cTnI)

As a control we next confirmed that the SBTI used in these experiments (Sigma Chemical Co., #T9003) was devoid of cTnI. Figure 3 is a Western blot in which 0–60 μg of commercially available purified SBTI was detected using the above mentioned SBTI antisera. Lane 1 of Figure 3A represents 40 μg of rat cardiac ventricle homogenate. Note the absence of Ponceau S staining (Figure 3A) and immunoreactivity (Figure 3B) for the ~ 20 kDa SBTI protein in this homogenate. In contrast, the homogenate yielded strong immunoreactivity for cTnI (Figure 3B, lane 1), but no cTnI was detected in samples containing only purified SBTI (Figure 3B, lanes 3–7). These results confirm the absence of cTnI

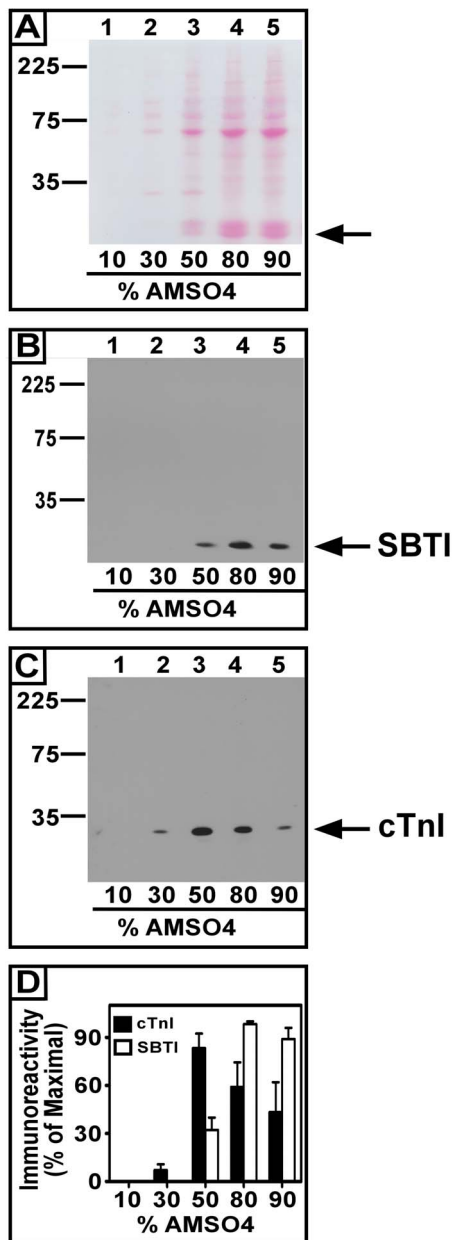


Figure 2. Optimization of ammonium sulfate (NH₄)₂SO₄ precipitation of cardiac troponin I (cTnI) and the utility of soybean trypsin inhibitor as a normalization tool. In each experiment homogenate protein (200 μg) from rat cardiac left ventricle was added to each of 5 different aliquots of Krebs' buffer (50 ml) on ice. The protease inhibitors leupeptin, aprotinin, phenylmethylsulfonyl-fluoride and soybean trypsin inhibitor (SBTI) were then added to each aliquot. Different amounts of (NH₄)₂SO₄ were then added to each 50 ml sample to determine the optimal percentage of (NH₄)₂SO₄ (0–90% (NH₄)₂SO₄-saturated solutions) for precipitation of cTnI. Panel A is a Ponceau S stain of a blot of the (NH₄)₂SO₄ precipitated proteins from each sample. The arrow in Panel A marks the position of the most prominent protein band in the gel, which we later determined to be SBTI. Panel B is a Western blot for exogenously added SBTI. Panel C is a similar blot, but anti-cTnI immunoreactivity was monitored instead of SBTI immunoreactivity. Data in the histogram of Panel D shows Mean±S.E. autoradiograph densitometry scores, expressed as the percentage of maximal densitometry, from 5 independent experiments. Note the optimal level of (NH₄)₂SO₄ saturation for the precipitation of SBTI was ≥80% whereas for cTnI it was 50%. doi:10.1371/journal.pone.0070580.g002

