

Epsilon PKC Increases Brain Mitochondrial SIRT1 Protein Levels via Heat Shock Protein 90 following Ischemic Preconditioning in Rats

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Abstract

Ischemic preconditioning is a neuroprotective mechanism whereby a sublethal ischemic exposure is protective against a subsequent lethal ischemic attack. We previously demonstrated that SIRT1, a nuclear localized stress-activated deacetylase, is vital for ischemic preconditioning neuroprotection. However, a recent study demonstrated that SIRT1 can also localize to the mitochondria. Mitochondrial localized SIRT1 may allow for a direct protection of mitochondria following ischemic preconditioning. The objective of this study was to determine whether ischemic preconditioning increases brain mitochondrial SIRT1 protein levels and to determine the role of PKC ϵ and HSP90 in targeting SIRT1 to the mitochondria. Here we report that preconditioning rats, with 2 min of global cerebral ischemia, induces a delayed increase in non-synaptic mitochondrial SIRT1 protein levels which was not observed in synaptic mitochondria. This increase in mitochondrial SIRT1 protein was found to occur only in neuronal cells and was mediated by PKC ϵ activation. Inhibition of HSP90, a protein chaperone involved in mitochondrial protein import, prevented preconditioning induced increases in mitochondrial SIRT1 and PKC ϵ protein. Our work provides new insights into a possible direct role of SIRT1 in modulating mitochondrial function under both normal and stress conditions, and to a possible role of mitochondrial SIRT1 in activating preconditioning induced ischemic tolerance.

Citation: Thompson JW, Dave KR, Saul I, Narayanan SV, Perez-Pinzon MA (2013) Epsilon PKC Increases Brain Mitochondrial SIRT1 Protein Levels via Heat Shock Protein 90 following Ischemic Preconditioning in Rats. PLoS ONE 8(9): e75753. doi:10.1371/journal.pone.0075753

Editor: Kah-Leong Lim, National University of Singapore, Singapore

Received: March 29, 2013; **Accepted:** August 18, 2013; **Published:** September 13, 2013

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Funding: This work was supported by National Institutes of Health grants NS45676-01, NS054147-01 and NS34773 (to M.A.P.P.), and NS073779 (to K.R.D.), by a Postdoctoral award from the American Heart Association, Greater Southeast Affiliate (to J.W.T.) and by a NIH F31 Predoctoral award NS080344-01 (to S.V.N.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Ischemic preconditioning (IPC) is an innate neuroprotective mechanism in which a mild ischemic stress protects against a subsequent lethal ischemic exposure. IPC activates both early (0-3h) and delayed (24-72 h) windows of protection [1-3]. The early window of protection is mediated by a rapid post-translational modification of proteins, whereas, the delayed window of protection is mediated by alterations in gene expression [4-6]. PKC epsilon (PKC ϵ) is a novel member of the protein kinase C family which has been demonstrated by our laboratory and others to be pivotal in IPC mediated neuroprotection [7,8]. Our laboratory has demonstrated that activation of PKC ϵ , in the absence of IPC, is sufficient to activate neuroprotection, whereas, inhibition of PKC ϵ blunts IPC mediated neuroprotection against cerebral ischemia [8-10].

Sirtuin 1 (SIRT1) is a member of the sirtuin family of NAD⁺ dependent deacetylases which is implicated as a metabolic

sensor of the cell [11-13]. SIRT1 is neuroprotective in numerous models of neurodegenerative diseases including ischemia/reperfusion [14-16]. In brain derived endothelial cells, SIRT1 protects against oxygen and glucose deprivation induced cell death [17]. SIRT1 is primarily localized to the nucleus where it regulates gene transcription through deacetylation of histone and non-histone proteins [18]. It has been demonstrated in both the brain and heart that IPC activates SIRT1 leading to ischemic tolerance. We have previously demonstrated that IPC activates nuclear SIRT1 deacetylase activity and SIRT1 mediated neuroprotection against cerebral ischemia [19,20].

Although SIRT1s function has been primarily characterized in the nucleus, a recent study has demonstrated that SIRT1 can also localize to the mitochondria. Aquilano et al. [21] demonstrated that in the mouse brain, liver and muscle SIRT1 was localized to the mitochondrial matrix where it interacted with mitochondrial DNA and the transcription factor TFAM. The

association of SIRT1 with mitochondrial DNA and transcription factors suggests a transcriptional regulatory role of SIRT1 in the mitochondria; similar to its described nuclear activities. However, it is currently not known if mitochondrial SIRT1 protein levels are altered during periods of stress which might have a direct effect on mitochondrial function. Since SIRT1 can localize to mitochondria, and IPC is known to protect brain mitochondria, we hypothesized that IPC increases mitochondrial SIRT1 protein levels. Therefore the objective of this study was to determine the effects of IPC on mitochondrial SIRT1 protein levels and the role of PKC ϵ and heat shock protein 90 (HSP90) in targeting SIRT1 to the mitochondria.

Materials and Methods

Ethics statement

All animal protocols were approved by the Animal Care and Use Committee of the University of Miami (assurance number: A-3224-01). All experiments were conducted in accordance to ARRIVE guidelines. Male (250-300g) and 16-17 day-pregnant Sprague-Dawley rats were purchased from Charles Rivers Laboratories and housed in a temperature controlled environment with 12 hr light -12 h dark cycle and *ad libitum* food and water.

Materials

Minimum Essential Medium (MEM), Hanks Balanced Salt Solution (HBSS) and Fetal Bovine Serum (FBS) were purchased from Gibco/Life Technologies (Grand Island, NY). The HSP90 inhibitor 17-AAG was purchased from TOCRIS Bioscience (Bristol, UK). The PKC ϵ activator, ψ RACK, and its inhibitor, ϵ V1-2 peptides, and TAT control peptide were purchased from KAI Pharmaceuticals Inc (San Francisco, CA). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Animal model of global cerebral ischemia

Global cerebral ischemia was induced in male Sprague Dawley rats by bilateral carotid occlusion with systemic hypotension as previously described [22]. In brief, rats were anesthetized with isoflurane and 70% nitrous oxide (with a balance of oxygen) and both common carotid arteries exposed and dissected free from the surrounding tissue. A ligature consisting of polyethylene (PE-10) tubing encased in SILASTIC tubing was placed around each artery. Sublethal ischemic stress (IPC) was induced by gradually withdrawing blood from the femoral vein into a heparinized syringe until systemic blood pressure reached 50 mmHg, followed by tightening of the carotid ligatures bilaterally for 2 min. Cerebral ischemia was terminated by removal of the carotid ligatures and replacement of the shed blood into the femoral vein thereby restoring the mean arterial blood pressure to pre-ischemic levels. Brain and body temperature were maintained at 37°C throughout the experiment. Sham animals underwent the same treatment as described above but without IPC induction. Animals were treated with buprenorphine for pain management. Hippocampal synaptic and non-synaptic mitochondria were isolated after 2 or

48 hrs of reperfusion as previously described [23]. In brief, hippocampi were homogenized in 250 mM sucrose, 1 mM BSA, 0.25 mM DTT and 1 mM EDTA, pH 7.4 and the resulting homogenate centrifuged at 500 x g for 5 min to remove the nuclear fraction. The resulting supernatant was layered onto a Percoll gradient and the synaptosomes and non-synaptic mitochondria isolated by centrifugation at 32,500 x g for 5 min. The synaptosomal layer and non-synaptic mitochondria were removed from the Percoll gradient, washed with homogenization buffer and resuspended in 0.25 M sucrose. Synaptosomal mitochondria were isolated by nitrogen cell bomb (1,200 psi for 7.5 min) rupturing of the synaptosomes. Synaptic mitochondria were pelleted at 20,000 x g for 20 min at 4°C.

