Published in final edited form as:

Neurosci Lett. 2008 August 15; 441(1): 120–124. doi:10.1016/j.neulet.2008.05.080.

εPKC confers acute tolerance to cerebral ischemic reperfusion injury

Rachel Bright, Ph.D.¹, Guo-Hua Sun, M.D.², Midori A. Yenari, M.D., Ph.D.⁴, Gary K. Steinberg, M.D. Ph.D.^{2,3}, and Daria Mochly-Rosen, Ph.D.^{1,2}

¹Dept. of Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA

²Dept of Neurosurgery, Stanford University School of Medicine, Stanford, CA

³Stanford Stroke Center, Stanford University School of Medicine, Stanford, CA

⁴Dept. of Neurology, Veterans Affairs Medical Center, University of California, San Francisco.

Abstract

In response to mild ischemic stress, the brain elicits endogenous survival mechanisms to protect cells against a subsequent lethal ischemic stress, referred to as ischemic tolerance. The molecular signals that mediate this protection are thought to involve the expression and activation of multiple kinases, including protein kinase C (PKC). Here we demonstrate that εPKC mediates cerebral ischemic tolerance *in vivo*. Systemic delivery of ψεRACK, an εPKC-selective peptide activator, confers neuroprotection against a subsequent cerebral ischemic event when delivered immediately prior to stroke. In addition, activation of εPKC by ψεRACK treatment decreases vascular tone *in vivo*, as demonstrated by a reduction in microvascular cerebral blood flow. Here we demonstrate the role of acute and transient εPKC in early cerebral tolerance *in vivo* and suggest that extra-parenchymal mechanisms, such as vasoconstriction, may contribute to the conferred protection.

Keywords

Ischemia; preconditioning; protein kinase C; cerebral blood flow

INTRODUCTION

A wide network of intracellular signaling pathways are activated in response to mild stresses such as sublethal ischemia or hypothermia to reduce metabolic demand, maintain basic cellular function, and increase survival in the event of a period of severe oxygen and glucose starvation such as stroke [8,13]. These endogenous neuroprotective events confer what is termed ischemic tolerance or preconditioning. Strategies to identify and harness the cellular and molecular mechanisms of this protection hold tremendous potential for therapeutic benefit.

Correspondence should be addressed to: Daria Mochly-Rosen, Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA 94305-5174, Tel: 650 725-7720; Fax: 650 723-2253, Email: mochly@stanford.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest statement:

Daria Mochly-Rosen is a founder of KAI Pharmaceuticals, whose goal is to bring peptide regulators of PKC to the clinic. However, the research described in this study was carried out in her lab with sole support from NIH grants to her university activities.

Preconditioning has been observed in multiple tissues, including the heart and brain, suggesting that conserved signaling pathways may mediate ischemic insult [8,23]. In response to a number of preconditioning-stimuli, changes in gene expression and protein activation initiate common signaling pathways including upregulation of free radical scavengers, opening of K_{ATP} channels, and activation of protein kinases including protein kinase C (PKC) [2,5,8,32]. Protein kinase C is a widely-expressed family of serine/ threonine kinases. The role of ϵ PKC has been well established in cardiac preconditioning [27], and is a point of signaling convergence following multiple types of cardiac preconditioning stimuli including transient sublethal ischemia, ethanol and adenosine [26]. More recently, a role for ϵ PKC in cerebral tolerance has been suggested using various *in vitro* models. These studies have demonstrated that ϵ PKC is activated following a preconditioning stimulus such as application of adenosine or NMDA, and is required for preconditioning- induced protection [26,31]. However, whether this protection correlates with the *in vivo* activity of ϵ PKC, in which the role of the vasculature, inflammation and other extra-parenchymal mediators are important, has not been addressed.