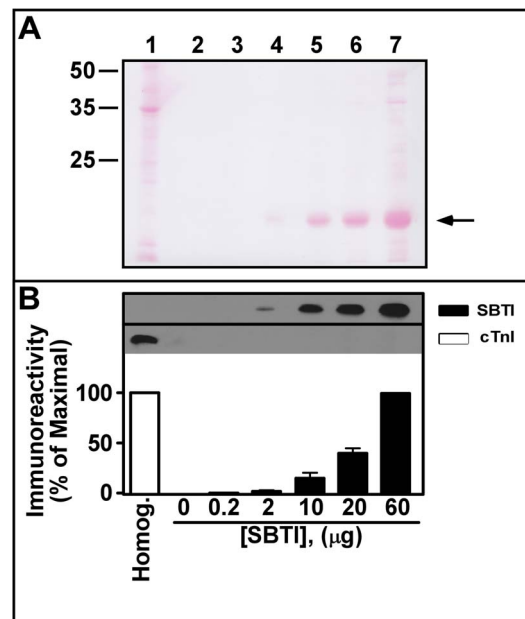


Figure 3. Commercially available soybean trypsin inhibitor (SBTI) preparations are devoid of cardiac troponin I (cTnI). Lane 1 contains 35 μg of rat left ventricle homogenate prepared as in Figure 1. Lanes 2–7 contain 0–60 μg of commercially available SBTI. All samples were subjected to SDS-PAGE and proteins were electro-transferred onto nitrocellulose paper (NCP). The resulting blots were stained for total proteins using Ponceau S stain (Panel A) and then subjected to Western blot analyses for the detection of SBTI and cTnI (Panel B). Note the absence of cTnI immunoreactivity in the commercially available SBTI samples. Typical autoradiographs are shown in the top of Panel B with the histogram representing Mean±S.E. densitometry values from all autoradiographs (expressed as the % of maximal densitometry scores) from 5 independent experiments. doi:10.1371/journal.pone.0070580.g003

in our SBTI preparations insuring that detectable cTnI in our experiments was not coming from the exogenously added SBTI.

Exogenously Added SBTI can be used to Normalize (NH₄)₂SO₄ Precipitations in Langendorff Heart Effluents Collected Following Ischemia/perfusion (IR)

The general treatment protocol for these experiments is shown in Figure 4A. Hearts were first given a 20 minute equilibration (Eq.) period to allow stable beating parameters to be achieved. Next, since the ultimate goal of these studies was to test the effects of our δPKC-dF₁Fo interaction inhibitor peptide on cTnI release after IR injury, we subjected hearts to a 1 hr perfusion, which normally would reflect the peptide loading (PepLoad) phase. However, no peptide was given during this period in the experiments represented in Figure 4. After the PepLoad phase, a 20 min global, no-flow ischemia was administered to hearts followed by a 150 min oxygenated reperfusion period. We collected 50 ml aliquots of perfusate from the hearts at the end of each of these protocol phases and several additional 50 ml collections were made during the 150 min reperfusion period (See arrows in Figure 4A). Effluents were then subjected to (NH₄)₂SO₄ precipitation (50% saturation level) and Western blot analyses for cTnI and SBTI as in Figure 2. Figure 4B shows a representative Ponceau S protein stain of the NCP used for the Western blot shown in Figure 4C. As expected, we observed some protein release immediately following the 20 min equilibration period, but very little protein was released after the 1 hr PepLoad period. In contrast, protein release increased immediately following the