Preparation of cortical neuronal and glial cell cultures

Primary cortical glial and neuronal only cultures were prepared as previously described [10]. In brief, neuronal only cultures were prepared from the cortices of E18-19 day pups. Embryos were harvested by caesarian section and the cortices rapidly removed and placed in ice-cold HBSS. The tissue was enzymatically digested for 15 min at 37°C. The resulting cellular suspension was filtered thru a 70 μ m cell strainer and plated at a density of 3.5 hemispheres per plate in MEM supplemented with 5% FBS, 5 mM GlutaMax (Invitrogen/Life Technologies, Grand Island, NY) and 15 mM glucose. Glial only cultures were prepared from the cortices of 1-2 day old pups. Glial cells were isolated as described above for neuronal only cultures and plated in the same media. The cells were used for experimentation after 14 days in culture.

Oxygen and glucose deprivation preconditioning

To mimic sublethal cerebral ischemia, cells were exposed to oxygen and glucose deprivation (OGD) for 45 min [10]. To induce OGD, cells were washed two times with glucose-free HBSS (CaCl₂ 1.26 mM, KCl 5.37 mM, KH₂PO₄ 0.44 mM, MgCl₂ 0.49 mM, MgSO₄·7H₂O 0.41 mM, NaCl 136.9 mM, NaHCO₃ 4.17 mM, Na₂HPO₄·7H₂O 0.34 mM, sucrose 15 mM, pH 7.4) and exposed to an oxygen-free environment (90% nitrogen, 5% hydrogen, and 5% CO₂, 37°C) using a COY anaerobic chamber (COY Laboratory Products Inc, Lake Charter Township, MI). OGD was terminated by placing the cells back into normal aerobic media.

Subcellular fractionation

Subcellular fractionation was performed as previously described [24-26]. For mitochondrial isolation, cells were suspended in 10 mM Tris, pH 7.4, and 320 mM sucrose, 1 mM EDTA, 1 mM DTT with protease and phosphatase inhibitors and homogenized using a teflon-glass homogenizer. The resulting homogenate was centrifuged at 1,500 x g for 5 min at 4°C. The supernatant was further centrifuged at 10,000 x g for 20 min at 4°C to pellet mitochondria. Mitochondrial pellets were washed three times with isolation buffer and suspended in RIPA lysis buffer for Western blot analysis or in isolation buffer for mitoplast generation and stored at -80°C. Mitoplasts were prepared by incubating mitochondria in 5 vol of hypotonic buffer (10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM DTT) for 1

hr on ice followed by incubation with 150 mM KCl for an additional 2 min. The mitoplasts were separated from postmitoplast supernatants by centrifugation at 20,000 x g for 20 min at 4°C. For alkali extraction, mitochondria were incubated for 30 min on ice with freshly prepared 0.1 M sodium carbonate (pH ~ 11.5). The samples were centrifuged at 40,000 x g for 1 hr at 4°C and the resulting pellet resuspended in sample buffer. To isolate the cytoplasmic fraction, cells were suspended in 10 mM Tris, (pH 7.4), 320 mM sucrose, 1 mM EDTA, 1 mM DTT with protease and phosphatase inhibitors and homogenized using a teflon-glass homogenizer. The cytoplasmic fraction was isolated from the nuclear and mitochondrial fractions by centrifugation at 10,000 g for 20 min to pellet nuclei and mitochondria. To isolate the nuclear fraction, cells were resuspended in buffer consisting of 20 mM Tris (pH 8.0), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 1 mM DTT for 10 min on ice. Cells were lysed with 1% Nonidet P-40, and vortexed vigorously for 10 secs followed by centrifugation at 10,000 x g. for 10 min [10,27]. The resulting pellet consisting of enriched nuclei was lysed with RIPA buffer.

Western blotting

Western blots were performed as previously described [10,27]. Cells and mitochondria were lysed in RIPA Buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄ and 1 mM PMSF). Protein concentration was determined by BCA protein assay and 40 µg of protein was loaded onto an 8 or 12% SDS-polyacrylamide gel and electroblotted to nitrocellulose. Membranes were blocked in 5% dry milk/TBST and hybridized with primary antibodies overnight at 4°C. Blots were probed with PKCε (Santa Cruz Biotechnology, Dallas, TX), SIRT1 (Santa Cruz Biotechnology), HSP60 (Cell Signaling Technology, Danvers, MA), acetylated lysine (Cell Signaling Technology), β-actin (Sigma), lamin-B (Cell Signaling Technology), cytochrome C (BD Pharmingen, San Jose, CA) and COXIV (InVitrogen/Life Technologies). Membranes were washed with TBST followed by incubation with secondary antibodies (Pierce, Thermo Scientific; Rockford, IL) for 1 hr at room temperature. Proteins were detected using enhanced chemiluminescence (ECL) system (Pierce, Thermo Scientific). Western blot densitometry was analyzed using ImageJ software from NIH.

SIRT1 activity assay

Mitochondrial SIRT1 activity was determined using a kit from Cayman Chemicals (Ann Arbor, MI) as previously described [28]. In brief, mitochondria were lysed in IP lysis buffer consisting of 1% Nonidet P-40, 20 mM Tris (pH 8.0) 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and protease and phosphatase inhibitors (Roche) and the lysate (500 µg) incubated with 2 µg of immunoprecipitating SIRT1 antibody (Santa Cruz Biotechnology) overnight at 4°C followed by precipitation with protein G Sepharose beads (Sigma) for 2 hrs at 4°C. The pellet was washed 4 x with IP lysis buffer followed by incubation with SIRT1 activity reaction mixture for 1 hr at room temperature with shaking. SIRT1

deacetylase activity was determined as per the manufacturer's instructions.

Mitochondrial respiration

Mitochondrial respiration studies were conducted as previously described [23]. In brief, cortical synaptic and non-synaptic mitochondria were isolated from either naïve rats or from rats treated with the PKCε activator peptide (ψεRACK; 0.2 mg/kg i.p.) [29,30] or the TAT carrier peptide. The isolated mitochondria were treated with the general sirtuin inhibitor, sirtinol (1 µM), the specific SIRT1 inhibitor, EX527 (10 µM [31]), or DMSO for 30 min on ice. The rate of mitochondrial oxygen consumption was determined using a Clark-type oxygen electrode in the presence of 0.5 mM ADP and the following substrates: 1) pyruvate and malate (induces mitochondrial respiration at complex I); 2) succinate and glycerol-3-phosphate (induces mitochondrial respiration at complex II); and 3) ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) (induces mitochondrial respiration at complex IV).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis between two groups was performed using the unpaired Student's *t*-test. Statistical analysis between more than two groups was performed using a one-way ANOVA with Dunnett's multiple comparison post hoc test. *P* < 0.05 was considered statistically significant.

Results

Ischemic preconditioning increases mitochondrial SIRT1 protein levels

To determine if IPC alters mitochondrial levels of SIRT1, we exposed rats to 2 min of global cerebral ischemia with systemic hypotension to induce IPC. SIRT1 protein levels were determined in hippocampal synaptic and non-synaptic mitochondria 2 and 48 hrs later. As demonstrated in Figure 1A and B, mitochondrial SIRT1 protein levels were unchanged 2 hrs following IPC exposure. However, at 48 hrs of reperfusion there was a significant increase (*p* < 0.05) in non-synaptic mitochondrial SIRT1 protein levels. In contrast, there was no change in synaptic mitochondrial SIRT1 levels at any of the time points examined. Mitochondrial purity was confirmed using the nuclear marker lamin-B (Figure 1C). The delayed increase in mitochondrial SIRT1 protein levels exhibited a temporal profile similar to that previously demonstrated in nuclear SIRT1 activity increase following IPC [19,20].

