Published in final edited form as:

J Neurosci Res. 2013 June ; 91(6): 799–807. doi:10.1002/jnr.23186.

Selective activation of PKC epsilon in mitochondria is neuroprotective in vitro and reduces focal ischemic brain injury in mice

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Abstract

Activation of PKC ϵ confers protection against neuronal ischemia/reperfusion. Since activation of PKC ϵ leads to its translocation to multiple intracellular sites, a mitochondrial-selective PKC ϵ activator was used to test the importance of mitochondrial activation to the neuroprotective effect of PKC ϵ . PKC ϵ can regulate key cytoprotective mitochondrial functions including electron transport chain activity, reactive oxygen species (ROS) generation, mitochondrial permeability transition, and detoxification of reactive aldehydes. We tested the ability of mitochondrial selective activation of PKC ϵ to protect primary brain cell cultures or mice subjected to ischemic stroke. Pre-treatment with either general PKC ϵ activator peptide, $\psi\epsilon$ RACK, or mitochondrial-selective PKC ϵ activator, $\psi\epsilon$ HSP90, reduced cell death induced by simulated ischemia/reperfusion in neurons, astrocytes, and mixed neuronal cultures. The protective effects of both $\psi\epsilon$ RACK and $\psi\epsilon$ HSP90 were blocked by the PKC ϵ antagonist, ϵ V₁₋₂, indicating protection requires PKC ϵ interaction with its anchoring protein, ϵ RACK. Further supporting a mitochondrial mechanism for PKC ϵ , neuroprotection by $\psi\epsilon$ HSP90 was associated with a marked delay in mitochondrial membrane depolarization and significantly attenuated ROS generation during ischemia. Importantly, $\psi\epsilon$ HSP90 reduced infarct size and reduced neurological deficit in C57/BL6 mice subjected to middle cerebral artery occlusion and 24 hours of reperfusion. Thus selective activation of mitochondrial PKC ϵ preserves mitochondrial function *in vitro* and improves outcome *in vivo*, suggesting potential therapeutic value clinically when brain ischemia is anticipated, including neurosurgery and cardiac surgery.

Keywords

mitochondria; astrocytes; acute stroke; cell culture; animal models

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Disclosure/Conflict of Interest: DMR co-founded KAI Pharmaceuticals, a company that develops PKC-regulating peptides as therapeutics. However, none of the work in her lab is supported by the company.

Introduction

Activation of PKC epsilon (PKC ϵ) is both necessary and sufficient to confer protection against neuronal ischemia/reperfusion, as demonstrated by our and others' prior work (Bright and Mochly-Rosen 2005; Bright et al. 2008; Chou and Messing 2005; Dave et al. 2008; Lange-Asschenfeldt et al. 2004; Raval et al. 2007; Wang et al. 2004). Treatment with the PKC ϵ isozyme-selective agonist ψ ERACK promotes survival in primary neuronal cultures (Wang et al. 2004) and hippocampal slices (Lange-Asschenfeldt et al. 2004; Raval et al. 2003) and reduces infarct size *in vivo* (Bright et al. 2008). Conversely, selective inhibition of PKC ϵ with the inhibitor ϵ V₁₋₂ abolishes neuroprotection induced by ischemic preconditioning (IPC) (DeFazio et al. 2009; Jia et al. 2007; Lange-Asschenfeldt et al. 2004; Raval et al. 2007). Thus, PKC ϵ appears to play a critical role in neuronal survival and increased understanding of the underlying mechanism(s) may identify novel targets for treatment of cerebral ischemia.

PKC ϵ activation leads to its translocation to multiple intracellular sites (Disatnik et al. 1995). Emerging evidence suggests PKC ϵ promotes cell survival by regulating mitochondrial function and signaling (Budass and Mochly-Rosen 2007; Dave et al. 2008; Raval et al. 2007; Yonekawa and Akita 2008) and it is hypothesized that PKC ϵ -mediated phosphorylation of mitochondrial substrates may underlie ischemic tolerance. Mitochondria are crucial for cellular energy and ionic homeostasis, as well as regulation of both necrotic and apoptotic cell death (Baines 2010; Newmeyer and Ferguson-Miller 2003).