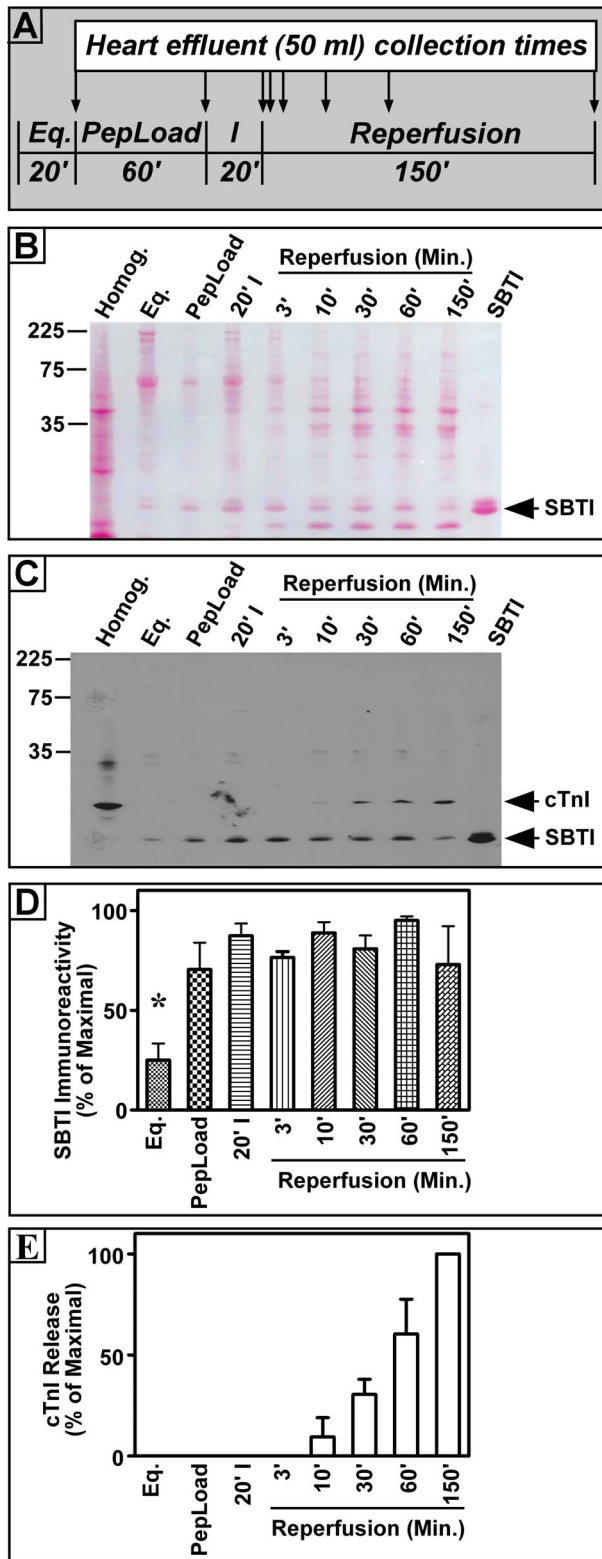


Figure 4. Detection of cardiac troponin I (cTnI) in effluents collected from Langendorff-perfused rat hearts following ischemia/reperfusion injury. Rat hearts were first given a 20 min equilibration (Eq) period, followed by a 1 hr perfusion (which will serve as the peptide loading phase (PepLoad), but no peptide was added for experiments in this figure). Next, hearts were given a 20 min global ischemia (I) exposure followed by 2.5 hr of perfusion with oxygenated Krebs' buffer (Reperfusion). Effluents (50 ml) were collected from each

of the protocol stages indicated by arrows in panel A. Proteins in each effluent were then precipitated by adding crystalline $(\text{NH}_4)_2\text{SO}_4$ to a level of 50% saturation and centrifuging them. Precipitated proteins were subjected to SDS-PAGE and transferred onto nitrocellulose paper (NCP). A representative Ponceau S stain of a typical NCP blot is shown in Panel B and autoradiographs from Western blots with anti-cTnI and anti-SBTI antisera are shown in Panel C. Mean \pm S.E. densitometry values for SBTI (Panel D) and cTnI (Panel E) are also shown. cTnI densitometry values were normalized to SBTI levels in the same blots and SBTI values were not statistically different between groups in which cTnI was released. Results shown are typical of the responses of effluents collected from 5 independent rat hearts and are represented in the histograms.

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20 min ischemia period, and with increasing reperfusion durations, we observed a time-dependent increase in total protein release, consistent with increasing cardiac cell injury (Figure 4B). Also of interest, we observed many more proteins being released in the reperfusion period than in any of the other stages of the protocol. Note that the SBTI recoveries observed in the Ponceau S stains (Figure 4B) were corroborated when we probed these same blots using the SBTI antisera (bottom, Figure 4C). Of interest, we observed significantly less SBTI precipitation in effluents collected from hearts immediately following the 20 min equilibration (Eq) period (Figure 4C and 4D) when compared to all other groups (Eq vs. 10 or 60 min $p < 0.001$, Eq. vs. all other groups $p < 0.05$). The reason for this is currently unknown, but could reflect excessive proteases released from the hearts as they were recovering from excision from the animals and perfused *ex vivo*. Since we did not intend to compare cTnI release under this equilibration condition these findings had minimal impact on our study. Of most importance we observed no significant differences in the Mean \pm S.E. SBTI levels precipitated by 50% saturated solutions of $(\text{NH}_4)_2\text{SO}_4$ between any of the other groups (Figure 4D) indicating that SBTI precipitation was not altered by the ischemia/reperfusion manipulations of our protocol. We therefore, propose that SBTI can be used as a normalization tool, not only when cTnI in freshly harvested homogenate proteins are assayed (Figure 2), but also in effluents collected from Langendorff hearts that were subjected to IR (Figure 4C–D).

