

# Mitochondrial import of PKC $\epsilon$ is mediated by HSP90: a role in cardioprotection from ischaemia and reperfusion injury

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## Aims

Protein kinase C epsilon (PKC $\epsilon$ ) is critical for cardiac protection from ischaemia and reperfusion (IR) injury. PKC $\epsilon$  substrates that mediate cytoprotection reside in the mitochondria. However, the mechanism enabling mitochondrial translocation and import of PKC $\epsilon$  to enable phosphorylation of these substrates is not known. Heat shock protein 90 (HSP90) is a cytoprotective protein chaperone that participates in mitochondrial import of a number of proteins. Here, we investigated the role of HSP90 in mitochondrial import of PKC $\epsilon$ .

## Methods and results

Using an *ex vivo* perfused rat heart model of IR, we found that PKC $\epsilon$  translocates from the cytosol to the mitochondrial fraction following IR. Immunogold electron microscopy and mitochondrial fractionation demonstrated that following IR, mitochondrial PKC $\epsilon$  is localized within the mitochondria, on the inner mitochondrial membrane. Pharmacological inhibition of HSP90 prevented IR-induced interaction between PKC $\epsilon$  and the translocase of the outer membrane (Tom20), reduced mitochondrial import of PKC $\epsilon$ , and increased necrotic cell death by ~70%. Using a rational approach, we designed a 7-amino acid peptide activator of PKC $\epsilon$ , derived from an HSP90 homologous sequence located in the C2 domain of PKC $\epsilon$  (termed  $\psi\epsilon$ HSP90). Treatment with this peptide (conjugated to the cell permeating TAT protein-derived peptide, TAT<sub>47–57</sub>) increased PKC $\epsilon$ –HSP90 protein–protein interaction, enhanced mitochondrial translocation of PKC $\epsilon$ , increased phosphorylation and activity of an intra-mitochondrial PKC $\epsilon$  substrate, aldehyde dehydrogenase 2, and reduced cardiac injury in *ex vivo* and *in vivo* models of myocardial infarction.

## Conclusion

Our results suggest that HSP90-mediated mitochondrial import of PKC $\epsilon$  plays an important role in the protection of the myocardium from IR injury.

## Keywords

Protein kinase C epsilon • Mitochondria • Protein–protein interaction • Ischaemia reperfusion • Heat shock protein 90

## 1. Introduction

Protein kinase C epsilon (PKC $\epsilon$ ) activation is required and sufficient to protect the heart from ischaemia and reperfusion (IR) injury.<sup>1–3</sup> We recently identified mitochondrial aldehyde dehydrogenase 2 (ALDH2) as an intra-mitochondrial substrate of PKC $\epsilon$ ,<sup>4,5</sup> whose phosphorylation and activation by PKC $\epsilon$  is required to confer

cardioprotection.<sup>4</sup> Additional mitochondrial substrates of PKC $\epsilon$  include cytochrome c oxidase subunit IV (COIV),<sup>6</sup> a PKC $\epsilon$  substrate in cardiac myocytes,<sup>6</sup> neuronal cells,<sup>7</sup> and the lens.<sup>8</sup> PKC $\epsilon$  activation also prevents opening of the mitochondrial permeability transition pore (MPTP)<sup>9</sup> and can promote mitochondrial ATP-sensitive K<sup>+</sup> channel (mitoK<sub>ATP</sub>) opening at the inner mitochondrial membrane (IMM),<sup>10</sup> although whether this reflects PKC $\epsilon$ -mediated

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phosphorylation of mitoK<sub>ATP</sub> awaits identification of the native channel protein.

A hallmark of PKC activation is translocation of the active enzyme from the cytosol to the cell particulate fraction and a variety of cytoprotective stimuli that activate PKC $\epsilon$ , including ischaemic preconditioning,<sup>11</sup> ethanol,<sup>4,5</sup> urocortin,<sup>12</sup> or transgenic expression of constitutively active PKC $\epsilon$ ,<sup>13</sup> result in elevated mitochondrial levels of PKC $\epsilon$ . However, not all studies support a role for mitochondrial PKC $\epsilon$  in cardioprotection.<sup>14,15</sup> Further, although PKC $\epsilon$  substrates have been shown to reside within mitochondria, the mechanism enabling mitochondrial import of PKC $\epsilon$  has not yet been described. Mitochondrial proteins encoded by nuclear DNA are imported into the mitochondria in a co-translational process,<sup>16</sup> using a 20–50-amino acid residue mitochondrial targeting signal that is recognized by the mitochondrial import receptor, Tom20. PKC $\epsilon$  does not contain a mitochondrial targeting sequence. However, proteins that lack this sequence can be imported into mitochondria in a process mediated by the stress chaperone protein, heat shock protein 90 (HSP90).<sup>17,18</sup> Here, we examined the role of HSP90 in mediating mitochondrial translocation of PKC $\epsilon$  and its effect on cardiac ischaemia/reperfusion.

## 2. Methods

### 2.1 Ex vivo model of cardiac ischaemia–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. Hearts from male Wistar rats (275–300 g) were perfused via the aorta at 10 mL/min with oxygenated Krebs–Henseleit buffer at 37°C. Hearts were subjected to 35 min global, no-flow ischaemia followed by 15 min reperfusion. HSP90 inhibitors, geldanamycin (GA; 1  $\mu$ M) or radicicol (RAD; 1  $\mu$ M), were perfused during the entire reperfusion period (Figure 1A). Cardiac damage was assessed by creatine phosphokinase (CPK) release into the perfusate (Equal Diagnostics, CT, USA). The  $\psi\epsilon$ -HSP90 peptide (1  $\mu$ M) was perfused for 10 min prior to ischaemia and during the reperfusion period, in the absence and presence of GA (1  $\mu$ M; Figure 5A).

### 2.2 Subcellular fractionation

Heart ventricles were homogenized in ice cold mannitol–sucrose (MS) buffer using a Polytron homogenizer. The heart homogenate was filtered through gauze then centrifuged at 700 g  $\times$  5 min. The resultant supernatant was filtered then centrifuged at 10 000 g  $\times$  10 min to pellet mitochondria. Mitochondrial pellets were washed three times and resuspended in 200  $\mu$ L MS buffer. Mitoplasts were generated as described.<sup>19,20</sup> Mitochondria (50  $\mu$ L of 10  $\mu$ g/ $\mu$ L) were resuspended in 450  $\mu$ L hypotonic buffer (5 mM Tris–HCl and 1 mM EDTA, pH 7.4) and incubated on ice for 15 min before centrifugation at 20 000 g for 10 min at 4°C. Mitoplasts were then resuspended in 450  $\mu$ L hypotonic buffer and sonicated on ice to disrupt the IMM. The solution was then spun at 100 000 g for 40 min with the resultant pellet containing the IMM-enriched fraction and the supernatant containing the matrix-enriched fraction. Submitochondrial particles (SMPs) were generated as described.<sup>19,21</sup> Mitochondria (10 mg/mL in MS buffer) were sonicated 3  $\times$  2 min on ice with 1 min intervals. The solution was spun at 10 000 g for 10 min to pellet unbroken mitochondria and the resultant supernatant spun at 100 000 g for 30 min to pellet SMPs.