A number of mitochondrial PKC ϵ substrates have been reported (Barnett et al. 2008; Chen et al. 2008; Dave et al. 2008; Guo et al. 2007; Jaburek et al. 2006; Ogbi et al. 2004; Ogbi and Johnson 2006). In the heart, PKC ϵ directly phosphorylates components of the respiratory chain, including cytochrome c oxidase (Ogbi et al. 2004; Ogbi and Johnson 2006) preserving oxidative phosphorylation and energy generation. PKC ϵ also regulates opening of the mitochondrial permeability transition pore (mPTP) and activation prevents mPTP opening in cardiomyocytes (Baines et al. 2003) and neurons (Agudo-Lopez et al. 2011). Activation of mitochondrial PKC ϵ also induces opening of the mitochondrial inner membrane ATP-sensitive K⁺ channel (Jaburek et al. 2006) promoting mitochondrial K⁺ flux and preventing mitochondrial matrix swelling. Another mitochondrial PKC ϵ substrate is aldehyde dehydrogenase 2 (ALDH2); phosphorylation at Thr412 leads to increased activity (Chen et al. 2008). ALDH2 is cytoprotective by metabolizing toxic reactive aldehydes such as 4-hydroxy-2-nonenal (4-HNE) which accumulate during ischemia/reperfusion (Chen et al. 2008).

Multiple neuroprotective treatments- ischemic preconditioning (Dave et al. 2008; Raval et al. 2007), treatment with neuroprotective levels of ceramide (Agudo-Lopez et al. 2011) and the PKC ϵ selective agonist ψ ERACK (Dave et al. 2008) induce mitochondrial PKC ϵ translocation. In heart, mitochondrial translocation of PKC ϵ is mediated by HSP90, which permits mitochondrial import of PKC ϵ by the import receptor Tom20 (Budass et al. 2010). We recently designed a mitochondrial selective peptide activator of PKC ϵ (Budass et al. 2010), based on the PKC ϵ regulatory C2 domain involved in protein-protein interactions (Kheifets and Mochly-Rosen 2007; Schechtman et al. 2004) which shares sequence homology with the domain of HSP90 involved in protein binding (Hawle et al. 2006; Mizuno et al. 2001). Treatment with this peptide, ψ EHSP90, selectively enhanced mitochondrial PKC ϵ translocation and protected against cardiac ischemia/reperfusion injury (Budass et al. 2010). Importantly, ψ EHSP90 did not induce PKC ϵ translocation to the plasma membrane (Budass et al. 2010).

In the present study, we investigated whether selective translocation of PKC ϵ to the mitochondria using ψ HSP90 is sufficient to protect neural cells against ischemia/reperfusion injury. We report that ψ HSP90 treatment promotes neuronal and astrocyte survival, preserves mitochondrial membrane potential, and reduces free-radical generation in primary neuronal cultures exposed to glucose deprivation. The peptide also reduces infarct size in an *in vivo* model of stroke.

Materials and Methods

Primary cultures

All procedures were carried out according to protocols approved by the Stanford University Animal Care and Use Committee, in accordance with the NIH guide for the care and use of laboratory animals.

Astrocytes cultures were prepared from the cerebral cortices of Swiss Webster mouse pups age 1–3 days (Charles River, Hollister, CA) as previously described (Dugan et al. 1995). Briefly, cortices freed of meninges were incubated in 0.05% trypsin/EDTA (Gibco-BRL, Life Technologies, Rockville, MD) for 30 min at 37 °C, mechanically dissociated, and plated in Falcon PRIMARIA 24-well plates (Becton Dickinson, Lincoln, IL) at 1–2 hemispheres per plate, in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 10% equine serum (Hyclone, Logan, UT), 21mM (final concentration) glucose (Sigma, St Louis, MO) and 10ng/ml epidermal growth factor (Sigma). Cultures were used for injury experiments after 21 days in culture.

Relatively pure neuronal cultures (Dugan et al. 1995) were prepared from the cortices of embryonic day 15 or 16 mice. Cortices collected in ice-cold Eagle's minimal essential medium (Gibco, Grand Island, NY), were digested with 0.05% trypsin/EDTA for 15 min at 37°C, triturated, then plated in medium containing 5% fetal bovine serum and 5% equine serum (HyClone, Logan, Utah). The culture medium was replaced with glial-conditioned medium containing 5% equine serum, and B-27 (2%, Gibco BRL, Rockville, MD). Cytosine arabinoside (3 μ mol/l) was added 24 h after plating to inhibit glial proliferation. Cultures were used after 13 days (DIV 13) for glucose deprivation (GD) or oxygen-glucose deprivation (OGD) experiments.

Neuronal–astrocyte mixed cultures were prepared by plating the cell suspension from the E15–16 brains on established DIV 12 or older astrocyte cultures. The cultures were maintained in a 37°C humidified incubator with 5% CO₂ in air atmosphere. Cultures were fed every 4 days with growth medium identical to the plating medium but lacking fetal serum and epidermal growth factor.