$(\text{NH}_4)_2\text{SO}_4$ Precipitation and Western Blot Analysis Reveal Progressive Release of cTnI from Perfused Rat Hearts

Figures 4C and E demonstrate significant detection of cTnI release from our Langendorff heart preparations following 20 min of ischemia and 30 min of reperfusion. We actually began to see modest cTnI release as early as 10 min of reperfusion in some experiments. The Mean \pm S.E. ($n = 5$) % of maximal cTnI release observed following 20 min of ischemia and 30, 60 or 150 min of reperfusion were determined to be 30 ± 7 ($p < 0.005$), 60 ± 17 ($p < 0.008$), and 100 (0.0001) %, respectively. Prior to developing this technique we had difficulty consistently estimating cardiac cell necrosis after IR using cTnI release with commercially available ELISAs, or by using triphenyltetrazolium chloride staining of heart sections, unless at least 90 min of reperfusion was given (not shown). This is an important point since the time course of increasing cTnI release, rather than levels at a single point, are often the most convincing evidence for confirming myocardial infarction in clinical situations [33]. Further, our results may suggest earlier events in the IR injury spectrum, which could be modulated by cardio-protective substances. Another interesting point with regard to the use of sample normalization with SBTI can be seen in the last (right most) effluent tested in Figure 4C. Note that there was substantial cTnI detected following 150 min of reperfusion in western blots. However, based on the low SBTI

immunoreactivity corresponding to that gel lane, we hypothesize that there was poor protein recovery in that group in this experiment. Consequently, with SBTI normalization the amount of cTnI released in that group is higher than that shown in the blot. These results demonstrated the utility of this approach for monitoring cTnI release following IR injury in this model.

Perfusion of Langendorff Rat Heart Preparations with the δ PKC-dF₁Fo Interaction Inhibitor Reduces cTnI Release Following Prolonged IR Injury

In Figure 5, hearts were perfused as in Figure 4A however, in this case, 50 nM concentrations of the cell-permeable, mitochondrially-targeted δ PKC-dF₁Fo interaction inhibitor, or a scrambled-amino acid sequence inactive control peptide [15,16], were administered during the 1 hr PepLoad phase of the protocol. Each of these peptides contains an HIV-Tat protein transduction sequence [34] and a second mitochondrial targeting sequence [35]. Following peptide loading, hearts were exposed to 20 min of global ischemia and 150 min of oxygenated reperfusion. Samples of perfusate (50 ml) were collected at different stages of the IR protocol as shown in Figure 4A. Released proteins were concentrated using (NH₄)₂SO₄ techniques (50% saturation) with SBTI normalization, and cTnI levels were monitored by Western blot. In the scrambled sequence control group we found the release of cTnI (Figure 5) to parallel that observed in hearts receiving no peptide (Figure 4). In contrast, hearts receiving the 1 hr preincubation with the δ PKC-dF₁Fo inhibitor peptide showed a reduction in normalized cTnI release at 60 and 150 min of reperfusion when compared to the scrambled sequence control peptide. On average the percent reductions of cTnI release by the δ PKC-dF₁Fo inhibitor at these time points were 80+7 ($p < 0.01$, $n = 4$) and 69+17 ($p < 0.05$, $n = 4$), respectively. These results indicate that this method can be used as a confirmatory marker of myocardial injury in Langendorff hearts subjected to IR. Further, it allows protection in Langendorff heart models to be detected at earlier reperfusion times than TTC staining or (in our experiments) cTnI ELISAs.

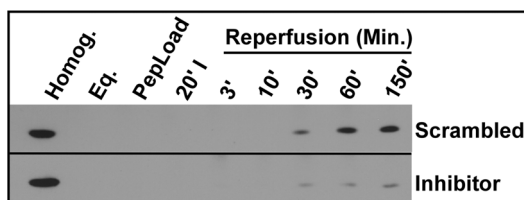


Figure 5. The δ PKC-dF₁Fo interaction inhibitor reduces cardiac troponin I (cTnI) release when perfused into Langendorff hearts. Experimental details were as described in the legend to Figure 4 except that during the peptide loading phase 50 nM concentrations of the scrambled sequence (inactive) control peptide or the δ PKC-dF₁Fo interaction inhibitor peptide were administered to hearts for 1 hr. Effluents were collected and Western blot analyses for cTnI were conducted. The left-most lane contains rat left ventricle homogenate used as a positive control for cTnI immunoreactivity. The remaining groups are described in the legend to Figure 4. Results shown are typical of the responses of 3 independent rat hearts in each peptide treatment group.