Here we determined the role of ϵ PKC in cerebral ischemia *in vivo*, using $\psi\epsilon$ RACK, an ϵ PKC-specific activator peptide. Multiple reports have demonstrated that PKCs play an important role in mediating vascular tone [6,19]. We therefore also examined the effects of ϵ PKC activation on cerebrovascular function. A clear understanding of the mechanisms which govern preconditioning holds great promise for therapies against diseases such as stroke, as well as conditions associated with reduced blood flow to the brain, such as during cardiac bypass surgery. Here we demonstrate that ϵ PKC may be an important target for acute neuroprotective therapies.

MATERIALS AND METHODS

Peptide preparation

$\psi\epsilon$ RACK (ϵ PKC activator peptide; ϵ PKC₈₅₋₉₂: C-HDAPIGYD) or ϵ V1-2 (ϵ PKC inhibitor peptide; ϵ PKC₁₄₋₂₁: C-EAVSLKPT) were synthesized and Tat, the protein transduction domain of the TAT protein (TAT₄₇₋₅₇; CYGRKKRRQRRR), was conjugated to the PKC peptides *via* a cysteine S-S bond. The ϵ PKC isozyme-selective activity of $\psi\epsilon$ RACK-Tat ($\psi\epsilon$ RACK) and ϵ V1-2-Tat (ϵ V1-2) has previously been demonstrated in both *in vitro* and *in vivo* models [3,31], and in particular, has been shown to alter ϵ PKC activity in the brain following systemic intraperitoneal delivery [3]. Peptide dose (0.2mg/kg) was based on previous studies [3,11,31].

MCA occlusion model

Transient ischemia (2hrs) was induced in male Sprague-Dawley rats (290–320g) using an intraluminal suture occluding the ostium of the middle cerebral artery (MCA) as previously described [18]. Animals were maintained and monitored under isoflurane anesthesia during all surgical procedures. Tat (vehicle control) or $\psi\epsilon$ RACK peptides were delivered as a bolus dose intraperitoneally at the following time points; I) 30 mins prior to ischemia and 3 mins into ischemia, II) 30 mins prior to ischemia, III) 3 mins into ischemia, IV) 24 hrs prior to ischemia, V) 3 mins following ischemia, at the onset of reperfusion (all injections 0.2 mg/kg in 1 mL saline). ϵ V1-2 peptide was delivered only at one time point; 30 mins prior to ischemia. Physiological parameters including body temperature (35°C–38°C) and respiration rate were monitored and maintained using a heat blanket and anesthetic adjustment. All animals were euthanized by anesthetic overdose. For PKC translocation assays, rats were sacrificed at 10 mins, 30 mins or 120 mins of ischemia (without removal of the suture to allow reperfusion). Sham control animals were treated with an identical protocol, however the suture was not advanced to occlude the MCA. The brain was sliced and striatum isolated and snap frozen for

tissue homogenization. All animal protocols were approved by the Institutional Animal Care and Use Committee of Stanford University.

Assessment of brain infarct size

Animals were euthanized and brains were quickly removed and sliced into five, 3 mm coronal sections. Slices were stained using 3% triphenyl tetrazolium chloride (TTC) and both faces of each slice were photographed for infarct assessment. Approximately 15% of animals were excluded from the analysis due to unsuccessful induction of ischemia, with no observable infarct. Relative stroke area (ratio of the infarct size relative to the ipsilateral hemisphere, corrected for edema based on measurement of the contralateral hemisphere) was measured to assess infarct size in the central three slices (two faces each; six faces total). Results were expressed as the mean $\pm$ SEM infarct size. All statistical analyses were performed using ANOVA followed by Bonferroni's post-hoc test.

Western blot analysis

Brains were quickly isolated, sliced, and striatal regions dissected from each central slice and frozen. Tissue fractionation was performed to collect soluble (cytosolic) and particulate (membrane) fractions, as previously described [12]. To compare ϵ PKC concentration in each fraction, total protein concentration was assessed using Bradford reagent, and 10 μ g of total lysate (40 μ g from hippocampal slice lysate) from each fraction was subjected to gel electrophoresis (10% bisacrylamide gel) and transferred to nitrocellulose membrane. Blots were blocked in 3% milk TBST (Tris-Buffered Saline Tween), probed with an anti- ϵ PKC antibody (Santa Cruz; 1:500) in 2% milk TBST and probed with an anti-rabbit secondary antibody (Amersham). All statistical analyses were performed using ANOVA followed by Fisher's posthoc tests.