Oxygen-Glucose Deprivation (OGD) and glucose deprivation (GD) in vitro ischemia

For OGD primary cultures were transferred to an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) with an atmosphere of 5% CO₂, 5% H₂ and 90% N₂. The culture medium was replaced three times with deoxygenated, glucose-free balanced salt solution (BSS₀), pH 7.4, containing Phenol Red (10 mg/l) and (in mM): NaCl 116, CaCl₂ 1.8, MgSO₄ 0.8, KCl 5.4, NaH₂PO₄ 1, NaHCO₃ 14.7, HEPES 10. BSS_{5.5} contains 5.5 mM glucose in BSS, and is the medium used for wash controls. Cultures were kept at 37°C in the anaerobic chamber. Oxygen tension was monitored with an oxygen electrode and kept under 0.02%. OGD was ended by adding glucose to the culture medium to a final concentration of 5.5 mM and returning the cultures to the normoxic incubator for 24 h prior to sampling the medium for lactate dehydrogenase (LDH) activity (Ouyang et al. 2007). This duration (2 h OGD) is sufficient to induce neuronal cell death but is not severe enough to induce astrocyte

death (Wang et al. 2004). Astrocytes require a longer duration of OGD to induce cell death; we used 6 h OGD followed by 24 h reperfusion to induce ischemia-reperfusion injury in astrocytes (Xu et al. 2001).

For GD the culture medium was replaced three times with BSS₀, pH 7.4, as above but lacking phenol red. Wash control was washed into BSS_{5.5} (Ouyang et al. 2007). GD was used in the imaging experiments because the slower kinetics allow better definition of changes in ROS and mitochondrial membrane potential.

Live cell epifluorescence imaging of mitochondrial membrane potential and ROS

Mitochondrial free radical production was monitored using hydroethidine (HEt 10uM). Cultures were loaded in the dark with 10 uM HEt in BSS_{5.5} (30 min, 37°C), and the same concentration of HEt was maintained in the culture medium throughout the experiment in BSS₀. Cells were excited at 495 nm, and emission monitored at 530 nm. Fluorescence measurements for each image (F_x) were normalized to the initial fluorescence intensity (F_0) of control wash. For assessment of changes in mitochondrial membrane potential, cultures were incubated for 30 min with tetramethylrhodamine ethylester (TMRE) (50 nM) at 37°C, and the same concentration of TMRE was maintained in all bathing solutions throughout the experiments. Cells were excited at 495 nm, emission was monitored at 530 nm every 30 min during GD for 2 hr. Changes in fluorescence were quantified by normalizing each image (F_x) to the initial fluorescence intensity (F_0) of control wash (Ouyang et al. 2007).

Peptide treatments

For injury experiments, cell cultures were washed three times into BSS₀ then incubated under OGD conditions for 2, 2, or 6 h for neuronal, mixed, and astrocyte cultures, respectively. O₂ and glucose were then restored and after 24 h the medium was sampled for LDH. The five groups, according to time of PKC peptide administration were: (1) No injury wash control. Cell cultures were washed three times with BSS_{5.5} medium and incubated in the normoxic incubator; the medium was sampled after 24 h. (2) OGD injury control. Cell cultures were exposed to OGD without peptide. (3) Treatment before OGD. Peptide was added to cultures 60 min prior to OGD. The media was changed before OGD to medium lacking peptide. (4) Treatment before and during OGD. The peptide was added for 60 min prior to OGD; after three washes with BSS₀, the same concentration of peptide was again added. (5) OGD injury control with TAT₄₇₋₅₇ (TAT), the short carrier peptide that is conjugated to our peptide of interest (cargo) to enable intracellular delivery. Control cultures had carrier peptide (TAT) added before and during OGD. Final peptide concentration was 1uM based on prior studies.

Lactate dehydrogenase (LDH) assay

LDH activity in the medium at 24 hr of reperfusion was measured as previously described (Koh and Choi 1987) to assess the extent of cell lysis. For pure neuronal cultures and astrocyte cultures, cells were then frozen/thawed to provide maximum LDH release values. For neuronal-astrocyte mixed cultures, medium was sampled after cells were exposed to NMDA for 24 h to provide maximum selective neuronal death values. The percent death (% of LDH release) was calculated by dividing the experimental time point by the maximum LDH values $\times 100$.