### 2.3 Western blotting

Ten micrograms of protein were separated by SDS–PAGE and then transferred to nitrocellulose. Membranes were blocked with 5% milk in Tris-buffered saline (pH 7.5) containing 0.05% Tween (TBS-T), incubated with primary antibody overnight, washed three times in TBS-T, and then incubated with IgG secondary antibody linked to horseradish peroxidase. Protein bands were visualized using chemiluminescence and quantified using ImageJ (NIH).

### 2.4 Immunoprecipitation

Five hundred micrograms of protein were suspended in 1 mL of immunoprecipitation (IP) buffer and incubated with 2  $\mu$ g PKC $\epsilon$ , HSP90, Tom20, or ALDH2 antibodies (Santa Cruz Biotechnology) for 2 h, followed by overnight incubation with Protein A/G beads at 4°C. Beads were washed three times in IP buffer and immunoprecipitated proteins detected by western blotting.

### 2.5 Immunogold electron microscopy

Isolated mitochondria were fixed in 4% paraformaldehyde/0.025% glutaraldehyde and 80 nm sections mounted on Ni grids. Mitochondria were incubated with blocking solution, followed by PKC $\epsilon$  antibody (1:100 in blocking solution), followed by goat anti-rabbit IgG conjugated to 10 nm gold particles (Ted Pella Inc.) (1:100 in blocking solution). Mitochondria were imaged using a JEOL 1230 electron microscope. Incubation with secondary antibody (IgG) alone served as controls.

### 2.6 In vitro mitochondrial translocation assay

Isolated mitochondria were incubated with recombinant PKC $\epsilon$  with or without diacylglycerol and phosphatidylserine (DAG/PS; 1 mM; PKC activators), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; 50  $\mu$ M), or rabbit reticulocyte lysate (RRL; source of HSP90), and with or without 1  $\mu$ M  $\psi\epsilon$ -HSP90. Mitochondrial translocation of recombinant PKC $\epsilon$  was assessed by western blotting.

### 2.7 Sequence alignments

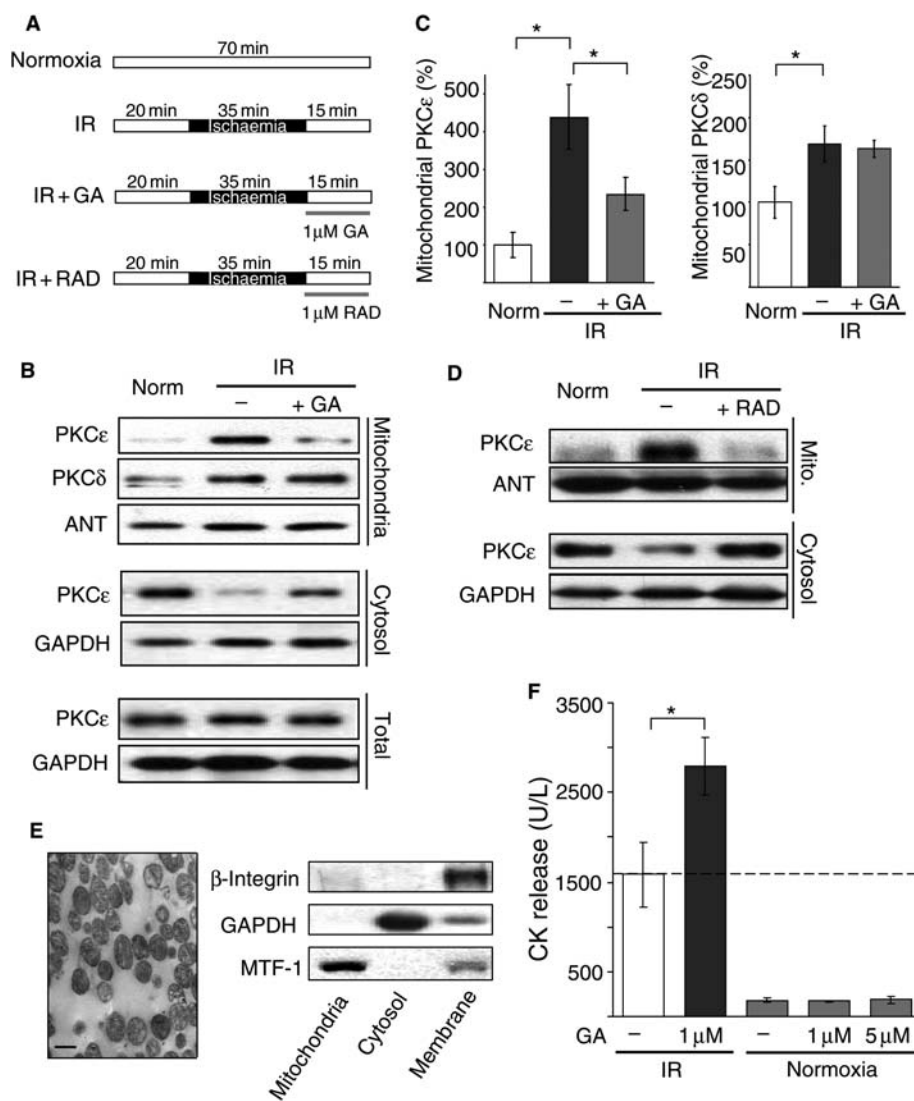
Sequences of the human PKC family members were aligned using ClustalW. Human PKC $\epsilon$  (accession no.: NP\_005391.1) was aligned with human HSP90 $\alpha$  (accession no.: NP\_005339) and HSP90 $\beta$  (accession no.: NP\_031381) using L-ALIGN, using the Blossum 80 scoring matrix.

### 2.8 In vivo model of cardiac ischaemia–reperfusion

Male Wistar rats (275–300 g) were anaesthetized with isoflurane and myocardial ischaemia was induced by ligation of the left anterior descending (LAD) coronary artery for 35 min, followed by 24 h of reperfusion. Control TAT or  $\psi\epsilon$ -HSP90 peptides (1 mg/kg in 400  $\mu$ L saline) were injected intraperitoneally 15 min prior to LAD ligation and 5 min before reperfusion onset. Fractional shortening was determined by echocardiography. Area at risk (AAR) was assessed by re-occlusion of the LAD at the previous suture site, followed by intravenous injection of Evans Blue (1.0 mg/kg). The heart was sectioned into transverse slices, which were incubated with 1% trimethyl tetrazolium chloride (TTC), weighed, and photographed by a digital camera. AAR (negative for Evans Blue) and infarct area (negative for TTC) were quantified using ImageJ and infarct size was calculated as (infarct area/AAR of infarction)  $\times$  100 (%).

### 2.9 Statistical analysis

All data are expressed as mean  $\pm$  SEM. Statistical analyses between two groups was performed using the unpaired Student's *t*-test. Statistical analysis of more than two groups was performed using one-way ANOVA with Dunnett's multiple comparisons *post hoc* test. A *P*-value of <0.05 was considered statistically significant.