Next, we determined whether changes in mitochondrial SIRT1 protein levels were neuronal-specific or whether it occurred in all brain mitochondria. To answer this question, we generated primary cortical neuronal-only or glial-only cultures and examined mitochondrial SIRT1 protein levels after exposure to *in vitro* IPC, as described in methods. Similar to our results *in vivo* in the hippocampus, mitochondrial SIRT1 protein levels were significantly (*p* < 0.05) increased at 48 hrs but not at 2 hrs following IPC exposure in neuronal-only

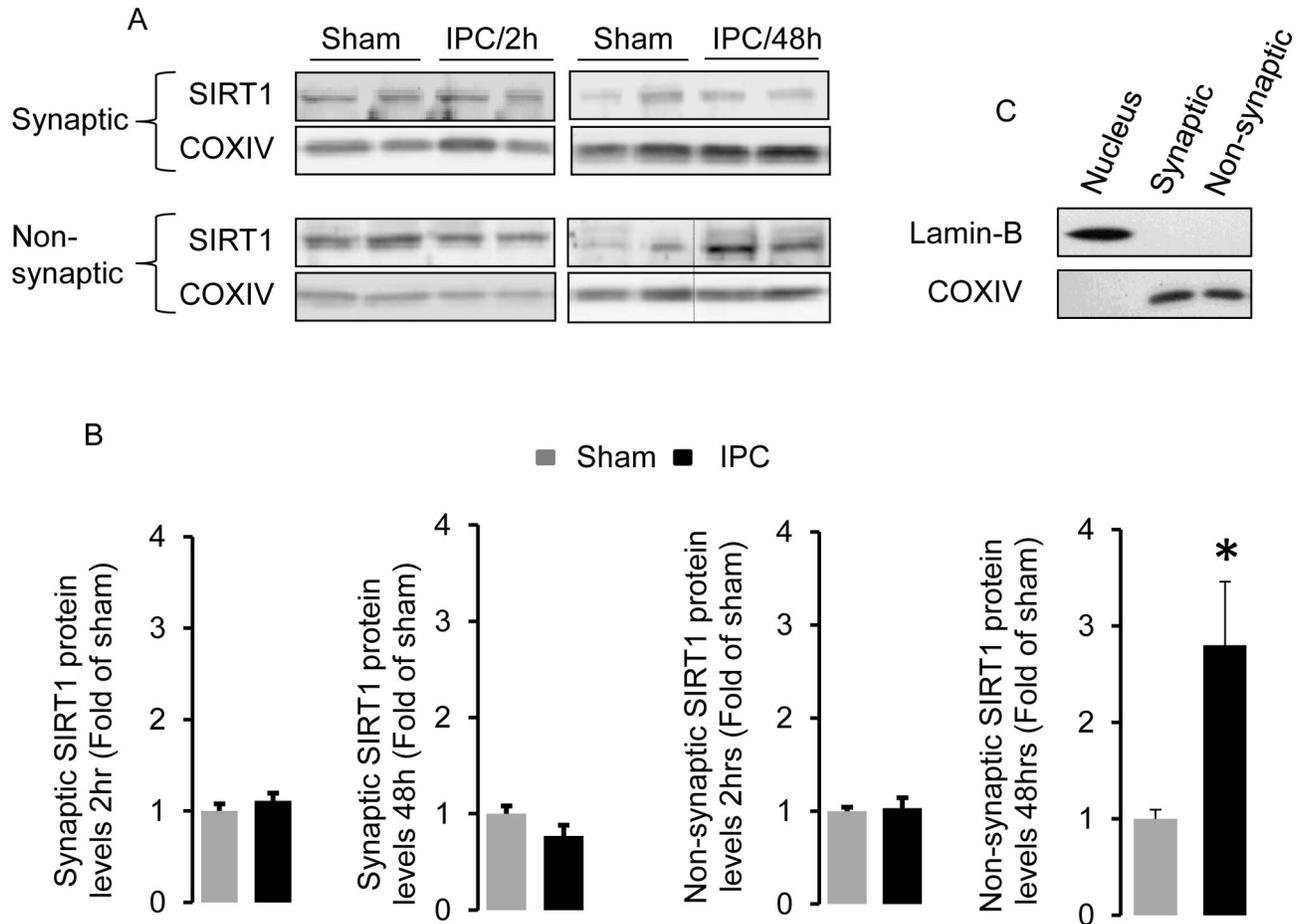


Figure 1. Mitochondrial SIRT1 protein levels increase in the hippocampus following IPC. In (A) a representative Western blot showing SIRT1 protein levels in hippocampal synaptic and non-synaptic mitochondria at 2 and 48 hrs following 2 min of global cerebral ischemia (IPC) from two different animals. Western blot quantitation of synaptic and non-synaptic mitochondrial SIRT1 protein levels is shown in (B) ($n = 8$). In (C) mitochondrial purity from nuclear contamination was determined by western blot analysis using antibodies to the nuclear localized protein Lamin-B and to the mitochondrial localized protein COXIV. Data are means \pm SEM compared to sham treated animals. * $p < 0.05$ increase from sham by Student's t -test.

doi: 10.1371/journal.pone.0075753.g001

cultures (Figure 2A and B). There was no change in mitochondrial SIRT1 protein levels in glial-only cultures at any of the time points examined. The increase in neuronal-mitochondrial SIRT1 protein levels correlated with an increase ($p < 0.05$) in mitochondrial SIRT1-specific deacetylase activity (Figure 2C) and with a reduction in acetylation of a 50 kDa mitochondrial protein (Figure 2D).

Next we determined if the increase in mitochondrial SIRT1 is the result of increased SIRT1 expression or the translocation of SIRT1 to the mitochondria from the nuclear or cytoplasmic compartments. Using subcellular fractionation we observed a significant ($p < 0.05$) reduction in nuclear SIRT1 protein levels 48 hrs following IPC exposure (Figure 3A and B). SIRT1 was not observed in the cytoplasm at any of the time points examined (Figure 3C). IPC exposure was also found to significantly ($p < 0.05$) increase total cellular SIRT1 protein levels in neuronal but not glial only-cultures (Figure 3D and E).

These results indicate that SIRT1 is specifically targeted to neuronal mitochondria when SIRT1 is activated by brief ischemia/reperfusion.

Epsilon PKC regulates mitochondrial SIRT1 protein levels

Next, we were interested in identifying the mechanism by which SIRT1 is targeted to the mitochondria. Our laboratory and others have demonstrated that a novel type protein kinase C, PKC ϵ , is both required and sufficient for IPC-induced ischemic protection [8,10,24,32]. We also demonstrated that PKC ϵ translocates to mitochondria following IPC and promoted mitochondrial protection against cerebral ischemia [9]. In the ischemia/reperfused heart, mitochondrial PKC ϵ protein levels are increased, and required for cardioprotection [24]. Therefore, we tested the hypothesis that PKC ϵ mediates the