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The δ PKC-dF₁Fo Interaction Inhibitor Partitions to the Mitochondrial Inner Membrane (IMM) and Matrix when Perfused into Rat Hearts

Our previous studies clearly demonstrated cardiac mitochondrial uptake of the δ PKC-dF₁Fo interaction inhibitor, but we did not provide evidence of its presence in the mitochondrial matrix or IMM where the F₁Fo complex exists. We felt that demonstration its presence at these submitochondrial sites would strengthen our overall hypothesis that the δ PKC-dF₁Fo binding interaction contributes to cardiac ischemia reperfusion injury by inhibiting the F₁Fo enzyme complex and that the δ PKC-dF₁Fo interaction inhibitor attenuates this interaction to protect against this injury. We therefore, conducted studies to determine if perfusion of rat hearts with this peptide allowed its targeting to these submitochondrial sites. In Figure 6 hearts were perfused in Langendorff mode with 50 nM concentrations of the FLAG epitope-tagged δ PKC-dF₁Fo inhibitor peptide for 1 hr at 37°C. Hearts were then perfused for 5 min with Krebs buffer without peptide and left ventricles were harvested for western blot analyses with anti-FLAG antisera. Figure 6A illustrates FLAG immunoreactivity in crude IMM, and mitochondrial matrix fractions isolated by hypotonic lysis and differential centrifugation techniques as described in Methods. Since the “d” subunit of F₁Fo ATP synthase (dF₁Fo) is found in the mitochondrial matrix our results suggest that following perfusion of hearts with the δ PKC-dF₁Fo inhibitor, sufficient FLAG epitope-tagged inhibitor peptide is delivered to the matrix to bind δ PKC and prevent its interaction with dF₁Fo. It is important to note that the protein concentration of the IMM fraction was 5 times greater than that of the matrix fractions. In the autoradiograph shown in Figure 6A we loaded all of the protein obtained for the matrix fractions from 0.5 mg of purified mitochondria. In contrast, only one-fifth of the total protein for the IMM in these analyses was loaded on gels to avoid protein overloading. Densitometry as shown in the Figure 6A was not corrected to reflect this. However, these data suggest the presence of our FLAG-tagged peptide inhibitor at the mitochondrial IMM and matrix where the F₁Fo ATP synthase exists.

To complement our western blot studies we next conducted transmission electron microscopy (TEM) experiments on left ventricle (Figure 6B–F) and mitochondria (Figure 6G and H) isolated from rat hearts perfused with 50 nM concentrations of the δ PKC-dF₁Fo inhibitor for 1 hr. In Figure 6C–H hearts were first perfused with this compound, perfused an additional 5 minutes with oxygenated Krebs buffer without the peptide, and then subsequently perfused with fixative (Methods). Cardiac ventricular tissue (as shown in Figure 6B) was then cut into 4 cross sections. From one of these sections (as shown by the white “X” in Figure 6B) we excised a 2 mm cube of tissue for embedding and the preparation of thin sections for TEM. Thin sections were blocked and probed with a 1:5 dilution of mouse monoclonal anti-FLAG antisera and then subjected to immunogold labeling using gold particle-labeled anti-mouse secondary antisera. Figure 6C shows a representative cross section of cardiac ventricle from hearts loaded with the FLAG epitope-tagged δ PKC-dF₁Fo interaction inhibitor peptide. Note the substantial areas of high density (gold particle) staining in most mitochondria (Figure 6C) compared to the “no FLAG antisera control” (Figure 6D). Figure 6E shows an enlargement of a mitochondria from a left ventricle section prepared from hearts loaded with our peptide. Note the substantial immuno-gold labeling observed in the mitochondrial cristae, and matrix and outer membrane and the scarcity of staining in extra-mitochondrial areas. As shown in Figures 6C, D and E we also observed some staining in the mitochondrial outer membrane. We hypothesize that the presence