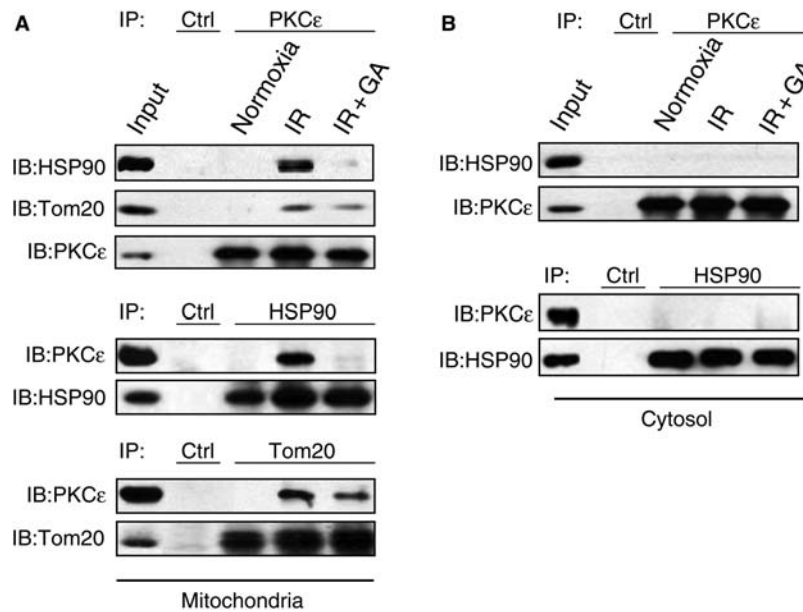
**Figure 1** Inhibition of HSP90 reduces IR-induced PKCε translocation to cardiac mitochondria. (A) Perfused heart protocols. (B) Western blot analysis of mitochondrial PKCε and PKCδ in hearts exposed to normoxia or IR (in the absence and presence of 1 μM GA). Also shown are cytosolic (middle panels) and total PKCε levels (lower panels). (C) Quantification of mitochondrial PKCε and PKCδ levels, normalized to adenine nucleotide translocase (ANT); mean ± SEM ( $n = 5$ ,  $*P < 0.05$ ). (D) IR-induced mitochondrial translocation of PKCε in the presence of another HSP90 inhibitor, RAD (1 μM). (E) Purity of the mitochondrial preparation confirmed by electron microscopy (scale bar = 1 μm) and western blotting for marker proteins of the cytosol (GAPDH), the mitochondria (MTF-1), or the plasma membrane (β-integrin). (F) GA effect on CPK release under normoxic and IR conditions; mean ± SEM ( $n = 5-7$ ,  $*P < 0.05$ ).

## 3. Results

### 3.1 HSP90 activity is required for IR-induced mitochondrial translocation of PKCε

Thirty-five-minute global ischaemia followed by 15 min reperfusion induced translocation of PKCε and PKCδ to the mitochondrial fraction (Figure 1A, B, and D;  $n = 5$ ;  $P < 0.05$ ). HSP90 inhibition with GA (1 μM), during reperfusion (Figure 1A), attenuated IR-induced mitochondrial translocation of PKCε by 54% (Figure 1B and C, left panel,  $n = 5$ ;  $P < 0.01$ ), but not mitochondrial translocation of PKCδ (Figure 1B and C,

right panel,  $n = 5$ ). GA had no effect on total PKCε levels (Figure 1B), indicating that reduced mitochondrial PKCε translocation was not due to increased PKCε degradation. HSP90 inhibition with RAD (1 μM), which is structurally unrelated to GA,<sup>22</sup> also blocked IR-induced mitochondrial translocation of PKCε (Figure 1D). Mitochondrial purity was confirmed by electron microscopy and western blot analysis with protein markers of cytosolic (GAPDH), plasma membrane (β-integrin), and mitochondrial fractions (mitofusion-1) (Figure 1E). GA treatment during reperfusion also increased CPK release by ~70% when compared with IR alone (Figure 1F;  $P < 0.05$ ;  $n = 7$ ), but caused no damage in normoxic perfused hearts, even when used at five-fold higher concentration (Figure 1F).



**Figure 2** IR-induced co-IP of mitochondrial PKC $\epsilon$  with HSP90 or Tom20 requires HSP90 chaperone activity. Co-IP of mitochondrial (A) and cytosolic (B) fractions from hearts exposed to normoxia or IR with and without GA (1  $\mu$ M); representative of five experiments. The presence of HSP90 or Tom20 in the PKC $\epsilon$  immunoprecipitate (top) was confirmed by the presence of PKC $\epsilon$  in the HSP90 (middle) or Tom20 (bottom) immunoprecipitates. None of the proteins could immunoprecipitate with IgG beads alone. Input (mitochondrial lysate) controls are also provided.

### 3.2 Mitochondrial translocation of PKC $\epsilon$ proceeds via the HSP90–Tom20 import system

Under normoxic conditions, there was no physical association between PKC $\epsilon$  and HSP90 or between PKC $\epsilon$  and Tom20 at the mitochondria (Figure 2A). However, PKC $\epsilon$  co-immunoprecipitated with both HSP90 and Tom20 in mitochondria after IR. IR-induced co-IP was observed when immunoblotting the PKC $\epsilon$  immunoprecipitate for the presence of HSP90 or Tom20, and confirmed by the presence of PKC $\epsilon$  in the HSP90 or Tom20 immunoprecipitate. Importantly, IR-induced physical association between PKC $\epsilon$ –HSP90 and PKC $\epsilon$ –Tom20 was inhibited by GA, suggesting that HSP90 activity was required. No association between PKC $\epsilon$  and HSP90 was found in the cytosol under any conditions (Figure 2B), suggesting that IR-induced PKC $\epsilon$ –HSP90 interaction occurs following mitochondrial translocation of PKC $\epsilon$ .