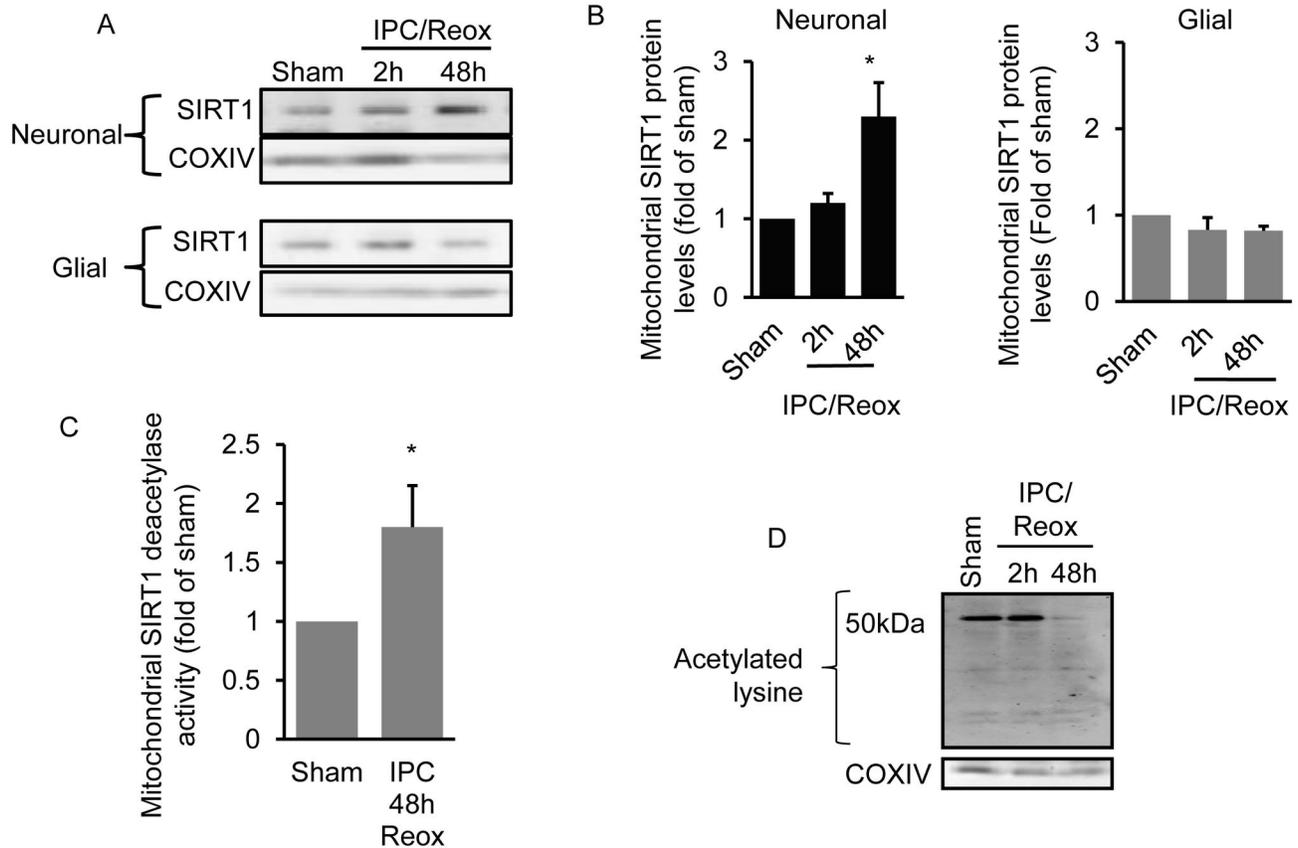


Figure 2. IPC increases mitochondrial SIRT1 protein levels specifically in neurons. Primary cortical glial and neuronal-only cultures were exposed to oxygen and glucose deprivation for 45 min (IPC) and the level of mitochondrial SIRT1 was determined 2 and 48 hrs later ($n = 6$) (A). Western blot quantitation is shown in (B). SIRT1-specific deacetylase activity was determined in mitochondrial lysates from neuronal cultures 48 hrs following exposure to sham or IPC ($n = 4$) (C). Neuronal mitochondria acetylation levels were determined at 2 and 48 hrs following IPC exposure ($n = 3$) (D). * $p < 0.05$ increase from sham by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison post hoc test.

doi: 10.1371/journal.pone.0075753.g002

increases in mitochondrial SIRT1 protein levels following IPC. To test this hypothesis, we activated PKC ϵ in cortical neuronal cultures with ψ RACK (100 nM), a specific PKC ϵ activator [33], and observed a significant ($p < 0.05$) increase in both mitochondrial SIRT1 protein levels and SIRT1 deacetylase activity 48 hrs later (Figure 4A-C). To confirm that PKC ϵ is required for increasing mitochondrial SIRT1 protein levels following IPC, we exposed neuronal cultures to the PKC ϵ inhibitor (ϵ V1-2, 100 nM [34]) or control TAT peptide during and following IPC exposure. As demonstrated in Figure 4 (D-F), 48 hours following IPC exposure, the increase in mitochondrial SIRT1 protein levels and SIRT1 deacetylase activity was significantly ($p < 0.05$) reduced by PKC ϵ inhibition. These results confirm our hypothesis that PKC ϵ increases the level and activity of mitochondrial SIRT1 in neurons.

Heat shock protein 90 is required for mitochondrial import of SIRT1

SIRT1 was shown to contain both nuclear import and export signal sequences [35], but not a mitochondrial targeting sequence. Therefore, we were interested in determining how SIRT1 was targeted into the mitochondria. In the heart, PKC ϵ which lacks a mitochondrial targeting sequence, requires the activity of the stress chaperone protein, heat shock protein 90 (HSP90), for mitochondrial import [24]. Therefore, we hypothesized that mitochondrial import of SIRT1 requires HSP90. To test this hypothesis, we treated neuronal cultures with the HSP90 inhibitor 17-AAG (100 nM) [36], following IPC exposure. As illustrated in Figure 5 (A-C), HSP90 inhibition significantly ($p < 0.05$) reduced mitochondrial SIRT1 and PKC ϵ protein levels 48 hrs following IPC exposure. 17-AAG treatment had no effect on total cellular SIRT1 or PKC ϵ protein levels (Figure 5D), indicating the reduction in mitochondrial SIRT1 and PKC ϵ was not the result of altered expression or

