Supplementary Material

Supplementary Methods

Materials

All antibodies and protein A/G beads were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), with the exception of the VDAC antibody (MitoSciences). The gold-conjugated secondary antibody for immunogold electron microscopy was from Ted Pella, Inc. The ψεHSP90 peptide (PKDNEER) was synthesized and conjugated to TAT_{47-57} by American Peptide Company, Inc (Sunnyvale, CA). The HSP90 inhibitor, geldanamycin, was purchased from InvivoGen.

Sequence Alignments

Sequences of the human PKC family members (GenBank™ accession numbers: αPKC; NP_002728.1, βPKC; NP_002729.2, γPKC; EAW72161.1, PKCδ; NP_997704.1, PKCε; NP_005391.1, θPKC; NP_006248.1, ηPKC; NP_006246.2 and ζPKC; CAA78813.1) and of PKCε of various species (human PKCε; NP_005391.1, rat; NP_058867.1 mouse; NP_035234.1 and Xenopus; NP_001107724.1) were aligned using ClustalW software. The sequences of human PKCε (accession number NP_005391.1) was aligned with human HSP90α (accession number NP_005339) and HSP90β (accession number; NP_031381) using LALIGN software, using the Blosum 80 scoring matrix.

Ex vivo **model of cardiac ischemia-reperfusion**

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All

protocols were approved by the Stanford University Institutional Animal Care and Use Committee. Male Wistar rats (275-300g) were administered heparin (2000 units/kg intraperitoneally) and anesthetized with sodium pentobarbital (100 mg/kg intraperitoneally). Excised hearts were perfused *via* the aorta at 10 ml/min with oxygenated Krebs-Henseleit buffer (120 mM NaCl, 5.8 mM KCl, 25 mM NaHCO3, 1.2 mM Na2PO4, 1.2 mM MgSO4, 1.2 mM CaCl2, and 10 mM dextrose, pH 7.4) at 37^{o}C. After 20 min equilibration, hearts were subjected to 35 min global, no-flow ischemia followed by 15 min reperfusion. HSP90 inhibitors, geldanamycin (GA) (1μM), or radicicol (RAD) (1μM) were perfused during the entire reperfusion period (Fig1A). Coronary perfusate was collected during reperfusion and cardiac damage assessed by creatine phosphokinase (CPK) activity (cumulative units/l for 15 min) (Equal Diagnostics, CT, USA). The ψεHSP90 peptide (1μM) was perfused for 10 min prior to ischemia and during the entire reperfusion period, in the absence and presence of GA (1μM) (Fig 5A).

Subcellular fractionation

Heart ventricles were homogenized in ice cold mannitol-sucrose (MS) buffer (210 mM mannitol, 70 mM sucrose, 5 mM MOPS and 1 mM EDTA containing Sigma Protease Inhibitor 1 and Sigma Phosphatase Inhibitors 1 and 2) using 3 x 5 sec strokes of a Polytron homogenizer on a medium setting. Heart homogenate was filtered through gauze then centrifuged at 700g for 5 minutes to remove cell debris and the nuclear/cytoskeleton fraction. The resultant supernatant was filtered through gauze and centrifuged at 10,000g to pellet mitochondria. The supernatant from this fraction was centrifuged at 100,000g to pellet the plasma membrane and the remaining

supernatant was the cytosolic fraction. Mitochondrial pellets were washed 3x and re-suspended in 200µl MS Buffer.