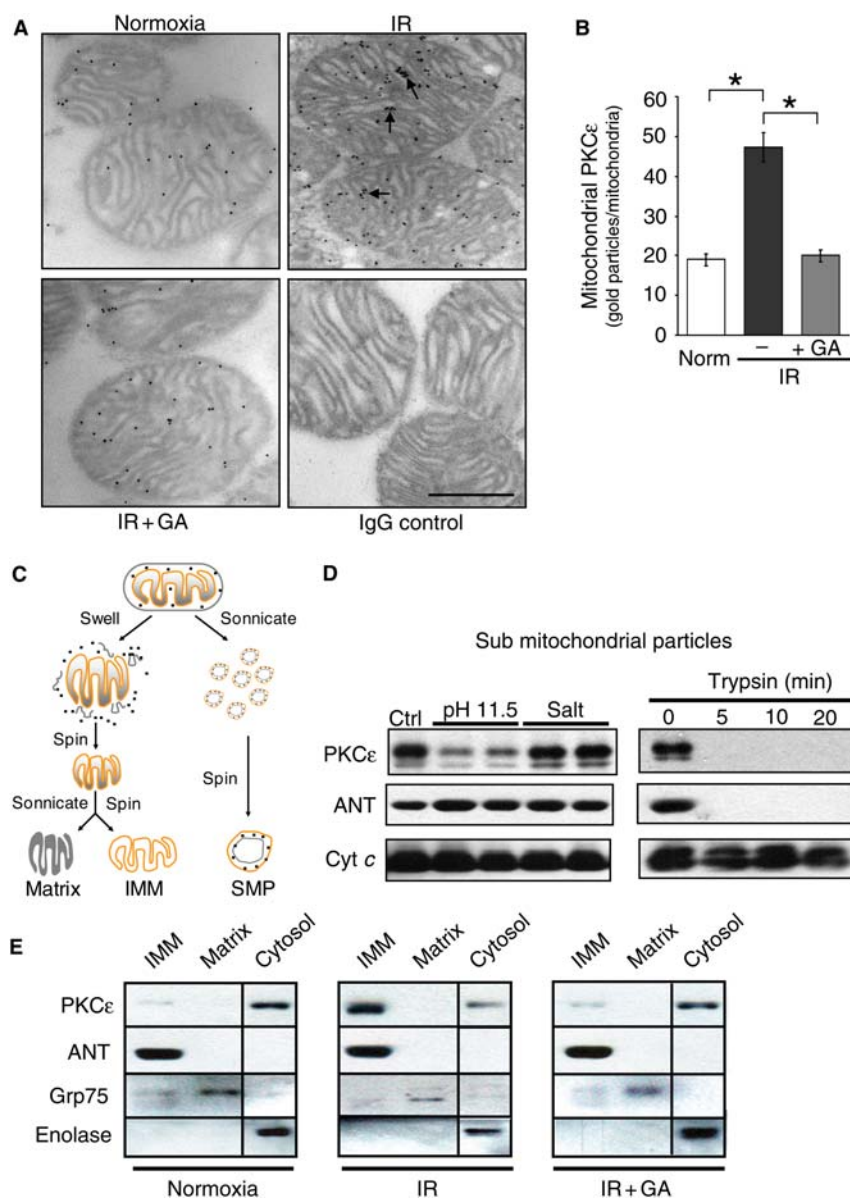
### 3.3 Mitochondrial PKC $\epsilon$ is bound to the IMM

Mitochondrial PKC $\epsilon$  location was examined by immunogold electron microscopy (EM) and mitochondrial subfractionation (Figure 3). Gold particles (representing bound PKC $\epsilon$  antibody) were found predominantly at the IMM (Figure 3A, right upper panel). Mitochondrial PKC $\epsilon$  levels increased by 2.3-fold when compared with normoxic conditions (Figure 3A, IR vs. normoxia, and Figure 3B,  $n = 3$ ; analysing 60 mitochondria/per group,  $P < 0.05$ ). The IR-induced increase in PKC $\epsilon$  levels was attenuated by GA (Figure 3A, IR + GA and Figure 3B,  $n = 3$ ,  $P < 0.05$ ). There was a complete absence of staining when mitochondria were incubated

with anti-rabbit IgG conjugated to immunogold, without prior incubation with the PKC $\epsilon$  antibody (IgG control), excluding non-specific binding due to IgG.

To further investigate the localization of PKC $\epsilon$  in the mitochondria, SMPs were prepared from hearts subjected to IR. SMPs were generated by sonication, creating inside-out mitochondrial vesicles, exposing IMM-associated proteins that face the matrix, and sequestering proteins that face the inner mitochondrial space, like cytochrome *c*, within the inverted mitochondrial vesicle<sup>21</sup> (Figure 3C). Exposure to a 200 mM Na<sub>2</sub>CO<sub>3</sub> carbonate wash at pH 11.5 (which removes strongly bound, but non-integral, membrane proteins) removed PKC $\epsilon$  from the IMM, whereas exposure to 400 mM KCl high-salt wash (which removes loosely associated proteins from membranes) did not dislodge PKC $\epsilon$  from this fraction (Figure 3D, upper left panel). Trypsin, which cannot cross membranes, completely removed PKC $\epsilon$  from these inside-out mitochondrial vesicles (Figure 3D, top right panel). That trypsin could access PKC $\epsilon$  suggests that PKC $\epsilon$  is present on the exposed (matrix) side of the IMM in the SMP preparation.<sup>21</sup> In contrast, the levels of cytochrome *c*, present in the space between the inner and the outer mitochondrial membranes (and therefore resides inside the SMP vesicles), were unaffected by trypsin digestion (Figure 3D, lower right panel), whereas the adenine nucleotide translocase (ANT), an integral IMM protein, showed a similar sensitivity to trypsin digestion as PKC $\epsilon$  (Figure 3D, middle right panel).

Mitochondria from hearts exposed to normoxia or IR were also subfractionated to yield IMM- and matrix-enriched components (Figure 3C, right scheme, and Figure 3E), which were then probed for PKC $\epsilon$  (Figure 3E, upper panels). [Purity was confirmed using antibodies against ANT (a marker of IMM), Grp75 (a mitochondrial matrix marker), and enolase (a cytosolic marker).] Similar to the



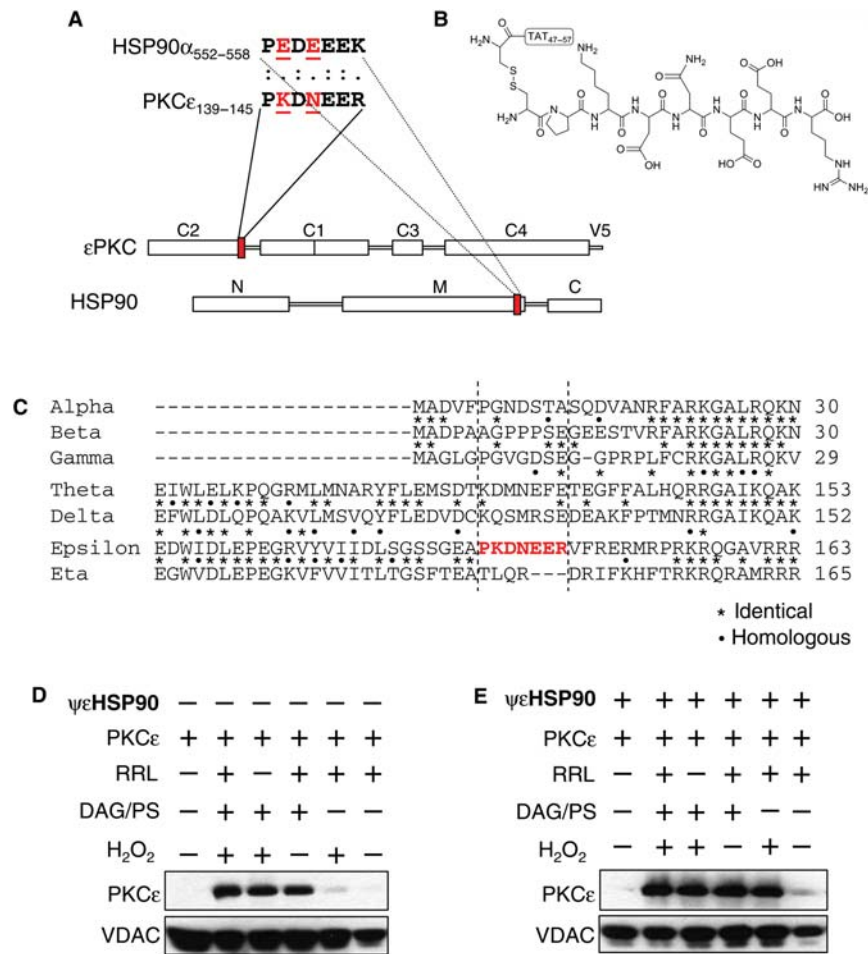
**Figure 3** PKC $\epsilon$  associates with the IMM. (A) Electron micrographs of PKC $\epsilon$  immunogold labelling in cardiac mitochondria. Localization on the IMM is indicated by arrows (scale bar = 0.5  $\mu$ m). Also included is an IgG control example. (B) Quantitation of mitochondrial PKC $\epsilon$  immunogold staining; mean  $\pm$  SEM ( $n = 3$  animals,  $*P < 0.05$ ). (C) Schematic of protocols used to fractionate mitochondria (SMPs). (D) SMPs from IR hearts were exposed to 200 mM Na<sub>2</sub>CO<sub>3</sub> (pH 11.5), 400 mM KCl (salt), or trypsin digestion and levels of PKC $\epsilon$ , the IMM protein, ANT, and the IMS protein, cytochrome c, were determined by western blotting. (E) IMM and mitochondrial matrix-enriched and cytosolic fractions were probed for PKC $\epsilon$ , ANT (a marker of IMM), glucose response protein (Grp75; a marker of matrix fraction), and enolase (a cytosolic marker) from hearts subjected to normoxia, IR, or IR in the presence of 1  $\mu$ M GA.