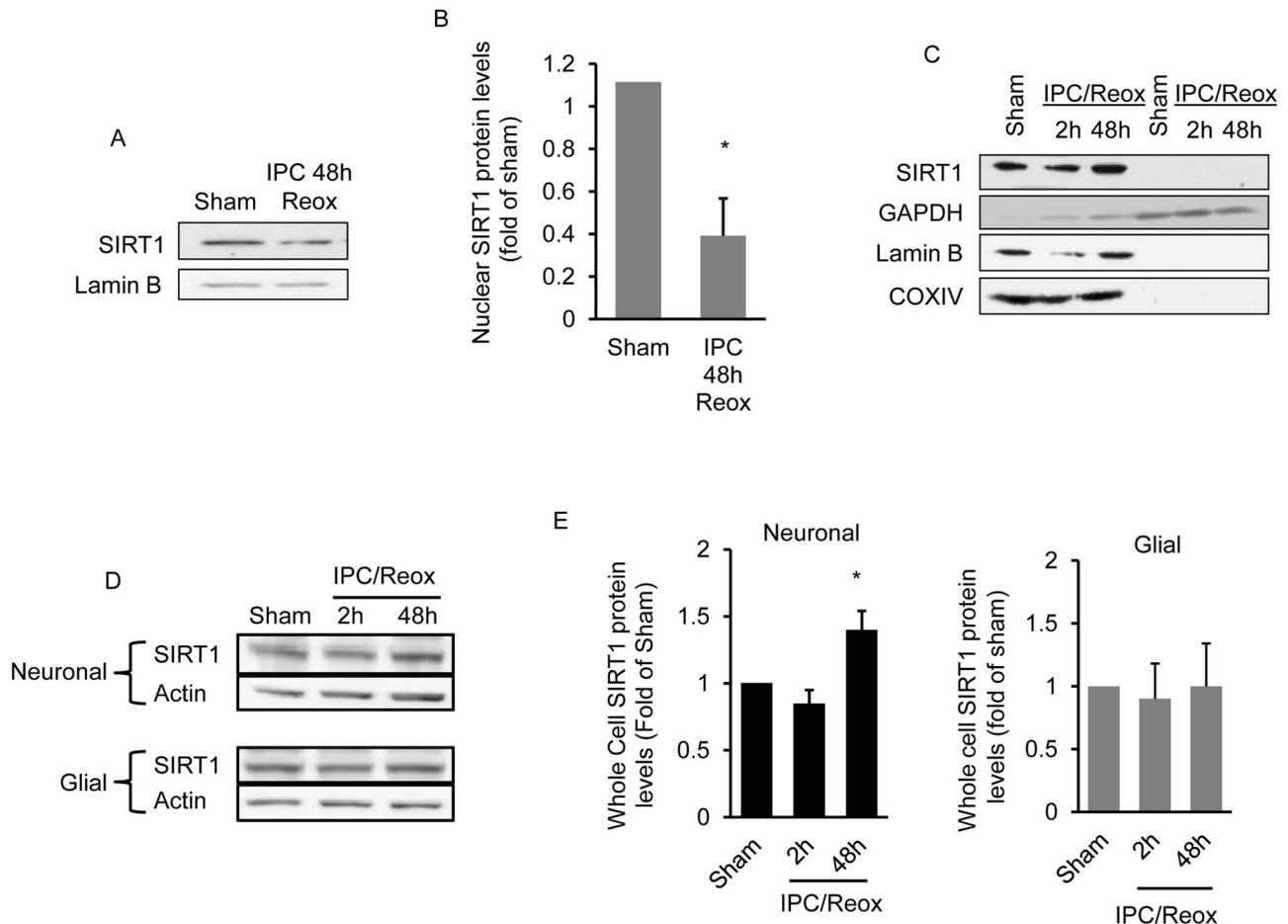


Figure 3. IPC increases SIRT1 expression and decreases nuclear SIRT1 protein levels. Cortical neuronal cultures were exposed to oxygen and glucose deprivation for 45 min (IPC) and nuclear SIRT1 protein levels determined 48 hrs later ($n = 5$) (A). Western blot quantitation of nuclear SIRT1 protein levels is shown in (B). The effect of IPC on cytoplasmic SIRT1 protein levels is shown in (C). The soluble cytoplasmic fraction was isolated from the nuclear and mitochondrial fractions as described in methods. The purity of the cytoplasmic fraction was determined by reprobating the blot for the cytoplasmic marker, GAPDH, with the nuclear marker lamin B and with the mitochondrial marker COXIV. In (D) whole cell SIRT1 protein levels in cortical glial and neuronal only cultures are shown at 2 and 48 hrs following IPC exposure ($n=8$). Western blot quantitation is shown in (E). Data are means \pm SEM compared to sham treated animals. * $p < 0.05$ increase from sham by one-way analysis of variance (ANOVA) and Dunnetts' multiple comparison post hoc test.

doi: 10.1371/journal.pone.0075753.g003

degradation of either protein, but rather inhibition of transport of both SIRT1 and PKC ϵ into the mitochondria.

SIRT1 and PKC ϵ localize within mitoplasts

To determine the submitochondrial localization of SIRT1, we exposed mitochondria to alkaline extraction which removes soluble and peripheral but not integral membrane proteins. SIRT1 from both sham and IPC treated cultures was found to primarily localize with cytochrome c and HSP60 in the supernatant following alkaline treatment (Figure 6A and B). A small portion of mitochondrial SIRT1 was also observed in the insoluble fraction. Similarly, PKC ϵ was found primarily but not exclusively in the supernatant (Figure 6A and C). Exposure to

IPC did not significantly alter the levels of SIRT1 or PKC ϵ found in the soluble or insoluble fraction when compared to sham control. These results suggest that the majority of SIRT1 and PKC ϵ are not strongly bound to mitochondrial membrane. Next, we used hypoosmotic lysis of the outer mitochondrial membrane to generate mitoplasts which consists of an intact mitochondrial inner membrane and matrix. After mitoplast generation the intermembrane space protein, cytochrome c, was found in the postmitoplast supernatant whereas SIRT1 was found to almost exclusively localize with the matrix protein, HSP60, in the mitoplast (Figure 6D and E). Exposing cultures to IPC did not significantly alter the submitochondrial localization of SIRT1 in the matrix or intermembrane space