Cerebral blood flow measurements

Male Sprague-Dawley rats (280–400g) were used. Animals were maintained under isoflurane anesthesia. Cerebral blood flow measurements were recorded using a Laser Doppler probe *via* a cranial burr hole (1mm posterior, 6 mm lateral of bregma on the left cortex). Flow measurements were taken for 20 mins to establish baseline flow, followed by intraperitoneal injection of $\psi\epsilon$ RACK, or Tat control peptide (0.2 mg/kg in 1ml saline), and flow was recorded for an additional 20–40 mins. Statistical analysis of cerebral blood flow was performed as follows; blood flow readings (at 1 minute intervals) for a period of 20–30 minutes were compared with blood flow measurements following delivery of $\psi\epsilon$ RACK, for a period of 20–40 mins. Statistical significance was assessed using Student's t-test.

RESULTS

ϵ PKC activity increases during ischemic injury *in vivo*

Protein Kinase C (PKC) intracellular translocation from the soluble to the membrane-enriched, particulate fraction is a standard method to measure activation state of the enzyme [15]. Using a time course of ischemic injury, ϵ PKC translocation was assessed in brain tissue homogenates. Within 10 mins of ischemia, the proportion of ϵ PKC in the membrane-bound fraction was significantly higher than in non-ischemic sham animals (45% increase, $p<0.05$). This translocation was maintained at 30 mins into ischemia (41% increase, $p<0.05$). Translocation of ϵ PKC was diminished by 120 mins of ischemia (Figure 1A, B).

Activation of ϵ PKC confers protection against ischemic injury through 24 hrs post-stroke

$\psi\epsilon$ RACK peptide was delivered by intraperitoneal-dose at various time points prior, during and following ischemia (0.2 mg/kg in 1 mL) (Figure 2A). Infarct size was assessed following

24 hrs of reperfusion. When delivered twice, both 30 mins prior to ischemia and 3 mins into ischemia, a significant reduction in infarct size was observed compared to Tat-carrier-treated controls. When $\psi\epsilon$ RACK was delivered at 30 mins prior to ischemia only, infarct size was also significantly reduced. When $\psi\epsilon$ RACK was delivered at 3 mins into ischemia, protection was observed, as seen by a reduction in infarct size; this reduction however did not reach statistical significance (Figure 2B and 2C; Tat-control treated (all groups combined); $27\pm 3\%$, 30 mins prior and 3 mins intra-ischemia ($n=9$); $14\pm 5\%$ $p<0.05$, 30 mins prior; ($n=14$); $15\pm 4\%$ $p<0.05$, 3 mins intra ($n=11$); $18\pm 4\%$ $p=0.07$). We next tested whether increasing ϵ PKC activity mediates long term protection against cerebral ischemic injury (delayed tolerance), by delivering $\psi\epsilon$ RACK peptide 24 hrs prior to stroke injury. In this protocol, $\psi\epsilon$ RACK treatment did not result in observable protection against ischemic injury (Figure 2C; $n=9$). $\psi\epsilon$ RACK peptide was also delivered immediately following 2 hours of ischemia, at the onset of reperfusion, to determine whether ϵ PKC activity mediates delayed injury processes. In these animals, $\psi\epsilon$ RACK did not alter infarct size compared to Tat-vehicle treated animals (Figure 2D; Tat vehicle control $33\pm 5\%$, $\psi\epsilon$ RACK post- $33\pm 6\%$, $n=8$ per group).

To determine whether inhibiting ϵ PKC activity is detrimental, ϵ V1-2 or Tat carrier was delivered 30 mins prior to the onset of transient focal ischemia. No significant difference in infarct size was observed (data not shown).