Western blot analysis

Primary neuronal cultures treated with ψ εHSP90-TAT peptide or TAT control before and during OGD, were harvested after 2 hr OGD and 1 h reperfusion for isolation of mitochondria. Cells were washed once with ice cold phosphate-buffered saline (pH 7.4), and

once with mitochondrial isolation buffer (0.32M Sucrose; 1mM EGTA and 10mM Tris-HCl, Sigma, pH 7.4) (Ouyang et al. 1997), and collected at $600 \times g$, 4°C for 5min. The cell pellet was resuspended in mitochondrial isolation buffer, homogenized with a Pyrex homogenizer on ice, centrifuged at $2600 \times g$, 4°C for 5min to pellet the nuclear fraction. The supernatant (soluble fraction) was collected and centrifuged at $13,000 \times g$, 4°C for 15min to separate the cytosolic fraction and crude mitochondrial fraction. The mitochondrial fraction was resuspended in ice cold lysis buffer (10 mM HEPES, 42 mM KCl, 5 mM MgCl_2 , 0.1 mM EDTA, 0.1 mM EGTA, and 0.1% Triton-100) with ProteaseARREST (G Biosciences, Maryland Heights, MO) for 30 min on ice. Following centrifugation at 13,000 rpm for 20min the supernatant was collected as the mitochondrial fraction. The cytosolic fraction was concentrated using an Amicon Ultra-0.5 centrifugal filter device from Millipore Corporation (Billerica, MA). Protein concentrations were determined by the bicinchoninic acid method (BCA Protein Assay Kit, Pierce, Rockford, IL). Equal amounts (20 μg) of protein were analyzed by Western blot using anti-PKC ϵ (sc-1681, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized with the Odyssey detection system (LI-COR Biosciences, Lincoln, NE). Anti-ANT (mitochondrial) and -Actin (cytosolic) antibodies were used for compartment specific loading controls. Densitometric analysis was performed using NIH ImageJ software.

Transient middle cerebral artery occlusion (MCAO), in vivo ischemia reperfusion

C57Bl6 mice purchased from Charles River Laboratory (Wilmington, MA, USA) were housed in the Stanford Medical School Animal Care Facility until use. Transient focal cerebral ischemia (MCAO) was induced in male mice, 8–10 weeks of age, under isoflurane anesthesia in 70% N_2O and O_2 by facemask. The MCA was occluded for 1 h with a silicone-coated 6-0 monofilament (Doccol Corp., Redlands, CA) which was then withdrawn for reperfusion as described (Han et al. 2009; Xiong et al. 2011). Rectal temperature was maintained at $37 \pm 0.5^{\circ}\text{C}$ with a heating pad (Harvard Apparatus, Holliston, MA). Heart rate, oxygen saturation and respiratory rate were monitored continuously (STARR Life Sciences Corp., Allison Park, PA). Animals with no observable deficits immediately after ischemia, those that died before 24 hr, and those with subarachnoid hemorrhage at the time of death were excluded; the exclusion rate was 15%.

Peptide treatment

Peptides $\psi\epsilon\text{HSP90-TAT}$ or TAT were injected IP at 1mg/kg 15 min prior to MCAO and 3 min before reperfusion. After assessing neurological score 24 hr after MCAO, mice were sacrificed under deep isoflurane anesthesia and cerebral infarction area was determined on 5 sections/brain after staining with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) by a blinded observer and corrected for edema using the NIH Image program (Image J 1.37v, Wayne Rasband, available through the NIH) as described previously (Han et al. 2009; Xiong et al. 2011). Brains were sectioned coronally with a rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA). The neurological grading score was 0, no observable neurological deficit to 4, unable to walk spontaneously and a depressed level of consciousness (Han et al. 2009; Xiong et al. 2011). The evaluator was blinded to experimental treatment.

Cerebral blood flow changes with MCAO

Transcranial regional cerebral blood flow (rCBF) in parietal cortex was monitored in separate groups of mice using a laser Doppler flow meter (TST Incorporated, ST. Paul, MN). A micro tip fiber optic probe (diameter 0.5 mm) connected through a Master Probe to the laser Doppler was attached to the skull 5 mm left lateral and 1 mm posterior to bregma. After induction of anesthesia blood flow velocity (mLD/min/100g) was measured. Peptides ($\psi\epsilon\text{HSP90-TAT}$ or TAT) were injected 15 min before and during MCAO as above. The

CBF was again measured during (30 min after occlusion) ischemia, and 5 min after beginning reperfusion.

Statistics—For comparisons of two groups t test was used, for experiments with more than 2 groups 1 way ANOVA with Student Newman Keuls post test was used. Values are mean $\pm$ SEM.