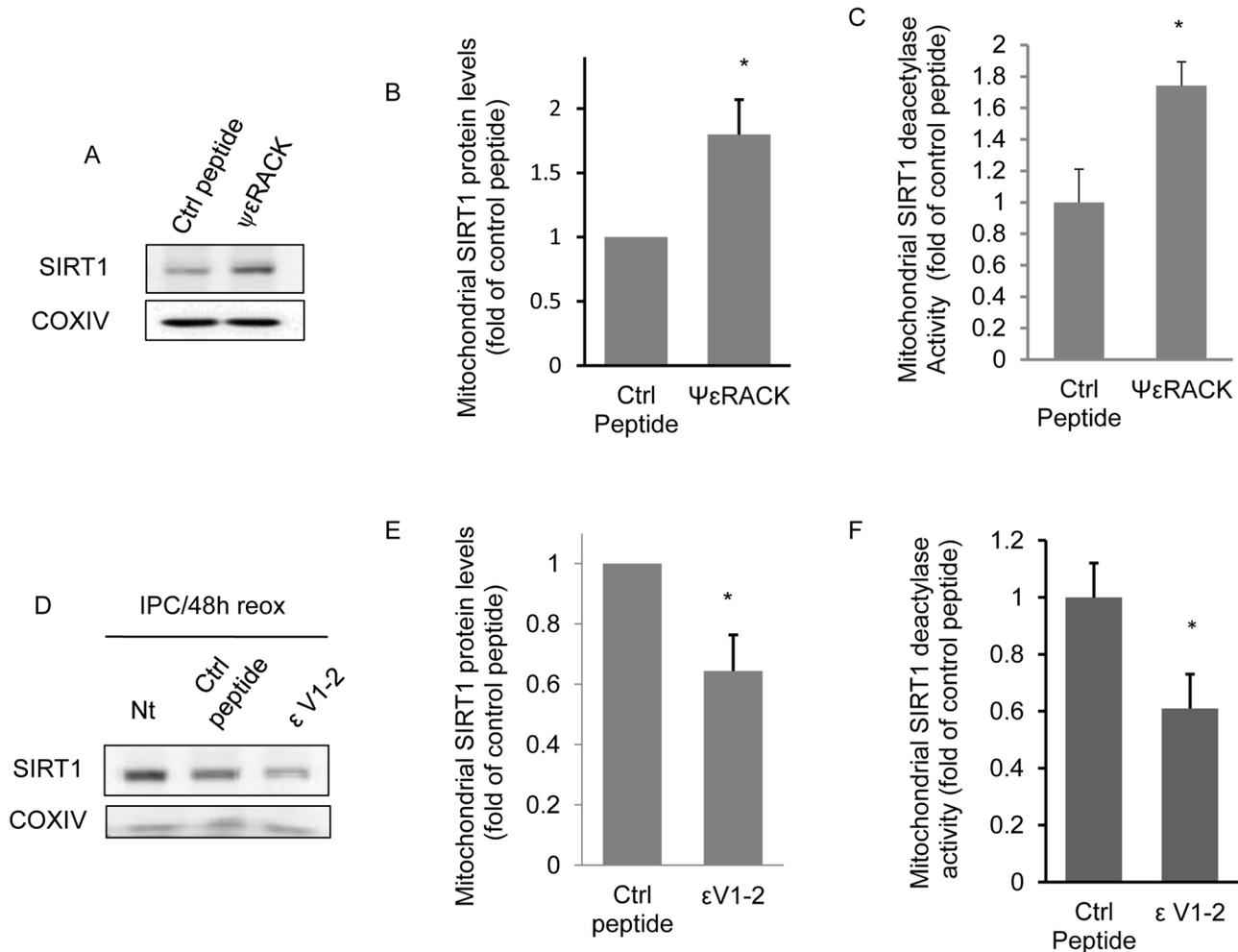


Figure 4. Epsilon PKC regulates mitochondrial SIRT1 protein levels. Neuronal cultures were treated with the PKC ϵ activator $\Psi\epsilon$ RACK (100 nM) or with the carrier TAT control peptide (100 nM) for 1 hr and mitochondrial SIRT1 protein levels (A and B) and deacetylase activity (C) determined 48 hrs later (n = 4). In (D) PKC ϵ activation was inhibited with the PKC ϵ specific inhibitor ϵ V1-2 (100 nM) during and following 45 min of oxygen and glucose deprivation (IPC) and the level of mitochondrial SIRT1 determined 48 hrs later (n = 3) in non-treated (Nt), control peptide and ϵ V1-2 treated samples. Western blot quantitation is shown in (E). The effect of ϵ V1-2 (100 nM) on IPC induced mitochondrial SIRT1 deacetylase activity is shown in (F) (n = 4). Data are means \pm SEM compared to control (Ctrl) TAT peptide treated cultures. * $p < 0.05$ increase from controls by Student's t -test.

doi: 10.1371/journal.pone.0075753.g004

compared to sham treated cultures. Similarly, PKC ϵ was found predominantly in the mitoplast fraction with a small portion of PKC ϵ protein observed in the postmitoplast supernatant (Figure 6D and F). The submitochondrial localization of PKC ϵ following IPC exposure was not significantly different than sham treated cultures. The finding of PKC ϵ in the intermembrane space is consistent with data from Costa et al. [37] which proposes that mitochondrial PKC ϵ exist in two populations, one in the intermembrane space and the other in the matrix. Both populations of PKC ϵ coordinate in the opening of the ATP-sensitive K $^+$ channels (mitoK $_{ATP}$) and mitochondrial neuroprotection.

Mitochondria respiration is not regulated by mitochondrial SIRT1 deacetylase activity

Recent research has demonstrated that numerous mitochondrial proteins associated with energy metabolism are acetylated [38] [39]. In the heart, caloric restriction, a known activator of the sirtuin family, primes mitochondria for ischemic stress by deacetylating specific proteins of the electron transport chain [40]. Since our results demonstrate that SIRT1 localizes within mitoplasts, which would place it in a position to interact with proteins of the electron transport chain, we hypothesized that mitochondrial SIRT1 may regulate mitochondrial respiration following preconditioning. As a first step to test this hypothesis we isolated cortical synaptic and

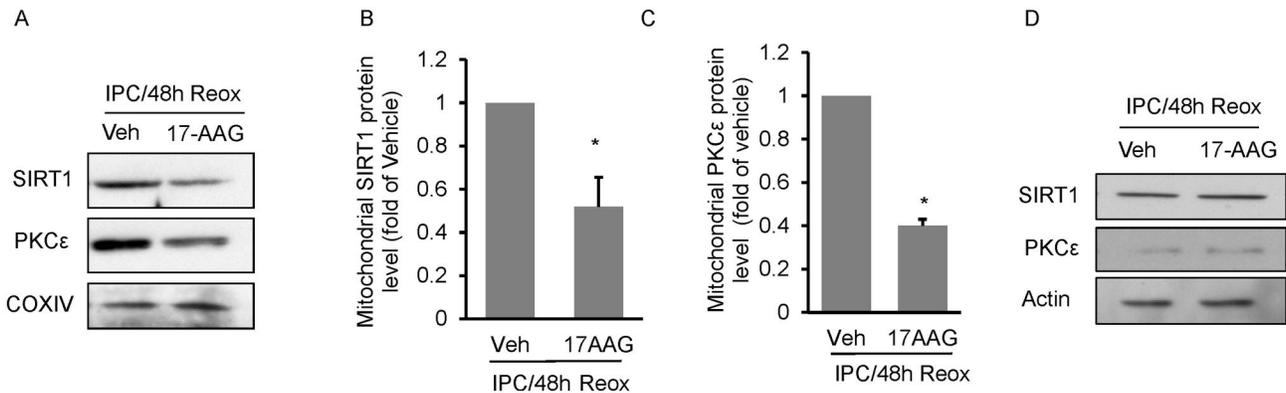


Figure 5. HSP90 is required for SIRT1 mitochondrial import. Mitochondrial SIRT1 and PKC ϵ protein levels were determined, 48 hrs following IPC exposure, in neuronal cultures treated with the HSP90 inhibitor, 17-AAG (100 nM) (A). Western blot quantitation is shown in (B and C; $n = 3$). The effects of 17-AAG treatment on whole cell SIRT1 and PKC ϵ are shown in (D). Data are means \pm SEM compared to vehicle (Veh) treated cultures. * $p < 0.05$ increase from controls by Student's t -test.

doi: 10.1371/journal.pone.0075753.g005

non-synaptic mitochondria from naive rats and treated the isolated mitochondria with the sirtuin deacetylase inhibitor, sirtinol (1 μ M). We chose to treat isolated mitochondria with sirtuin inhibitors so as to avoid any indirect effects of nuclear SIRT1 inhibition on mitochondrial function. As illustrated in Figure 7A and B, although not significant, sirtinol treatment was observed to reduce the average non-synaptic mitochondrial respiration 40%, 19%, and 52% in the presence of pyruvate plus malate, succinate plus glycerol-3-phosphate and ascorbate plus TMPD, respectively, when compared to vehicle controls. The effect of sirtinol treatment on synaptic mitochondrial respiration was less pronounced as that observed in non-synaptic mitochondria; with a reduction in mitochondrial respiration of 10% and 5% in the presence of pyruvate plus malate and succinate plus glycerol-3-phosphate, respectively and with a significant ($p = 0.019$) 22% reduction in mitochondrial respiration in the presence of ascorbate plus TMPD, when compare to vehicle controls. This data indicates a trend for reduced respiration in non-synaptic mitochondria in the presence of a sirtuin inhibitor. Therefore we hypothesized that the increase in non-synaptic mitochondrial SIRT1 protein levels observed following IPC or PKC ϵ activation may have a greater and thus significant effect in regulating non-synaptic mitochondrial respiration during conditions of ischemic tolerance. To test this hypothesis we pretreated rats with the PKC ϵ activator, ψ ϵ RACK, which induces a preconditioning response, or control peptide [10] [8], and isolated non-synaptic mitochondria 48 hrs later. SIRT1 specific deacetylase activity was inhibited in the isolated mitochondria by treatment with EX527 (10 μ M) and the rate of mitochondrial respiration determined. As demonstrated in Figure 7C-E, EX527 treatment reduced mitochondrial respiration to similar levels in both control peptide and PKC ϵ treated animals when compared to vehicle controls. However the reduction in mitochondrial respiration in the presence of EX527 was not significantly different in any of the conditions examined.

Discussion

In this study, we demonstrate a novel regulatory mechanism in targeting SIRT1 to the mitochondria which we believe represents an expansion of the mechanisms and targets by which SIRT1 mediates neuroprotection. We also demonstrate that changes in mitochondrial SIRT1 protein levels were dependent upon PKC ϵ activation, suggesting a novel interaction between the PKC ϵ and SIRT1 signaling pathways.