ϵ PKC activation reduces cerebral blood flow

We next examined whether delivery of $\psi\epsilon$ RACK alters vascular tone by measuring microvascular cerebral blood flow using laser Doppler flowmetry. Delivery of $\psi\epsilon$ RACK peptide (0.2 mg/kg) significantly decreased flow in all animals tested by an average of $15\pm 6\%$ (Figure 3; $n=10$, $p<0.05$). Delivery of Tat peptide alone did not significantly alter cerebral blood flow (data not shown.) The onset of flow reduction following $\psi\epsilon$ RACK delivery was variable between animals, from 5–30 minutes. Importantly, delivery of $\psi\epsilon$ RACK peptide does not alter mean arterial blood pressure, blood oxygen or carbon dioxide (data not shown).

DISCUSSION

The role of ϵ PKC in cerebral ischemia *in vivo* has been unclear, in part due to conflicting reports on the expression and activity of ϵ PKC following stroke [4]. We demonstrate here that ϵ PKC is activated during cerebral ischemia, and may play an important role in mediating the early cellular response to ischemic stress.

ϵ PKC activity during cerebral ischemia

Following ischemic stress, ion channel dysregulation and influx of calcium into the cell leads to the activation of multiple protein kinases including PKC. We recognize that individual PKC isozymes play unique roles in mediating both survival and cell death pathways in the brain. However, until now, we have had only a limited understanding of the activation pattern and activity of individual PKC isozymes.

Conflicting reports exist on ϵ PKC activity during and following ischemic injury. Some studies suggest that ϵ PKC is activated following oxygen/ glucose deprivation, or in response to kainic acid (a glutamate analog) treatment in *in vitro* and *ex vivo* models [20,28]. Other reports suggest that ϵ PKC does not respond to ischemia or ischemia-like insults [9,14,30]. Differences in the insult duration and severity in these models, cell types involved, and time points selected for assessing ϵ PKC activity, may have contributed to these conflicting findings. Importantly, many of these studies assess ϵ PKC activity during the period of reperfusion, and not early during ischemia, or acutely during or following preconditioning.

Here, we show that ϵ PKC is activated during transient focal ischemia. Within 10 minutes of ischemia, an approximately 40% increase in membrane translocation of ϵ PKC was observed. ϵ PKC activation at early timepoints during ischemia suggests that this isozyme is activated following even short periods of ischemia, similar to a tolerizing ischemic period. Indeed, in models of focal preconditioning, 10–15 minutes of transient MCA occlusion is sufficient to induce tolerance [24]. These results are consistent with *ex vivo* studies, in which ϵ PKC is activated within 1 hour following a preconditioning stress [26]. Therefore, it is likely that ϵ PKC is activated, and may mediate the initial cellular response to brief or mild ischemia such as preconditioning stress, *in vivo*. Sustained ϵ PKC activation at 30 minutes of ischemia also implies that ϵ PKC activity contributes to the ischemic response under more severe ischemic insults.

ϵ PKC activity promotes cell survival prior to ischemia

Following our finding that ϵ PKC is activated in response to ischemic insult, we examined whether activation or inhibition of ϵ PKC altered outcome following ischemic stroke. Delivery of $\psi\epsilon$ RACK activator peptide prior to ischemia conferred a significant reduction in infarct size compared to Tat control. Activation of ϵ PKC did not confer protection against a delayed ischemic insult when $\psi\epsilon$ RACK was delivered 24 hrs prior to transient focal ischemia, indicating that ϵ PKC activity may be involved only in acute protection paradigms. Importantly, delivery of $\psi\epsilon$ RACK during reperfusion resulted in no change in lesion size at 24 hrs. This suggests that ϵ PKC specifically mediates protective signaling prior to, or early during ischemia, however, may not be involved in promoting cell survival during the reperfusion period (Figure 2). There was no significant difference in the infarct size between ϵ V1-2 treated- and carrier-control treated animals (data not shown), suggesting inhibiting ϵ PKC activity does not worsen ischemic injury. Significant progress has been made in understanding ϵ PKC function in ischemic tissue. Most recent work has demonstrated the involvement of ϵ PKC in regulation of heat shock protein signaling, preservation of mitochondrial function, and reduction of free radical generation [7,16,22,25]. ϵ PKC signaling in ischemic tissue has been the subject of a number of reviews [4,22].