Results

ψ εRACK and ψ εHSP90 peptides reduce neuronal OGD cell death in mixed cultures

We previously demonstrated that PKC ϵ agonist ψ εRACK attenuates neuronal OGD death when administered prior to ischemia, but not when applied during reperfusion (Wang et al. 2004). Therefore, we administered PKC ϵ activators ψ εRACK or ψ εHSP90 according to two treatment regimens: “Before + During” OGD, in which the activator peptides were added to the culture medium 60 min prior to OGD and also to the OGD media (Fig. 1A) and “Before” only, in which the agonist peptides were added to the media for 60 min prior to ischemia, but were not present in the OGD medium (Fig. 1B). As we have shown before (Wang et al. 2004), using a short duration of OGD that only caused neuronal but not astrocyte cell death, ψ εRACK applied before and during OGD was neuroprotective reducing neuronal death in mixed cultures by 29% compared to the Control peptide group ($p < 0.05$, Fig. 1A). Treatment with the novel mitochondrial-selective PKC ϵ activating peptide, ψ εHSP90, conferred similar protection to that with ψ εRACK reducing death by 36% ($p < 0.01$; Fig. 1A). The neuroprotective effects of both ψ εRACK and ψ εHSP90 through activation of PKC ϵ were abolished by pre-incubation with the PKC ϵ antagonist ϵ V₁₋₂, indicating that protection for both peptides requires PKC ϵ interaction with its anchoring protein, ϵ RACK (Csukai et al. 1997). When mixed cultures were treated with peptide only before OGD (Fig. 1B), both ψ εRACK and ψ εHSP90 reduced LDH release, however, the protection was not as pronounced as when the agonists were present both before and during and did not reach statistical significance in the ψ εRACK group. Treatment with TAT carrier (control) peptide had no effect on injury.

ψ εRACK and ψ εHSP90 reduce neuronal OGD cell death

To investigate whether neuroprotection induced by ψ εRACK and ψ εHSP90 required the presence of astrocytes, we repeated the OGD-reperfusion experiment using relatively pure neuronal cultures. Similar to the observations on neuronal injury in mixed cultures, treatment with either ψ εRACK and ψ εHSP90 peptides reduce neuronal cell death induced by OGD-reperfusion when applied before and during OGD, by 23% and 29%, respectively ($p < 0.01$; Fig. 2A). A neuroprotective effect was also obtained when peptides were added before OGD only (Fig. 2B). Again, the protective effect of either ψ εRACK or ψ εHSP90 was abolished by pre-incubation with ϵ V₁₋₂. These data demonstrate that ψ εRACK and ψ εHSP90 protect neurons directly in the absence of astrocytes.

Treatment with ψ εRACK or ψ εHSP90 reduces OGD astrocyte cell death

We next investigated whether ψ εRACK or ψ εHSP90 treatments had any effect on astrocytic cell death induced by OGD-reperfusion. When applied before and during OGD, either ψ εRACK or ψ εHSP90 exerted a neuroprotective effect and reduced LDH release by 29% and 34%, respectively (Fig. 3A). The protective effect of both ψ εRACK and ψ εHSP90 was blocked by ϵ V₁₋₂ (Fig. 3A). Neither ψ εRACK nor ψ εHSP90 had any effect on astrocyte survival when the peptides were only added before OGD but were not present during OGD (Fig. 3B). The TAT carrier peptide had no effect on astrocyte survival.

ψ εHSP90 enhances PKCε mitochondrial translocation during OGD/reperfusion

To assess the mitochondrial component of PKCε activation Western blot analysis was carried out on the cytosolic and mitochondrial fractions from neuronal cultures (12 DIV) exposed 2 h OGD and 1 h reperfusion (Fig. 4). ψ εHSP90 treatment before and during OGD prevented the reduction in mitochondrial PKCε that is seen in control cultures subjected to OGD/reperfusion but treated with TAT peptide. That significant amounts of PKCε are present in mitochondria of neurons under normal growth conditions was previously reported (Wang et al. 2004).

The mitochondrial/cytosolic ratio, indicating the extent of activation of PKCε (translocated PKC), was significantly greater with ψ εHSP90 compared to control peptide TAT in neuronal cultures subjected to OGD (Fig. 4, B; $p < 0.01$).

ψ εHSP90 decreases neuronal ROS formation induced by GD

Previous studies demonstrated that PKCε activation can reduce H_2O_2 production in hippocampal synaptosomes (Dave et al. 2008) whereas PKCε inhibition with εV_{1-2} prevents the attenuation in ROS generation conferred by ceramide preconditioning (Agudo-Lopez et al.). To examine whether selective activation of PKCε in the mitochondria, using ψ εHSP90, can attenuate free-radical production in pure neuronal cultures, ROS generation was monitored in real time using the fluorescent dye HET during exposure to GD (Fig. 5A). As expected, ROS levels increased throughout GD exposure and were ~3-fold higher after 2 hr compared to the control (wash) group ($p < 0.05$). Treatment with 1 μ M ψ εHSP90 attenuated the GD-induced ROS generation almost to control wash levels and was significantly different than GD alone after 60, 90, and 120 min. ($p < 0.001$). The TAT carrier peptide had no effect on ROS generation induced by GD.