Sub-mitochondrial fractionation

Sub-mitochondrial particles (SMP) were generated by sonication based on previously described protocols [1, 2]. Mitochondria (10mg/ml in MS Buffer) were sonicated at 3 × 2 min on ice with 1 min intervals. The solution was spun at 10,000g for 10 min to pellet unbroken mitochondria and resultant supernatant spun at 100,000g for 30 min to pellet SMPs. For trypsin digestion, SMPs were incubated with 1µg trypsin protease in 100µl MS buffer for 0, 5, 10 and 20 minutes at 37ºC. Trypsin digestion was quenched by protease inhibitor cocktail (1µl) and SMPs pelleted by centrifugation at 100,000g for 10 min. For high salt treatment, SMPs were incubated with 400mM KCl on ice for 10 minutes followed by centrifugation. For high pH treatment, SMPs were incubated with 200mM Na_2CO_3 (pH 11.5) for 10 minutes followed by centrifugation. SMPs were re-suspended in 50µl sample buffer and proteins separated by SDS-PAGE. Mitoplasts were generated based on previously described methods [2-4]. Mitochondria (50µl of 10µg/µl) were re-suspended in 450µl hypotonic buffer (5mM Tris-HCl, 1mM EDTA pH 7.4) and incubated on ice for 15 minutes. The resultant solution was centrifuged at 20,000g for 10 min at 4ºC to pellet mitoplasts (consisting of the IMM and matrix). Mitoplasts then resuspended in 450µl of hypotonic buffer and sonicated for 2 min (150W 30 secs on followed by 30 secs off x 2) on ice to disrupt the IMM. The solution was then spun at 100,000g for 40 min. The resultant pellet contains the IMM-enriched fraction while the matrix-enriched fraction is the supernatant [2].

Western Blotting

Protein concentration was determined by Bradford Assay. 10 μg protein was separated by 10% SDS-PAGE then transferred to nitrocellulose. Blots were blocked with a 5% milk solution in Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 (TBST) for 1 hour and incubated overnight with primary antibody followed by 3 x 15 min washes in TBST. Membranes were then incubated with anti-IgG secondary antibody linked to horseradish peroxidase (Amersham Biosciences) for 1 hour followed by 3 x 15 min washes in TBST. Protein bands were visualized using ECL and quantitated using NIH ImageJ software.

Immunoprecipitation

500μg of mitochondrial or cytosolic protein was diluted into 1ml of IP Lysis Buffer (150mM NaCl, 10mM Tris-HCl, 5mM EDTA, 0.1% Triton X-100, pH=7.4, containing Sigma Protease Inhibitor 1 and Sigma Phosphatase Inhibitors 1 and 2). Proteins were incubated with PKCε antibody (2μg) for 2 hours at 4ºC with inversion mixing. 10μg of Protein A/G Beads (Santa Cruz Biotechnology) were added and the mixture was incubated overnight at 4ºC with inversion mixing. Beads were then centrifuged, washed 3x in IP Lysis Buffer, then re-suspended in sample buffer. Immunoprecipitated proteins were separated on 10% SDS-PAGE gels. The presence of associated proteins was determined by western blotting using antibodies specific for HSP90α/β, TOM20 and ALDH2. Protein immunoprecipitation was then repeated in reverse (immunoprecipitation with HSP90, TOM20 or ALDH2 antibodies followed by western blotting with PKCε antibody). An IgG-conjugated beads alone group was also included for each sample set to assess any non-specific protein binding to the beads.

2-D IEF/SDS gel electrophoresis

For 2-D IEF/SDS polyacrylamide gel electrophoresis, rats hearts subjected to *ex vivo* model of cardiac ischemia-reperfusion injury were homogenized in buffer consisting of 7M urea, 2M thiourea and 4% CHAPS in the presence of protease inhibitor and phosphatase inhibitor cocktails (Sigma-Aldrich Chemicals, MO, USA) and total homogenate was pulled from 3 separate experiments. After sonication total lysate were subjected to a first dimensional separation by an IPG-phor isoelectric focus power supply using pre-cast Immobilin DryStrip pI 3-10 strips according to the manufacturer's instruction manual (Amersham Biosciences, NJ, USA). SDS gel electrophoresis, Western blotting and immuno-detection were carried out using standard methods. Anti-phospho serine or ALDH2 antibodies were used at a 1:1000 dilution.