EM analysis, PKC $\epsilon$  in the IMM-enriched fraction increased following exposure to IR and was reduced by GA.

### 3.4 A rationally designed peptide activator of HSP90 and PKC $\epsilon$ interaction ( $\psi\epsilon$ HSP90) increases PKC $\epsilon$ translocation to cardiac mitochondria

We previously identified peptide activators of PKC $\epsilon$  that induce protein–protein interaction between PKC $\epsilon$  and its anchoring protein,  $\epsilon$ RACK.<sup>3</sup> Specifically, an 8-amino acid peptide corresponding

to a sequence in the C2 domain of PKC $\epsilon$  that is homologous to a sequence in  $\epsilon$ RACK<sup>3</sup> (hence termed pseudo- $\epsilon$ RACK or  $\psi\epsilon$ RACK peptide) was found to be an allosteric agonist of PKC $\epsilon$ .  $\psi\epsilon$ RACK interferes with the auto-inhibitory *intra*-molecular interaction within PKC $\epsilon$ , stabilizing PKC $\epsilon$  in a conformational state in which the  $\epsilon$ RACK-binding site on PKC $\epsilon$  is available for *inter*-molecular interaction with  $\epsilon$ RACK.<sup>3,23</sup> The observation that PKC $\epsilon$  only binds HSP90 upon activation (Figure 2), as is the case with PKC $\epsilon$ - $\epsilon$ RACK binding, led us to reason that the HSP90-binding site on PKC $\epsilon$  may also participate in an auto-inhibitory *intra*-molecular interaction when PKC $\epsilon$  is inactive. Using L-ALIGN sequence alignment software, we identified

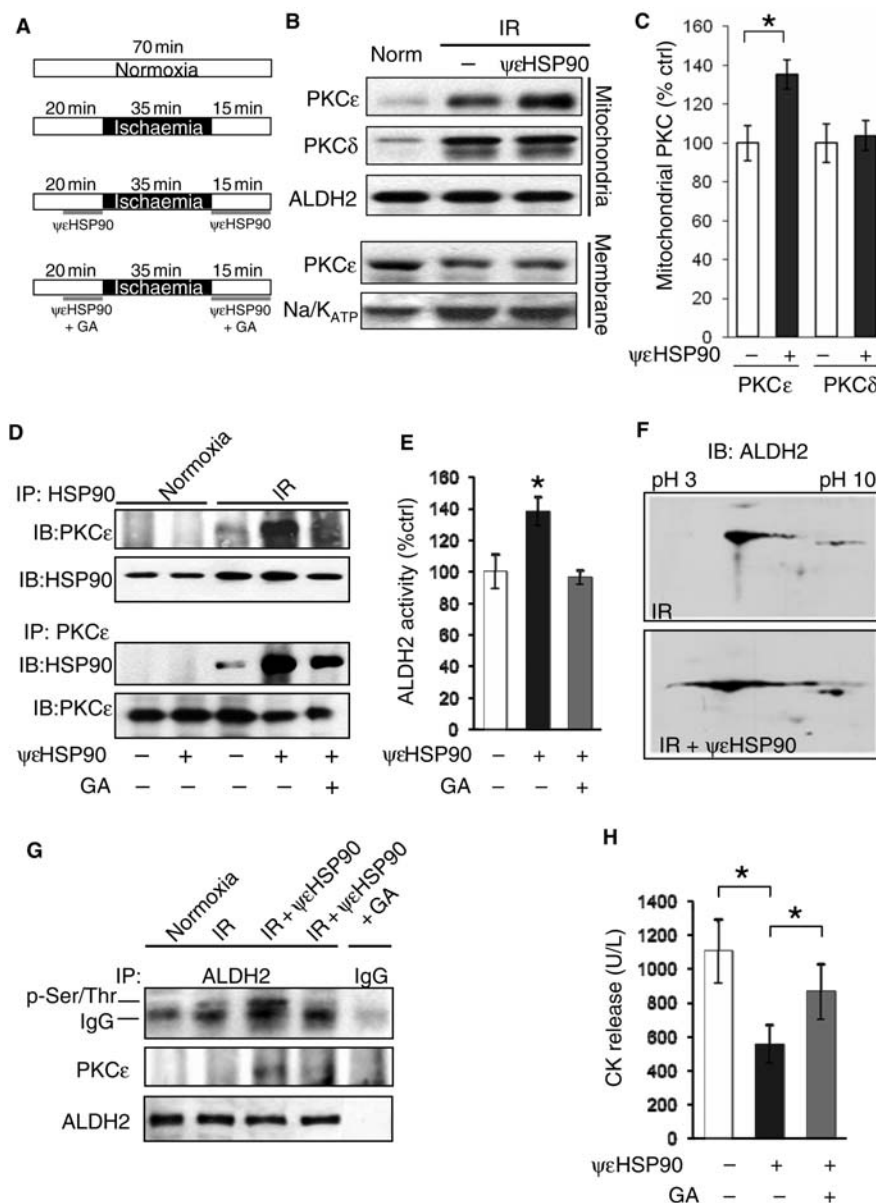


**Figure 4** A rationally designed peptide activator of HSP90 and PKC $\epsilon$  interaction ( $\psi\epsilon$ HSP90) increases mitochondrial PKC $\epsilon$  translocation. (A) Homologous sequence between PKC $\epsilon$  and HSP90 displaying charge differences (Lys<sup>140</sup> and Asn<sup>142</sup> in human PKC $\epsilon$  and Glu<sup>553</sup> and Glu<sup>555</sup> in human HSP90 $\alpha$ ). Also shown is a scheme indicating the location of the homologous sequences (red) on PKC $\epsilon$  and HSP90. (B)  $\psi\epsilon$ HSP90 peptide conjugated to cell penetrating TAT<sub>47-57</sub> peptide. (C) Alignment of human PKC isozymes, indicating that the  $\psi\epsilon$ HSP90 sequence is unique for the PKC $\epsilon$  isozyme. Identical and homologous sequences are indicated by asterisks and filled circles, respectively. Mitochondrial association of recombinant PKC $\epsilon$  after activation with DAG/PS (1 mM) and/or H<sub>2</sub>O<sub>2</sub> (50  $\mu$ M) and with RRL in the absence (D) and presence (E) of 1  $\mu$ M  $\psi\epsilon$ HSP90. VDAC is used as a loading control.