ψ εHSP90 protects neuronal mitochondrial membrane potential during GD

PKCε activation can preserve mitochondrial membrane potential ($\Delta\psi_m$) following simulated ischemia (McCarthy et al. 2005). We measured $\Delta\psi_m$ in real time using TMRE in pure neuronal cultures exposed to glucose deprivation (GD). After 90 min GD in both the GD control- and carrier peptide TAT-treated cells the TMRE fluorescence intensity decreased significantly, and the TMRE mitochondrial fluorescence was below the detection threshold of the microscope after 120 min. Treatment with ψ εHSP90 significantly attenuated GD-induced mitochondrial membrane depolarization, (Fig. 5B) ($p < 0.001$ at 30, 60, and 90 min).

Infarction volume and neurological deficit are decreased by ψ εHSP90

To determine whether protection observed in ψ εHSP90 peptide-treated astrocyte and neuronal cultures subjected to OGD could be translated to an animal model of stroke, mice were given IP injections of ψ εHSP90 or carrier peptide TAT. ψ εHSP90 administration significantly reduced infarct volume from 53.95 ± 2.84 to 40.33 ± 2.27 ($P < 0.001$, figure 6A, B) and reduced the neurological deficit score from 2.75 ± 0.11 to 2.24 ± 0.11 ($P < 0.002$, figure 6C). To rule out an effect of the peptides on cerebral blood flow, we monitored transcranial regional cerebral blood flow (rCBF) before, during and after MCAO within the parietal cortex. Blood flow prior to occlusion was 103.0 ± 6.7 ml/min/100g for the ψ εHSP90 group, 104.8 ± 3.2 for the TAT group. CBF dropped to 20.7 ± 6.3 ml/min/100g for ψ εHSP90 treated mice and 21.2 ± 4.5 for TAT mice after suture placement and recovered to 58.5 ± 5.1 for ψ εHSP90 and 53.3 ± 5.2 for TAT after 5 min reperfusion. Therefore, there were no differences between ψ εHSP90 and TAT treated groups ($n=6$) at any of the time points measured.

Discussion

The current study demonstrates that selective activation of PKC ϵ in mitochondria alone can reduce cell death induced by ischemia/reperfusion both *in vivo* and *in vitro*. Both the general PKC ϵ activator peptide, ψ ERACK, and the mitochondrial PKC ϵ activator, ψ EHSP90, conferred protection in primary neuronal and astrocytic cultures when applied before the onset of ischemia. As the ψ EHSP90 peptide selectively augments translocation of PKC ϵ to the mitochondria but not to the plasma membrane (Budás et al. 2010), our results suggest that a major component of the neuroprotective mechanism of PKC ϵ is mediated by regulation of mitochondrial function. In support of this hypothesis, ψ EHSP90 prevented the reduction in mitochondrially localized PKC ϵ induced by OGD, and prevented mitochondrial depolarization, and reduced ROS generation by GD.

ψ ERACK and ψ EHSP90 both reduced neuronal injury, whether the neurons were co-cultured with astrocytes or not (Fig. 1 and 2). However, we also found PKC ϵ activation with ψ ERACK or ψ EHSP90 when given both before and during OGD could protect astrocytes. In our previous study we observed a trend towards astrocyte protection after both 4.5 and 6 hr OGD with ψ ERACK which however did not reach significance (Wang et al. 2004). Taken together with our current findings, it appears that PKC ϵ does indeed have a role in astrocyte cell survival, which likely contributes to the *in vivo* neuroprotective effects of PKC ϵ . It is noteworthy that in contrast to neurons, treatment with agonist before OGD alone was clearly ineffective in astrocytes, while it was still effective for neurons, though not as potent as treatment both before and during.