ALDH2 assay

Enzymatic activity of mitochondrial ALDH2 was determined spectrophotometrically by monitoring the reductive reaction of NAD^+ to NADH at 340 nm as previously described [5, 6]. Mitochondria were isolated by differential centrifugation in MS buffer (210 mM mannitol, 70 mM sucrose, 5 mM MOPS and 1 mM EDTA containing Sigma Protease Inhibitor 1 and Sigma Phosphatase Inhibitors 1 and 2), then re-suspended in lysis buffer (0.1M Tris HCl pH 8.0, 10 mM DTT, 20% glycerol, 1% Triton, containing Sigma Protease Inhibitors 1 and Sigma Phosphatase Inhibitors 1 and 2). Protein concentration was determined by Bradford Assay. ALDH2 assays were carried out at 25°C in 50 mM sodium pyrophosphate buffer, pH=9.5. To this volume, 10 mM acetaldehyde and 400 μg of mitochondrial protein lysate were added. To start the reaction, 2.5 mM NAD was added and the accumulation of NADH was monitored for 5

min with measurements being taken every 30 s. ALDH2 reaction rates were measured as μmol NADH/min/mg protein and were expressed as % untreated control.

Immunogold Electron Microscopy

Isolated mitochondria were fixed in 4% paraformaldehyde and 0.025% gluteraldehyde. Sections of 80 nm were mounted on Ni grids which were incubated in blocking solution [140mM NaCl, 3mM KCl, 8mM Na2HPO4, 1.5mMKH2PO4, 0.05% Tween-20, pH7.4 containing 0.5% (w/v) ovalbumin, 0.5%(w/v) BSA (Sigma)] for 1 hour followed by PKCε antibody (1:100 in blocking solution) followed by 3 x 15 min washes in PBS, followed by 1 hour incubation with goat anti-rabbit IgG conjugated to 10nm gold particles (Ted Pella Inc) (1:100 in blocking solution). Secondary antibody (IgG) alone controls were prepared in the absence of PKCε antibody. Grids were washed 3 x 15 min in PBS and stained for 20 s in 1:1 saturated uranylacetate (7.7%) in acetone followed by staining in 0.2% lead citrate for 3 min. Mitochondria were imaged using JEOL 1230 electron microscope.

***In vitro* mitochondrial translocation assay**

Activation of recombinant PKCε was performed as described previously [7]. Recombinant GST-PKCε (5μl of 10ng/μl stock) (Cell Signaling Technology) was aliquoted into assay buffer (20mM Tris HCl, 50mM KCl, 1mM DTT, 0.1mg/ml BSA Mg^{2+} (5mM), ATP (100μM), pH 7.4) containing different combinations of activation factors including phosphatidylserine/diacylglycerol (1mM), H_2O_2 (50μM) and ψεHSP90 (1μM) in a final volume of 50μl. PKCε was activated by incubation in assay buffer at 37ºC for 20 min. For some groups,

rabbit reticulocyte lysate (10µl) was added to the incubation mixture for 10 minutes after PKCε activation. The activated recombinant PKCε mixture was then added to freshly isolated cardiac mitochondria (1mg/ml) in 500µl mitochondrial translocation assay (MTA) buffer (250mM sucrose, 80mM KCl, 5mM $MgCl_2$, 2mM KH_2PO_4, 10mM MOPS-KOH, 10mM succinate, 2mM ATP, 3% BSA, pH 7.2) and incubated for 20 minutes at 37ºC with shaking (for ψεHSP90 treated groups, 1µM ψεHSP90 was also present in the MTA buffer). The reaction was halted by the addition of 50µM dinitrophenol (DNP). Mitochondria were then centrifuged at 10,000g x 10 min, re-suspended in 50µl sample loading buffer and mitochondrial PKCε levels measured by western blotting using VDAC as a loading control. Recombinant GST-PKCε has a molecular weight of ~115kD and was thus distinguished from any endogenous PKCε (molecular weight ~ 95kD).