Mitochondrial increases in SIRT1 protein levels following IPC were only observed during the late phase or delayed window of neuroprotection. The increase in mitochondrial SIRT1 protein is also correlated with our previously reported findings of a late phase but not early increase in nuclear SIRT1 activity [19,20]. Activation of SIRT1 during IPC is known to be protective against ischemia in both the heart and brain [19,20,41-43]. In our laboratory pharmacological inhibition of SIRT1, which would presumably antagonize both nuclear and mitochondrial SIRT1 activity, blocked IPC induced ischemic tolerance [19,20].

Although a basal level of SIRT1 protein was observed in all of the mitochondrial preparations investigated, we found that IPC only increased mitochondrial SIRT1 protein levels in non-synaptic mitochondria and in mitochondria from neuronal-only cultures. These results suggest that IPC alters SIRT1 levels primarily in mitochondria localized to neuronal cell bodies. This specific targeting of SIRT1 to a subpopulation of neuronal mitochondria may be explained by our findings that HSP90 is required for mitochondrial import of SIRT1. In the adult rat brain, HSP90 is reported to primarily localize to the neuronal cell body [44]. Gass et al. [45] showed that HSP90 immunoreactivity was found in all neuronal populations of the hippocampus and neocortex, and that it was found to be primarily perikarya in location and was found to be absent from glia and from synaptic sites. These findings on HSP90 localization correlate well with our findings in mitochondrial SIRT1 protein levels following IPC.

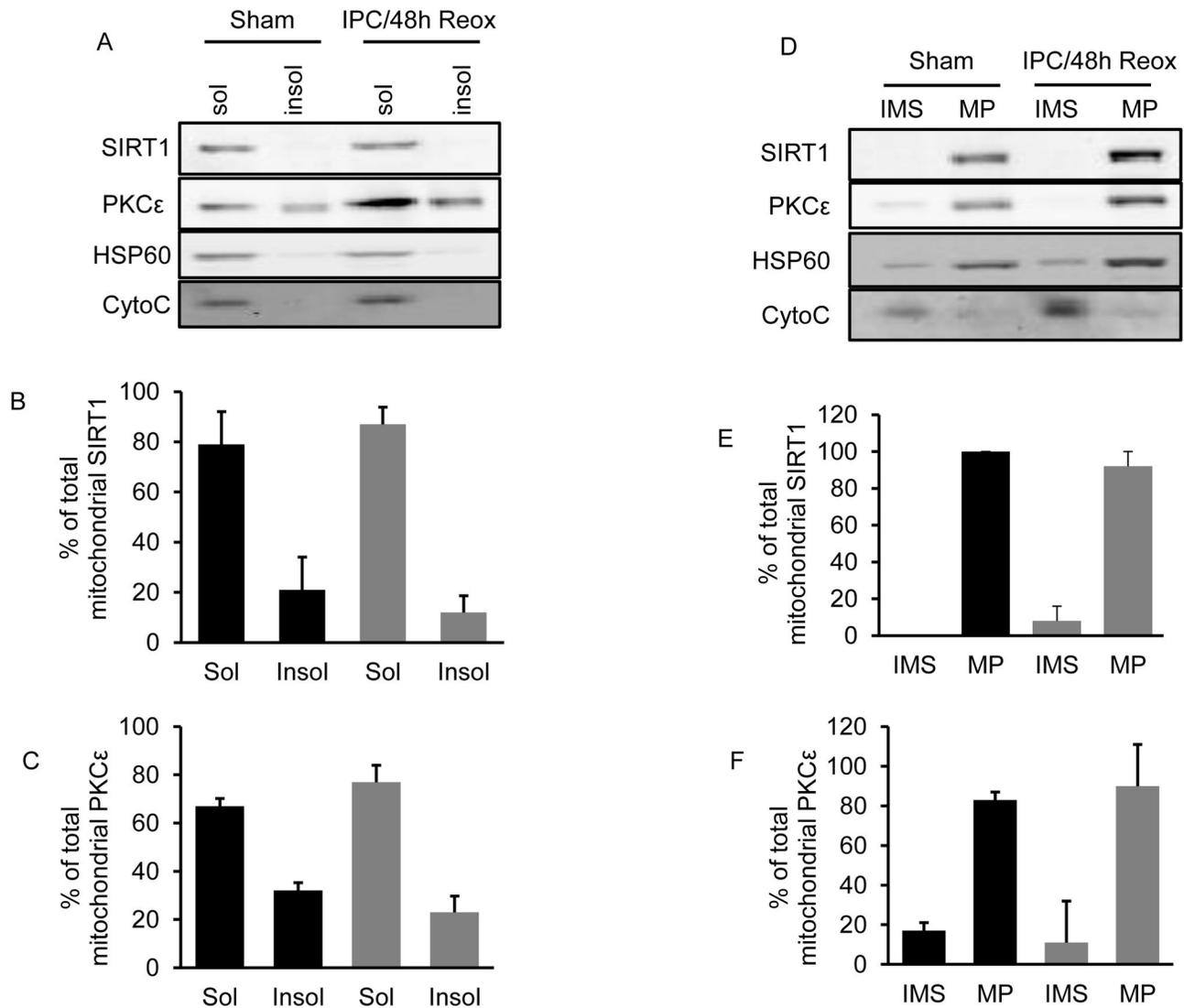


Figure 6. SIRT1 localizes to the mitoplast fraction. Mitochondria from sham or IPC-treated neuronal cultures were exposed to alkaline extraction and the localization of SIRT1, PKC ϵ , cytochrome c (an intermembrane space marker), and HSP60 (a matrix marker) was determined by Western blot analysis (A). Western blot quantitation of SIRT1 and PKC ϵ levels in the soluble (sol) and insoluble (Insol) fractions are shown in (B and C) ($n = 3$). In (D), SIRT1 and PKC ϵ localizations were determined in mitoplasts (MP) and intermembrane space (IMS) fractions generated by hypoosmotic lysis of the outer mitochondrial membrane. Western blot quantitation of SIRT1 and PKC ϵ levels in MP and IMS fractions are shown in (E and F) ($n = 3$). No significant difference ($p > 0.05$) was observed in the localization of SIRT1 or PKC ϵ following IPC exposure when compared to sham controls. Data are means \pm SEM.

doi: 10.1371/journal.pone.0075753.g006

We also demonstrate that the total cellular SIRT1 protein levels were increased following IPC exposure in neuronal but not glial only-cultures which correlates with the observed increases in neuronal but not glial mitochondrial SIRT1 protein levels. We also observed a reduction in nuclear SIRT1 protein levels 48 hrs following IPC exposure. These findings suggest that the increase in mitochondrial SIRT1 protein levels may be the result of both increased SIRT1 expression, as well as, nuclear to mitochondrial shuttling of SIRT1. Our data also

demonstrate that SIRT1 is targeted to mitochondria by mechanisms mediated by PKC ϵ . This is based upon our findings that activation of PKC ϵ , in the absence of IPC, increases mitochondrial SIRT1 protein levels and deacetylase activity, whereas inhibition of PKC ϵ prevents increases in mitochondrial SIRT1 protein levels and deacetylase activity following IPC exposure. The subcellular localization of SIRT1 is known to be regulated by posttranslational phosphorylation and sumoylation [35,46,47]. In the heart, desumoylation of SIRT1