Treatment with ψ EHSP90 markedly delayed mitochondrial depolarization and reduced ROS accumulation in purified neuronal cultures subjected to GD (Fig. 5). These data are consistent with previous reports that PKC ϵ activation can reduce mitochondrial ROS generation in neuronal mitochondria (Dave et al. 2008) and preserve mitochondrial membrane potential in cardiac mitochondria (McCarthy et al. 2005). One mechanism may be regulation of mitochondrial electron transport activity, in particular regulation of cytochrome *c* oxidase (CO), which catalyses the last step in electron-transport, transferring electrons from cytochrome *c* to molecular oxygen. Alterations in cytochrome *c* oxidase activity influence mitochondrial membrane potential, ATP generation, and ROS generation (Ogbi et al. 2004). Since during ischemia the ETC complexes are in a more reduced state, superoxide production is favored (Moncada and Erusalimsky 2002). Enhanced CO activity should minimize ROS production by improving electron flow (Ogbi et al. 2004). PKC ϵ physically associates with cytochrome *c* oxidase subunit IV (COIV) in neonatal cardiomyocytes (Ogbi et al. 2004; Ogbi and Johnson 2006) and adult myocytes (Guo et al. 2007). PKC ϵ activation increases PKC ϵ -COIV interaction and enhances CO activity ~2 fold, with more rapid recovery of ATP following cardiac ischemia/reperfusion *in vivo* (Guo et al. 2007). Others have confirmed PKC ϵ -mediated phosphorylation of COIV in murine lens epithelial cells (Barnett et al. 2008), and rat hippocampal synaptosomes (Dave et al. 2008), demonstrating that this also occurs in neurons.

PKC ϵ may reduce dissipation of the mitochondrial membrane potential by opening mitoK_{ATP} channels thereby helping regulate mitochondrial matrix volume and ROS production and protecting cells by increasing K⁺ flux across the inner mitochondrial membrane (Murphy and Steenbergen 2007). In isolated mitochondria, selective activation of PKC ϵ with ψ ERACK promotes mitoK_{ATP} channel opening, which is prevented by ϵ V₁₋₂ or by the mitoK_{ATP} channel antagonist 5-HD (Costa et al. 2006; Jaburek et al. 2006). Opening of mitoK_{ATP} in mitoplasts by ψ ERACK can be prevented by phosphatase PP2A (Jaburek et al. 2006), suggesting regulation by direct phosphorylation. In hippocampal neurons,

mitoK_{ATP} appears to be downstream of PKC ϵ as neuroprotection induced by either IPC or PKC ϵ activation is blocked by 5-HD (Raval et al. 2007).

Another potential target of mitochondrial PKC ϵ is mitochondrial permeability transition pore (MPTP) opening, a primary cause of cell death following ischemia/reperfusion (Newmeyer and Ferguson-Miller 2003). Recombinant PKC ϵ directly inhibited MPTP opening induced by Ca²⁺ in cardiac mitochondria (Baines et al. 2003) and neuroprotection by ceramide depended on PKC ϵ mediated inhibition of MPTP (Agudo-Lopez et al. 2011). These data are consistent with our current data that ψ HSP90 (which selectively enhances mitochondrial PKC ϵ) prevents both mitochondrial membrane depolarization and ROS generation. The mechanism by which PKC ϵ reduces MPTP opening has yet to be determined. PKC ϵ can directly interact with both VDAC and ANT (Baines et al. 2003), however it is not clear whether these proteins are required components of the MPTP (Baines 2009). Alternatively, PKC ϵ -mediated inhibition of MPTP opening may be indirect and related to mitoK_{ATP} opening (Costa et al. 2006) or by reduced mitochondrial ROS generation.

While further work is required to identify the detailed molecular mechanism, the results of the present study demonstrate that selective activation of PKC ϵ in the mitochondria (using ψ HSP90 peptide) preserves mitochondrial function and reduces neuronal and astrocytic cell death induced by ischemia. Further, peripheral administration of ψ HSP90 protects the brain from stroke *in vivo*. Development of this approach for clinical applications would likely be limited to settings in which pretreatment or at least administration during a potential ischemic event was possible, such as during neurosurgery or cardiac surgery.

Acknowledgments

Supported in part by NIH grants HL52141 to DMR and GM49831 to RGG.

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Fig. 1. ψεRACK and ψεHSP90 reduce OGD neuronal death in mixed cortical cultures

Mixed primary cultures containing neurons and astrocytes were exposed to OGD for 2 hr and 24 h reperfusion. This duration of OGD does not kill astrocytes. Cells were treated with the PKCε activator ψεRACK, or the PKCε activator ψεHSP90 which selectively promotes mitochondrial translocation of PKCε. The carrier peptide TAT was used as the control (Ctrl Peptide). Peptides were added either 60 min before OGD and during OGD (A) or before but not during OGD (B). LDH activity was measured to quantify cell death. Both ψεRACK and ψεHSP90 reduced neuronal death, and this protection was prevented by treatment with the PKCε-selective antagonist εV₁₋₂. (n=10–12 independent cultures, * indicates p<0.05, ** p<0.01, *** p<0.001 compared to OGD Ctrl Peptide or OGD Ctrl)