***In vivo* model of cardiac ischemia reperfusion**

Male Wistar rats (275-300g) were anaesthetized by isofluorane inhalation (3% for induction and 1.5% for maintenance of anesthesia). Myocardial ischemia was induced by transient ligation of the left anterior descending (LAD) coronary artery with 5-0 polyester suture. Animals were subjected to 35 minutes of ischemia, followed by 24 hours of reperfusion, induced by suture removal. Buprenorphine (0.05mg/kg, subcutaneous injection) was administered for post-operative analgesia. The control TAT or ψεHSP90 peptides (1 mg/kg in 400µl saline for each) were injected intraperitoneally 15 min prior to LAD ligation and 5 min before the onset of reperfusion. At the end of the 24-hour reperfusion period, fractional shortening was determined by echocardiography using a GE i13L probe (GE Healthcare). For infarct size measurements, area at risk (AAR) was assessed by re-occlusion of the LAD at the previous suture site, followed

by intravenous injection of Evan's Blue (1.0mg/kg). The heart was sectioned into 4-5 transverse slices, which were then incubated in a 1% solution of trimethyl tetrazolium chloride (TTC), weighed, then photographed by digital camera. Area at risk of infarction (negative for Evans Blue), and infarct area (negative for TTC), were assessed using Image J. Infarct size was calculated as [infarct area/area at risk of infarction] x 100 (%).

Supplementary References

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Supplementary Figure 1.

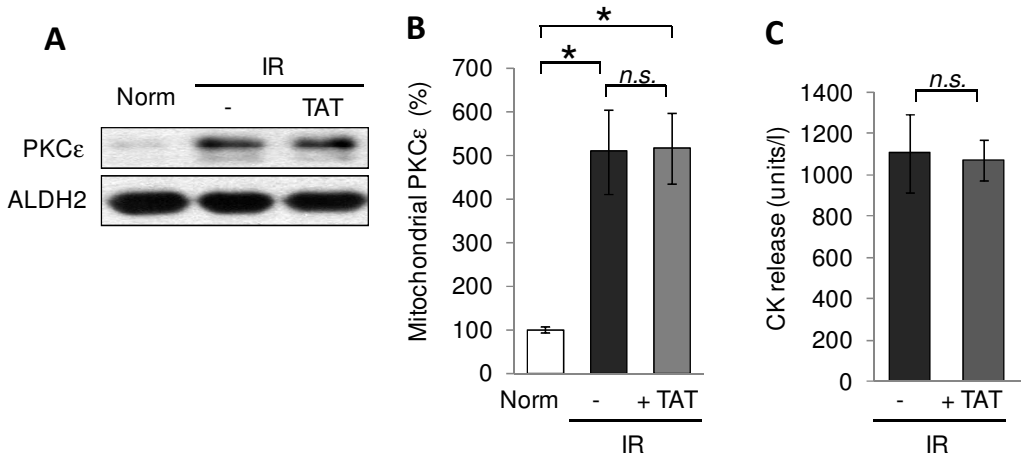

Figure 1. Effect of TAT peptide on mitochondrial PKCε translocation and IR injury.

A) Mitochondrial translocation of PKCε in response to 35 minutes ischemia followed by 15 minutes reperfusion (in the absence and presence of 1μM TAT carrier protein, applied 10 minutes prior to ischemia and during reperfusion). Mitochondrial ALDH2 was used as a loading control. Western blot shown is representative of 3 independent experiments. B) Quantification of IR-induced PKCε translocation, demonstrating lack of effect of TAT carrier peptide relative to buffer control (n=3, $p<0.05$). C) Creatine Kinase (CK) release in response to IR in the absence and presence of 1μM TAT peptide (n=5). TAT peptide had no effect on CK release in comparison to the buffer control IR group.