PKDNEER at the end of the C2 domain of PKC $\epsilon$  (amino acids 139–145; Figure 4A) to be homologous to PEDEEEK, found at the end of the middle domain of HSP90 (amino acids 552–558 on HSP90 $\alpha$  and 544–550 on HSP90 $\beta$ ; Figure 4A). There are two charge differences between these homologous sequences (Lys<sup>140</sup> and Asn<sup>142</sup> on PKC $\epsilon$  compared with Glu<sup>553</sup> and Glu<sup>555</sup> on HSP90 $\alpha$ ; red and underlined in Figure 4A). Such charge differences between the sequences were found in each of the other previous pseudo-site-derived peptides.<sup>24,25</sup> Further, the HSP90 homologous sequence on PKC $\epsilon$  is not found in any other members of the PKC family (Figure 4C), but is evolutionary conserved in PKC $\epsilon$  in a variety of species, including human, rat, mouse, and *Xenopus*. We synthesized a peptide corresponding to the HSP90 homology region in PKC $\epsilon$  (termed  $\psi\epsilon$ HSP90) and conjugated it, via cysteine–cysteine bond, to the cell permeating TAT protein-derived peptide, TAT<sub>25</sub><sup>47-57</sup> (Figure 4B).

The effect of  $\psi\epsilon$ HSP90 on mitochondrial PKC $\epsilon$  translocation was initially determined, *in vitro*. Isolated cardiac mitochondria were

incubated with recombinant PKC $\epsilon$  in the absence or presence of the PKC activators, DAG/PS (1mM), and/or with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; 50  $\mu$ M Figure 4D and E). Unstimulated recombinant PKC $\epsilon$  did not associate with the mitochondria. However, on activation with DAG/PS, there was substantial mitochondrial PKC $\epsilon$  association (Figure 4D). H<sub>2</sub>O<sub>2</sub> induced only a limited PKC $\epsilon$  translocation and did not increase the mitochondrial PKC $\epsilon$  translocation induced by DAG/PS (Figure 4D). RRL, used as an exogenous source of HSP90,<sup>26</sup> did not increase PKC $\epsilon$  association with the mitochondria, suggesting that HSP90 is already present at the mitochondria. This was also demonstrated by the data in Figure 2A (see also Figure 5D), in which HSP90 was detected at the mitochondria under normoxic conditions, and in agreement with previous data.<sup>27</sup> In the presence of  $\psi\epsilon$ HSP90 peptide (1  $\mu$ M, Figure 4E), the H<sub>2</sub>O<sub>2</sub>-induced mitochondrial association of PKC $\epsilon$  was enhanced (Figure 4E, second to last lane), suggesting that  $\psi\epsilon$ HSP90 enhances oxidative stress-induced mitochondrial translocation of PKC $\epsilon$ .



**Figure 5**  $\psi\epsilon$ HSP90 enhances mitochondrial PKC $\epsilon$  translocation, increases ALDH2 activity, and reduces cardiac damage by ischaemia/reperfusion, *ex vivo*. (A) IR protocols in the presence or absence of  $\psi\epsilon$ HSP90 peptide (1  $\mu$ M) and GA (1  $\mu$ M). (B)  $\psi\epsilon$ HSP90 peptide increased IR-induced mitochondrial translocation of PKC $\epsilon$  but was without effect on mitochondrial translocation of PKC $\delta$ . (C) Quantification of data in (B); mean  $\pm$  S.E.M ( $n = 7$ , \* $P < 0.05$ ). (D) Co-IP of mitochondrial HSP90 and PKC $\epsilon$  in normoxic and IR hearts in the absence and presence of  $\psi\epsilon$ HSP90 peptide or GA. (E) Mitochondrial ALDH2 activity in hearts exposed to IR in the absence and presence of  $\psi\epsilon$ HSP90 and GA. (F) Shift of immunoreactive ALDH2 on 2D IEF gels towards acidic pI by  $\psi\epsilon$ HSP90 when compared with IR alone, suggesting increased ALDH2 phosphorylation. (G) ALDH2 phosphorylation detected by immunoblotting the ALDH2 immunoprecipitate with anti-phospho Ser/Thr antibody (upper band on blot, distinct from IgG). PKC $\epsilon$  co-IP was also assessed by immunoblotting the ALDH2 immunoprecipitate (middle lane). (H) CK release following IR injury, in hearts treated with and without  $\psi\epsilon$ HSP90 and GA; mean  $\pm$  SEM ( $n = 6$ , \* $P < 0.05$ ).

### 3.5 $\psi\epsilon$ HSP90 promotes mitochondrial PKC $\epsilon$ translocation, activates mitochondrial ALDH2, and reduces IR injury

We determined whether  $\psi\epsilon$ HSP90 peptide increased mitochondrial translocation of PKC $\epsilon$  in *ex vivo* hearts. Mitochondria isolated from hearts subjected to IR in the presence of 1  $\mu$ M  $\psi\epsilon$ HSP90 (see protocols in Figure 5A) had a  $35 \pm 7\%$  increase in PKC $\epsilon$  ( $n = 5$ ,  $P < 0.05$ ;

Figure 5B and C), when compared with the IR alone group. Mitochondrial PKC $\delta$  translocation was unaffected by  $\psi\epsilon$ HSP90 treatment (Figure 5B and C), indicating specificity for PKC $\epsilon$ . Further,  $\psi\epsilon$ HSP90 did not affect PKC $\epsilon$  translocation to the plasma membrane (Figure 5B, lower panels), indicating a selective increase in mitochondrial PKC $\epsilon$ . There was no effect of the TAT carrier peptide alone on mitochondrial translocation of PKC $\epsilon$  or CK release (see Supplementary material online, Figure S1). Treatment with  $\psi\epsilon$ HSP90 also enhanced IR-induced physical interaction between HSP90 and

PKC $\epsilon$  and the  $\psi\epsilon$ HSP90-induced increase in PKC $\epsilon$ –HSP90 association was substantially reduced by pharmacological inhibition of HSP90 with GA (Figure 5D).