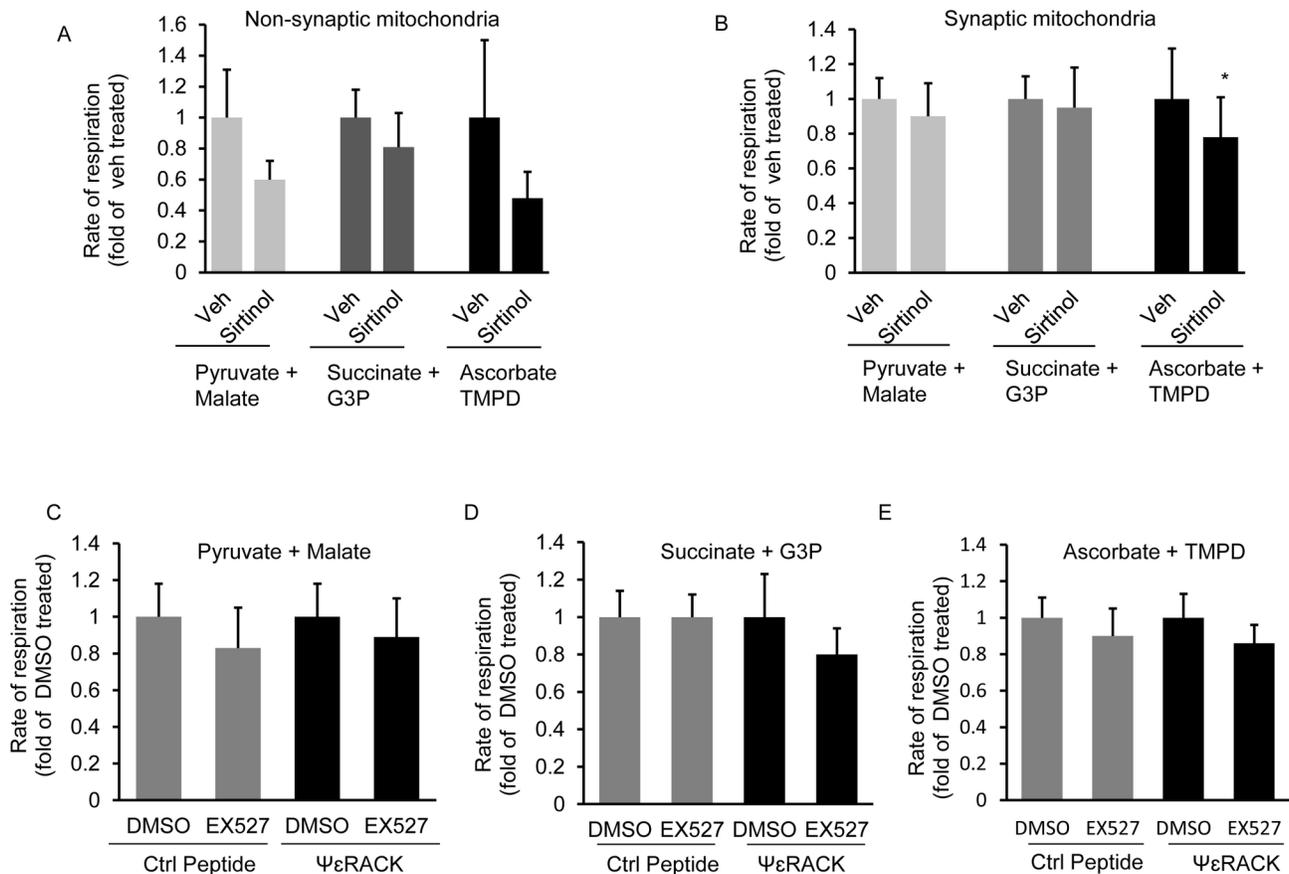


Figure 7. Inhibition of mitochondrial SIRT1 deacetylase activity does not alter mitochondrial respiration rate. Cortical non-synaptic (A) and synaptic (B) mitochondria were isolated from naive rats and treated with either DMSO (Veh) or the sirtuin inhibitor, sirtinol and the rate of mitochondrial substrate oxidation determined ($n = 5$). In C-E, rats were treated with $\psi\epsilon$ RACK, to induce PKC ϵ preconditioning, or control peptide (Ctrl) and the non-synaptic mitochondria isolated 48 hrs later. Non-synaptic mitochondria were treated with the specific SIRT1 inhibitor, EX527, or DMSO and the rate of mitochondrial substrate oxidation determined ($n = 5$). Data are means \pm SEM compared to veh treated groups. * $p = 0.019$ increase from vehicle controls by paired Student's t -test.

doi: 10.1371/journal.pone.0075753.g007

induces a nuclear-to-cytoplasmic shuttling of SIRT1, whereas phosphorylation of SIRT1 by phosphoinositide 3-hydroxykinase (PI3K)-AKT in C2C12 cells induces cytoplasmic-to-nuclear translocation of SIRT1 [46]. In the above mentioned studies, the neuroprotective function of SIRT1, against ischemia/reperfusion injury and oxidative stress, was only observed when SIRT1 was located in the nucleus but not in the cytoplasm. Similarly, in endothelial cells, cytoplasmic-to-nuclear shuttling of SIRT1 is required for protection against high glucose induced apoptosis [48]. Therefore, targeting of SIRT1 to the mitochondria by PKC ϵ may be required for IPC mediated neuroprotection. This hypothesis is supported by the fact that PKC ϵ activation, which increases mitochondrial SIRT1 protein levels, is critical for IPC neuroprotection against ischemic exposure [7,8,10]. The mechanism by which PKC ϵ regulates mitochondrial SIRT1 protein levels is unclear, but most likely involves either direct phosphorylation of SIRT1 by PKC ϵ or posttranslational modification of SIRT1 by a PKC ϵ

dependent signaling cascade. Future studies are required to better delineate the interactions of SIRT1 and PKC ϵ . The physiological role of SIRT1 in the mitochondria under both normal physiological conditions and under stress conditions remains undefined. In these studies, we found that acute inhibition of mitochondrial SIRT1 deacetylase activity had no significant effect on non-synaptic mitochondrial respiration, suggesting a possible role for SIRT1 in regulating mitochondrial gene expression. In the nucleus SIRT1 directly regulates the activity of peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α); a major transcriptional co-activator of genes involved in energy metabolism [49-51]. A similar association of SIRT1 and PGC-1 α was also observed in the mitochondria by Aquilano et al. [21]. Therefore, we reason that IPC-induced changes in both nuclear and mitochondrial SIRT1 activity may allow for a coordination of mitochondrial protein expression from both the nuclear and mitochondrial genomes. Further support for this hypothesis comes from Aquilano et al.

[21] studies that showed mitochondrial SIRT1 was associated with mitochondrial DNA and the transcription factor TFAM. Two important nuclear transcription factors, PGC-1 α and nuclear respiratory factor-1 (NRF-1) when upregulated, lead to transcription of TFAM, which plays a key role in mitochondrial DNA replication (reviewed in [52]). Thus, we propose that IPC-induced SIRT1 translocation to the nucleus and mitochondria serves as a signaling pathway that orchestrates upregulation of electron transport subunits that may be incorporated into preexisting mitochondrial network (reviewed in [52]). This fact may also explain why our results point to SIRT1 translocation to mitochondria occurs primarily in the somatic fraction of neurons and not the synaptosomal fraction.

In summary, it has been demonstrated in both the heart and brain that SIRT1 is an important mediator of IPC-induced ischemic tolerance. Based upon the data presented here, part

of SIRT1 protection may stem from the ability of IPC to regulate SIRT1 mitochondrial protein levels, which may allow for a direct regulation of mitochondrial function by SIRT1. Our discovery that SIRT1 is targeted to mitochondria by PKC ϵ following IPC may offer new therapeutic directions in the targeting of metabolic dysfunction, which is associated with the pathophysiology of numerous diseases such as ischemia/reperfusion.

Author Contributions

Conceived and designed the experiments: JWT, KR, MPP. Performed the experiments: JWT, KR, IS, SVN. Analyzed the data: JWT, KR. Wrote the manuscript: JWT, KR, MPP. Critical review of the article: SVN SI.

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