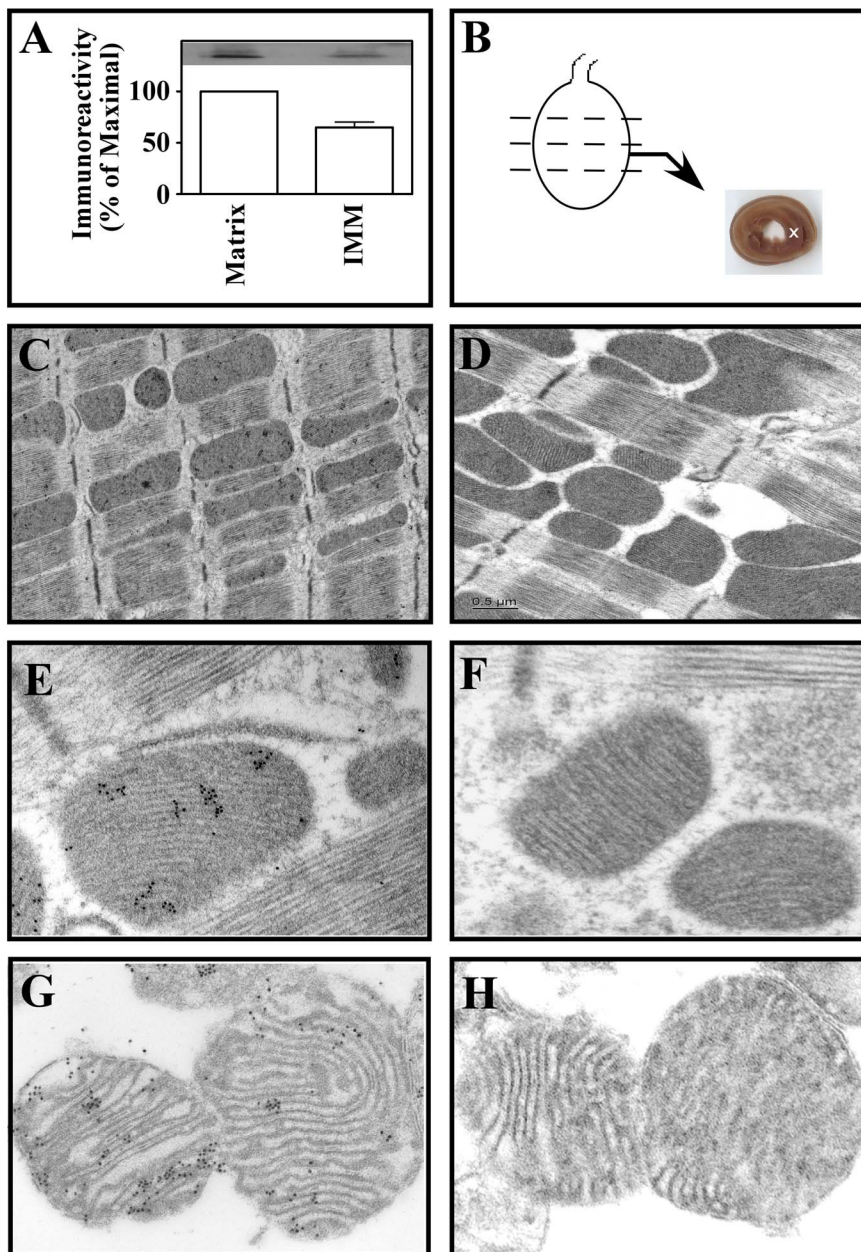


Figure 6. Sub-mitochondrial Localization of the δ PKC-dF₁Fo peptide inhibitor after its perfusion into Langendorff rat hearts. Adult rat hearts were first perfused with 50 nM concentrations of the FLAG-epitope tagged δ PKC-dF₁Fo inhibitor peptide for 1 hr. Hearts were then washed for 5 min with Krebs buffer that contained no peptide. In Panel A mitochondria were then isolated from the left ventricle and subfractionated into mitochondrial inner (IM) and matrix fractions as described in Methods. Sub-mitochondrial fractions were then probed in Western blots with monoclonal mouse anti-FLAG antisera at 1:500 dilution. Results are expressed as the mean \pm S.E. percentage of the maximal densitometry scores from 3 independent experiments. Extensive FLAG immunoreactivity was observed in the matrix and IMM fractions where δ PKC would be predicted to interact with the "d" subunit of F₁Fo ATP synthase. Panels B–H refer to results from immuno-gold transmission electron microscopy studies. Results shown are typical analyses conducted in 3 independent experiments in which random fields were selected from embedded sections and a total of 364 mitochondria were analyzed.