We previously found that selective PKC $\epsilon$  activation results in ALDH2 phosphorylation and increases ALDH2 activity,<sup>4</sup> and that ALDH2 activity correlates with cardioprotection from IR [ $R^2 = 0.97$ ].<sup>4</sup> When compared with control-treated hearts,  $\psi\epsilon$ HSP90 treatment caused a  $38 \pm 9\%$  increase in ALDH2 activity (Figure 5E;  $n = 6$ ,  $P < 0.05$ ), an effect that was blocked by HSP90 inhibition with GA (Figure 5E;  $n = 6$ ,  $P < 0.05$ ). To determine whether increased ALDH2 activity was due to increased ALDH2 phosphorylation, we performed two-dimensional gel immunoelectrophoresis (2D IEF) followed by immunoblotting with anti-ALDH2 antibodies. Similar to our study using  $\psi\epsilon$ RACK,<sup>4</sup> immunoreactive ALDH2 shifted to more acidic pI ranges after  $\psi\epsilon$ HSP90 treatment when compared with IR alone (Figure 5F), suggesting increased ALDH2 phosphorylation. This was also confirmed directly by immunoblotting the ALDH2 immunoprecipitate with anti-phospho Ser/Thr antibody (Figure 5G). The  $\psi\epsilon$ HSP90-induced increase in ALDH2 phosphorylation and activity was prevented by GA, demonstrating the requirement of HSP90. In addition,  $\psi\epsilon$ HSP90 treatment increased ALDH2–PKC $\epsilon$  co-IP in these samples (Figure 5G, lane 3). Finally, we determined the effect of  $\psi\epsilon$ HSP90 peptide on cardiac damage following IR. Hearts treated with  $\psi\epsilon$ HSP90 peptide had  $\sim 50\%$  reduced IR-induced cardiac damage as assessed by CPK release (Figure 5H;  $P < 0.05$ ,  $n = 6$ ), which was attenuated by HSP90 inhibition (Figure 5H;  $P < 0.05$ ,  $n = 5$ ).

### 3.6 $\psi\epsilon$ HSP90 peptide reduces infarct size and increases functional recovery following ischaemia and reperfusion injury, *in vivo*

We used an *in vivo* model of acute myocardial infarction in adult male rats that consisted of 35 min ischaemia followed by 24 h reperfusion. Rats were injected intraperitoneally with either  $\psi\epsilon$ HSP90 or with the control carrier peptide, TAT, (1 mg/kg in 400  $\mu$ L saline, each), which were administered 15 min before LAD occlusion and 5 min before the onset of reperfusion (Figure 6A). [The dose and route of administration were chosen based on our previous work using other TAT-conjugated peptides, such as  $\psi\epsilon$ RACK and  $\epsilon V_{1-2}^{28,29}$ .] Treatment with  $\psi\epsilon$ HSP90 reduced infarct size by  $\sim 30\%$  relative to control animals (Figure 6B;  $n = 6$ ,  $P < 0.05$ ), which had equivalent AAR for infarction (Figure 6C). Treatment with  $\psi\epsilon$ HSP90 also led to improvement in cardiac function; fractional shortening increased from  $31 \pm 2\%$  in TAT control-treated rats to  $40 \pm 3\%$  in rats treated with  $\psi\epsilon$ HSP90 (Figure 6D;  $n = 6$ ,  $P < 0.05$ ).

## 4. Discussion

PKC $\epsilon$  is critical for cardioprotection from IR injury. A number of studies have demonstrated that PKC $\epsilon$ -mediated protection is due to phosphorylation of mitochondrial proteins.<sup>4,6,8,13,30</sup> Here, we report that mitochondrial import of PKC $\epsilon$  is mediated by HSP90 and plays a crucial cardioprotective role. Although previous studies have demonstrated PKC $\epsilon$  activity within mitochondria,<sup>6–8,10,30</sup> to our knowledge, this study is the first description of interaction between PKC $\epsilon$  and the mitochondrial import machinery and suggests a possible mechanism for mitochondrial translocation of PKC $\epsilon$ . We also describe a peptide, designed based on PKC $\epsilon$ –HSP90

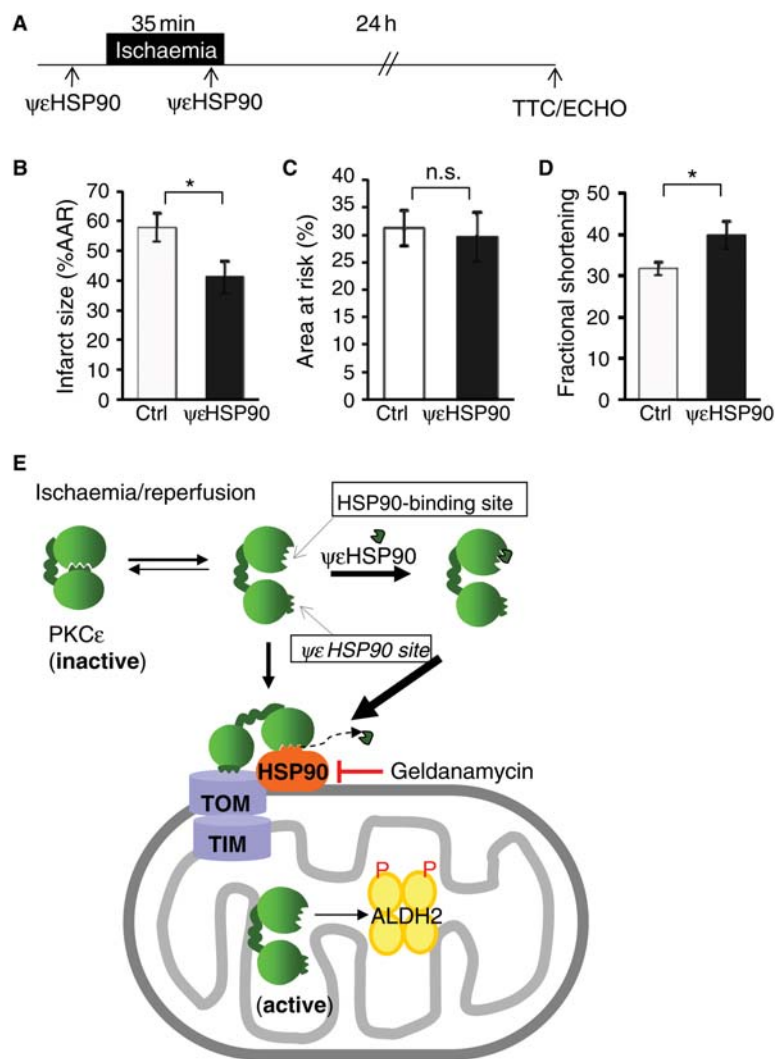
protein–protein interaction sites ( $\psi\epsilon$ HSP90), which increases mitochondrial PKC $\epsilon$ –HSP90 interaction, promotes mitochondrial translocation of PKC $\epsilon$ , and reduces infarct size *ex vivo* and *in vivo*.