Fig. 2. $\psi\epsilon$ RACK and $\psi\epsilon$ HSP90 reduce OGD induced neuronal death in pure neuronal cultures Relatively pure neuronal cultures were exposed to OGD for 2 hr and 24 hr reperfusion. LDH activity was measured to quantify cell death. A. $\psi\epsilon$ RACK and $\psi\epsilon$ HSP90 reduced neuronal death when applied before and during OGD (n=20). B. Neuroprotection was also seen when peptides were only added before OGD (n=20 for all conditions except + ϵV_{1-2} n=12). The protective effect of both $\psi\epsilon$ RACK and $\psi\epsilon$ HSP90 was abolished by pre-incubation with ϵV_{1-2} . (* indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to OGD Ctrl Peptide or OGD Ctrl)

Fig. 3. PKC ϵ peptides reduce OGD astrocyte death only if given before and during OGD
Astrocyte cultures were exposed to OGD for 6 hr followed by 24 hr reperfusion; LDH activity was measured to quantify cell death. A. $\psi\epsilon$ RACK and $\psi\epsilon$ HSP90 reduced astrocyte cell death when applied before and during OGD (n=7 for control wash, n=12 all other conditions). The protective effect of both $\psi\epsilon$ RACK and $\psi\epsilon$ HSP90 was abolished by pre-incubation with ϵV_{1-2} . B. Neither $\psi\epsilon$ RACK nor $\psi\epsilon$ HSP90 reduced cell death when only applied before OGD. Ctrl Peptide alone had no effect on cell death. (n=12 all conditions except wash control n=7). * indicates $p<0.05$, *** indicates $p<0.001$ when compared to OGD Ctrl Peptide or OGD control.

Fig. 4. $\psi\epsilon$ HSP90 maintains PKC ϵ mitochondrial translocation during OGD/reperfusion PKC ϵ translocation to mitochondria in cortical neurons is reduced by OGD, and this reduction is prevented by treatment with $\psi\epsilon$ HSP90. Both total PKC ϵ and the fraction in mitochondria is reduced by OGD. A. The level of mitochondrial and cytosolic PKC ϵ was determined by western, a representative western is shown. B. The percent translocation to mitochondria was calculated as mitochondrial PKC ϵ /(mitochondrial PKC ϵ + cytosolic PKC ϵ) $\times$ 100. The graph shows values from three individual experiments. We again observed that under normal growth conditions there is significant presence of PKC ϵ in neuronal mitochondria, about 70% (Wang et al. 2004). **= $p < 0.01$

Fig. 5. $\psi\epsilon$ HSP90 preserves mitochondrial membrane potential ($\Delta\psi_m$) and prevents ROS generation induced by glucose deprivation (GD)

(A) ROS generation was monitored in real-time using the fluorescent dye HET during 2 hr GD. Treatment with $1\mu\text{M}$ $\psi\epsilon$ HSP90 significantly attenuated GD-induced ROS generation (* $p < 0.05$ at 30min, $p < 0.01$ at 60min to 120min) compared to Ctrl Peptide-GD ($n = 40-60$ cells). Ctrl Peptide (TAT) had no effect on GD-induced ROS generation at any time point. (B) Fluorescence images recorded from Ctrl Peptide and $\psi\epsilon$ HSP90 treated neurons at 0 min and 120 min GD. (C) TMRE measurement of mitochondrial membrane potential ($\Delta\psi_m$) in neurons exposed to GD. Treatment with $1\mu\text{M}$ $\psi\epsilon$ HSP90 significantly attenuated GD-induced $\Delta\psi_m$ depolarization (* $p < 0.01$). (D) Fluorescence images of representative fields of cells recorded from Ctrl Peptide treated and $\psi\epsilon$ HSP90 treated neurons at 0 min and after 90 min GD. $n = 40-60$ cells, * indicates $p < 0.05$, *** indicates $p < 0.001$ compared to the GD-Ctrl Peptide group.

Fig. 6. $\psi\epsilon$ HSP90 protects in an *in vivo* stroke model

Mice received two intraperitoneal injections with $\psi\epsilon$ HSP90 (1mg/kg) or TAT Ctrl Peptide (1mg/kg) administered 15 min prior to 1 hr MCAO and again 5 min before the onset of reperfusion. After 24 hr reperfusion neurological score was evaluated and the animals sacrificed for analysis of infarct volume. Representative TTC-stained coronal sections are shown (A). Ischemic infarct area (B) and neurological score (C) were significantly decreased in $\psi\epsilon$ HSP90-treated animals (n=12) compared to Ctrl Peptide treated mice (n=11), * indicates $p > 0.01$.