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of the δ PKC-dF₁Fo inhibitor in the OM reflects its interaction with TIM/TOM mitochondrial protein uptake machinery as it is taken up into mitochondria and ultimately delivered to the IMM or matrix. In contrast, in thin sections prepared identically to those in Figure 6E we observed virtually no immunogold labeling when the anti-FLAG primary antisera (but not the gold-labeled secondary antisera) was omitted. Panels G and H of Figure 6 represent similar analyses to those in Figure 6E and F except

density gradient-purified mitochondria, rather than left ventricle tissue, were fixed, sectioned and then probed for anti-FLAG immunoreactivity followed by gold particle-labeled secondary antibody. Similar to our results with anti-FLAG immunogold stained sections from intact ventricle, Figure 6G shows substantial labeling of the mitochondrial matrix, IMM, and OM. Figure 6H represents the control staining in purified mitochondria when the anti-FLAG antisera was omitted. Collectively these data represent

the first demonstration that the δ PKC-dF₁F_o interaction inhibitor partitions to sub-mitochondrial sites when perfused into rat hearts. Of most importance, we observed substantial presence of the peptide in the mitochondrial matrix where the “d” subunit of F₁F_o ATP synthase exists and hence the proposed site of the δ PKC-dF₁F_o interaction which we hypothesize inhibits F₁F_o functions during cardiac IR. Our peptide therefore, appears to be delivered to the correct submitochondrial loci when perfused into Langendorff rat hearts to antagonize this interaction.

Discussion

Considerable effort has gone into the development of rapid ELISA assays for the detection of cTnI released into the bloodstream as a confirmatory marker of myocardial infarction. cTnI can be quite stable in human blood with increases in its levels being readily detectable at 6–8 hr after ischemia and it can remain elevated for days [8]. Further, newer “high sensitivity cTnI assays may allow detection of “normal”, (non-MI) cTnI levels and also allow a more rapid detection of elevated cTnI levels in patients having an MI [4,33,36]. Many recent studies have used ELISA detection of cTnI release from perfused hearts as an indicator of cardiac injury [37–41]. Therefore, the reasons for our lack of consistent cTnI detection using a high sensitivity rat cTnI ELISA are not fully understood. Many factors can alter cTnI detection in ELISA-based assays, some of which may help to explain our results. These include: poor cTnI recoveries during sample volume reduction or centrifugation steps; the use of different cTnI antisera in the ELISA kits [5,8], cTnI proteolysis [5,8,30,42–44]/phosphorylation [30,32,42]/complexing with other proteins [30], and the concentrations of fibrin or anticoagulants in samples [44]. In most cases, the results from different ELISA kits are not even directly comparable, with up to 20-fold variations in cTnI values being reported using different cTnI ELISAs on the same samples [42,44]. There is also currently no cTnI standard to normalize the results from one cTnI ELISA to another [4,36]. Also of importance, cTnI levels can be elevated under numerous non-myocardial infarction (MI) conditions such as in marathon runners [45], hypertensive emergencies [46] toxicity due to chemotherapeutic agents [47,48], and chest trauma [49]. Therefore, even though cTnI is currently the preferred confirmatory biochemical marker of MI, and is often very helpful in this context, obtaining consistent cTnI values, and the interpretation of cTnI ELISA results, can be complicated. For this reason, clinical cTnI levels are used in conjunction with other indicators of MI such as electrocardiograms and echocardiography. Finally, and of direct relevance to this study, different animal species have been reported to have variable responses in ELISA-based cTnI detection and cTnI can also be elevated in those models under non-ischemic stress conditions [50,51].

Indeed, numerous sources of variability exist with current cTnI detection assays. Therefore, to confirm the cardio-protective actions of our δ PKC-dF₁F_o interaction inhibitor using a biochemical marker we found it necessary to use a non-ELISA-based approach. We first piloted concentration of 50 ml Langendorff effluents using Centriprep-10 concentrators and found very sporadic and often non-existent cTnI detection. This was not improved if Centriprep concentrated samples were subjected to chloroform-methanol precipitation. Since the concentration of these samples required long centrifugation periods and often the rate of sample concentration varied between samples, we believe that the time required for these concentrations may have allowed excessive proteolysis, oxidation or non-selective adherence of cTnI protein to the concentrator. Next, we

attempted acid protein precipitation of rat heart homogenate protein (e.g. perchloric and others) which were also unsuccessful. Essentially, we observed normal protein band patterns in our (acid precipitation) blots by Ponceau S. staining, but we could not detect cTnI or SBTI in these Western blots. We experimented with a range of concentrations of total heart homogenate protein that was extracted with perchloric or other acids. We also neutralized homogenates to pH 7, and even performed a second precipitation step using chloroform/methanol. None of these manipulations allowed us to detect cTnI or SBTI in Western blots. It is possible that the acid extraction or one of the subsequent steps altered crucial epitopes in the cTnI or SBTI proteins such that our antisera could no longer detect them in Western blots. We then tested the (NH₄)₂SO₄ protein precipitation method and found that it obviated the above mentioned problems encountered with other concentration methods.