A number of cardioprotective stimuli have been found to enhance mitochondrial translocation of PKC $\epsilon$ ,<sup>4,5,9,11,12,30</sup> whereas other studies did not support a role for mitochondrial PKC $\epsilon$  in cardioprotection.<sup>14,15</sup> This discrepancy may be explained by differences in stimulation or models used, the time point at which the translocation analyses were performed, or differences in cellular fractionation techniques. Here, we found that IR-induced mitochondrial translocation of both PKC $\epsilon$  and PKC $\delta$  when analysed 15 min after reperfusion began. HSP90 inhibition during reperfusion attenuated mitochondrial translocation of PKC $\epsilon$ , but not that of PKC $\delta$  (Figure 1). Electron microscopy and mitochondrial subfractionation analyses confirmed that intra-mitochondrial PKC $\epsilon$  levels are increased by IR in an HSP90-dependent manner and demonstrated that mitochondrial PKC $\epsilon$  is localized at the matrix side of the IMM (Figure 3). These data are consistent with recent studies reporting PKC $\epsilon$  at the IMM,<sup>10,30</sup> and with data demonstrating that PKC $\epsilon$  phosphorylates a number of intra-mitochondrial proteins.<sup>4–6,8,13,30</sup> Because mitochondrial translocation of PKC $\epsilon$  occurs rapidly, with a corresponding decline in cytosolic PKC $\epsilon$  levels, and since the total cellular PKC $\epsilon$  levels do not change (Figure 1B), our data suggest that HSP90 enables dynamic mitochondrial translocation of PKC $\epsilon$  in response to IR. HSP90-mediated mitochondrial import of proteins proceeds via the translocase of the outer membrane (TOM) multiprotein complex through recognition of the chaperoned protein by the import receptors, Tom20, Tom22, or Tom70.<sup>16</sup> We found that IR induced physical association between PKC $\epsilon$  and mitochondrial Tom20, which was prevented by GA. These data suggest that stimulus-induced mitochondrial import of PKC $\epsilon$  proceeds via an HSP90-dependent interaction with the TOM import complex. HSP90 inhibition did not affect IR-induced mitochondrial translocation of PKC $\delta$ . Other chaperones including HSP70, HSC70, and HSP40 mediate mitochondrial import of proteins;<sup>17,31</sup> therefore, it is possible that another chaperone mediates mitochondrial import of PKC $\delta$ .

HSP90 inhibition with GA during reperfusion resulted in a 70% increase in CK release, indicating that HSP90 mediates a cytoprotective function during reperfusion of ischaemic myocardium. However, HSP90 mediates a number of functions, including mitochondrial translocation of other cytoprotective proteins;<sup>18</sup> therefore, increased damage due to GA was not exclusively due to effects on mitochondrial PKC $\epsilon$ . We therefore sought a means to selectively modulate PKC $\epsilon$ –HSP90 interaction. Because PKC $\epsilon$  is regulated by multiple *intra*-molecular interactions,<sup>23,32</sup> we reasoned that an inhibitory *intra*-molecular interaction may exist between the HSP90-binding site in PKC $\epsilon$  and a sequence within PKC $\epsilon$  that shares homology with a region on HSP90 (Figure 6E). The  $\psi\epsilon$ HSP90 peptide corresponds to such a 7-amino acid sequence homology between the C2 domain of PKC $\epsilon$ , which is homologous with a sequence in the middle domain of HSP90. The C2 domain of PKC $\epsilon$  is known to mediate PKC $\epsilon$  protein–protein interactions,<sup>33</sup> and the corresponding sequence on HSP90 resides within a region that is essential for HSP90 protein–protein interaction.<sup>34</sup> Importantly, charge differences exist between these homologous sequences, characteristic of interaction sites within PKC.<sup>25,33</sup>

Our data demonstrate that  $\psi\epsilon$ HSP90 treatment enhanced IR-induced protein–protein interaction between PKC $\epsilon$  and HSP90 (Figure 5D), enhanced mitochondrial PKC $\epsilon$  translocation (Figures 4D





**Figure 6**  $\psi\epsilon$ HSP90 reduces infarct size and improves functional recovery following ischaemia/reperfusion injury *in vivo*. (A) An *in vivo* model of myocardial infarction (B)  $\psi\epsilon$ HSP90 reduced infarct size from  $57 \pm 5\%$  in control (TAT carrier treated) to  $41 \pm 6\%$  ( $n = 6$ , \* $P < 0.05$ ). (C) AAR was equal in both experimental groups. (D)  $\psi\epsilon$ HSP90 improved fractional shortening from  $31 \pm 2\%$  in controls, to  $40 \pm 3\%$  ( $n = 6$ , \* $P < 0.05$ ). Histograms show mean  $\pm$  SEM ( $n = 6$ , \* $P < 0.05$ ). (E) A proposed scheme of mitochondrial PKC $\epsilon$  translocation. On activation, PKC $\epsilon$  undergoes a conformational change, exposing the HSP90-binding site, enabling HSP90–PKC $\epsilon$  interaction at the mitochondria and PKC $\epsilon$  interaction with TOM, promoting mitochondrial import of PKC $\epsilon$ . The  $\psi\epsilon$ HSP90 peptide binds to the open form of PKC $\epsilon$ , stabilizing the enzyme in a conformation that promotes PKC $\epsilon$ –HSP90 interaction. Because the affinity of PKC $\epsilon$  for the  $\psi\epsilon$ HSP90 peptide is lower than that of the native PKC $\epsilon$ -binding site on HSP90, the peptide is displaced when PKC $\epsilon$  binds HSP90 at the mitochondria.

and E, and 5B–D), and decreased cardiac injury (Figures 5H and 6B). The  $\psi\epsilon$ HSP90-induced effects were attenuated by GA, demonstrating that  $\psi\epsilon$ HSP90 requires HSP90 (Figure 5E–H). The  $\psi\epsilon$ HSP90 peptide did not affect PKC $\delta$  mitochondrial translocation, demonstrating selectivity for PKC $\epsilon$ . Since PKC $\epsilon$  does not associate with HSP90 until activation with IR, it is likely that PKC $\epsilon$ –HSP90 interaction is dependent on a conformational change that occurs upon PKC $\epsilon$  activation, which exposes the HSP90-binding site. We propose that  $\psi\epsilon$ HSP90 stabilizes the activated PKC $\epsilon$  in a transient conformation that promotes its binding to HSP90, resulting in enhanced mitochondrial import of PKC $\epsilon$  (Figure 6E).

We recently identified mitochondrial ALDH2 as a PKC $\epsilon$  substrate, whose activity correlates with cardioprotection from IR.<sup>4</sup> Here, we showed that  $\psi\epsilon$ HSP90 increased phosphorylation and activity of

ALDH2. Although the current study focused on ALDH2, PKC $\epsilon$  can regulate other mitochondrial functions that mediate cytoprotection, including regulation of mitochondrial respiration and ROS production (mediated by phosphorylation of COIV by PKC $\epsilon$ ),<sup>6,7,30</sup> regulation of mitochondrial K<sup>+</sup> flux and mitochondrial matrix swelling,<sup>10</sup> and inhibition of MPTP opening.<sup>9</sup>

In summary, our results demonstrate that mitochondrial import of PKC $\epsilon$  is mediated by HSP90 and is required for cardiac protection against IR. Our data suggest a possible mechanism by which PKC $\epsilon$  can access cytoprotective substrates located within the mitochondria to confer cardioprotection. We also describe a novel peptide activator of PKC $\epsilon$ ,  $\psi\epsilon$ HSP90, which promotes mitochondrial PKC $\epsilon$ –HSP90 interaction and may have therapeutic use in the treatment of cardiac IR injury.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

**Conflict of interest:** D.M.-R. is a founder and shareholder of KAI pharmaceuticals. None of the work was supported by, or performed in collaboration with the company.

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