Another important point is that *in vivo*, mostly cTnI fragments, not the holo-protein are found in serum after an MI. This has been found in many Langendorff heart studies as well. We found the release of only a ~25 kDa cTnI protein species after ischemic injury (Figures 4–5). We therefore, cannot rule out the possibility that additional cTnI fragments were not detectable using our approach and therefore, the effects of the δ PKC-dF₁F_o inhibitor on the release or stability of these fragments is unknown. Numerous studies have reported cTnI proteolysis or other modifications, in cardiac myocytes or serum, following different periods of ischemia and reperfusion [8,30,42,43]. For example, studies by McDonough et. al subjected rat hearts to 0, 15 or 60 min of ischemia followed by 45 min of reperfusion and applied a TnC affinity column, cTnI antisera and spectrometric analyses to demonstrate progressive degradation of cTnI as the intensity of ischemic insult was increased [30]. These authors found that rat holo-cTnI has 210 amino acids (AAs) and was progressively degraded to fragments containing AAs 1–193, 63–193 and 73–193, respectively. Degraded cTnI fragments also formed complexes with troponin C (TnC), and troponin T (TnT), some of which may occur because of crosslinks induced by transglutaminase (30). There were numerous methodological differences between their study and ours. For example, we did not use a TnC affinity column to purify cTnI. We observed a 25 kDa species of cTnI that was released from perfused rat hearts following IR injury and did not determine if this was holo-cTnI or a cTnI fragment, only that it was detected in western blots by an antisera directed at a cTnI epitope in the center of the protein. We selected this antisera because previous studies have suggested that, following IR injury, cTnI is progressively proteolyzed from the N- and C-termini and antisera directed towards an epitope in the center of the protein should detect most of these fragments. It is possible that the other fragments detected by McDonough et. al were generated in our experiments, but not released because the duration of ischemia used in our experiments was different, or we used different electrophoresis conditions.

In another study, Labugger et. al developed Western blot techniques to detect holo-cTnI and 11 fragments of cTnI in the serum of patients undergoing acute MI [42]. These patients however, did not have the precisely controlled durations of ischemia and reperfusion found in our experiments and insults were likely longer. These authors also spiked human serum with recombinant cTnI and incubated the serum with alkaline phosphatase. Their results indicated that much of the cTnI found in human serum from MI patients is phosphorylated or proteolyzed [42]. Additionally, one study reported the loss of at least some cTnI fragments in Langendorff perfused hearts after ischemia if reperfusion is conducted for >1 min [8], much shorter

than the reperfusion points used in our study. In addition, cTnI has also been reported to be proteolyzed inside of cardiac myocytes after 15–25 min of global ischemia [8] or in ventricular biopsies from patients undergoing cardiac bypass surgery [43]. Further, the C-terminus of cTnI can be degraded when recombinant cTnI is exogenously added to human serum [44]. Also, covalent complexes between cTnI/cTnC and cTnI/cTnT have been reported after brief ischemia and may be lost with prolonged ischemia [30]. Finally, PKA-induced phosphorylation of cTnI at its N-terminus may prevent cTnI degradation [30]. It is therefore, possible that the ~25 kDa cTnI species we detected was somewhat protected from degradation because it was either complexed with other proteins or phosphorylated.

Our previous studies demonstrated improved energetics and survival in neonatal cardiac myocytes pretreated with the δ PKC-dF₁Fo interaction inhibitor just prior to prolonged hypoxia [16]. In work to be published elsewhere we have also demonstrated that Langendorff hearts incubated with the δ PKC-dF₁Fo inhibitor enhanced ATP levels following a prolonged IR protocol. We propose that excessive inhibition of the F₁Fo synthase by a δ PKC interaction with the dF₁Fo subunit plays a significant role in cardiac IR injury, and that our novel peptide could provide a mechanism for attenuating these effects. This study also demonstrates for the first time that our FLAG epitope-tagged δ PKC-dF₁Fo interaction inhibitor peptide could be detected at the mitochondrial matrix and IMM where the F₁Fo ATP synthase complex exists. We have further shown the protective actions of our peptide against cardiac IR injury by demonstrating reduced release of the clinically relevant biochemical marker of cardiac cell necrosis, cTnI in hearts perfused with the δ PKC-dF₁Fo inhibitor.

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