Neurovascular activity of ϵ PKC

Multiple studies have shown that PKC enzymes are important in mediating vascular tone in a variety of models, in *in vitro* primary endothelial and smooth muscle cells, isolated arteries, and *in vivo* cerebrovascular responses [1]. Inhibition of PKC isozymes with pan-specific PKC inhibitors such as staurosporine and calphostin C induce vasodilation, whereas activation of PKC using phorbol esters such as PMA causes potent vasoconstriction [1,13]. However, the specific PKC isozymes which mediate these responses are largely unknown.

Here we show that acute delivery of $\psi\epsilon$ RACK causes a mild, but significant reduction of cerebral blood flow, by approximately 15%, suggesting ϵ PKC activity promotes microvascular vasoconstriction. Interestingly, this reduction in flow occurs in the absence of any ischemic stimulus, (however measurements were made while the animal was under isoflurane anesthesia, a known vasodilator). This vasoconstriction was maintained for the duration of the study (up to 60 minutes post- $\psi\epsilon$ RACK delivery).

An ϵ PKC-mediated reduction in cerebral blood flow is an unexpected finding. Indeed, agents that induce cerebral *vasodilation*, such as arginine (*via* nitric oxide production) or adenosine, confer neuroprotection during stroke by *increasing* flow to 'at-risk' areas *via* collateral supply arteries [21,33]. It is possible that a decrease in blood flow may cause a preconditioning-like effect by mildly reducing the amount of oxygen and glucose supplied to tissue and suppressing metabolic rate, similar to a mild ischemic event. Indeed, hypothermia, a well known mechanism to protect the brain against damage under ischemic conditions, confers protection in part by

suppressing metabolic demand in tissue [17]. Interestingly, it has recently been shown that hypothermia may preserve ϵ PKC activity in the brain after stroke [29], suggesting a link between these two neuroprotective pathways.

The role of ϵ PKC in mediating CBF is unknown; $\psi\epsilon$ RACK may have direct effects on vascular cell function, or may play an indirect role on neuronal regulation of cerebral blood flow. Interestingly, recent work has demonstrated that inhibition of PKC using Ro-32-0432 suppresses vascular smooth muscle cell endothelin-B receptor upregulation in response to ischemia, and contractile response in arterial segments [10], suggesting the direct role of ϵ PKC on vascular cell contractile function. However, previous studies have demonstrated that delivery of $\psi\epsilon$ RACK in *in vitro* and *ex vivo* cerebral ischemia models, in which blood flow does not play a role, confers neuroprotection [26,31]. The neuroprotection observed following delivery of $\psi\epsilon$ RACK peptide may not therefore be solely or directly attributable to the reduction in cerebral blood flow. Whether this reduction in blood flow in the observed neuroprotective effect of $\psi\epsilon$ RACK contributes to preconditioning-like effects observed *in vivo* is a subject of future studies.

References

1. Akopov SE, Sercombe R, Seylaz J. Endothelial dysfunction in cerebral vessels following carotid artery infusion of phorbol ester in rabbits: the role of polymorphonuclear leukocytes. *J Cereb Blood Flow Metab* 1994;14:1078–1087. [PubMed: 7929651]
2. Armstrong SC. Protein kinase activation and myocardial ischemia/reperfusion injury. *Cardiovasc Res* 2004;61:427–436. [PubMed: 14962474]
3. Begley R, Liron T, Baryza J, Mochly-Rosen D. Biodistribution of intracellularly acting peptides conjugated reversibly to Tat. *Biochem Biophys Res Commun* 2004;318:949–954. [PubMed: 15147964]
4. Bright R, Mochly-Rosen D. The role of protein kinase C in cerebral ischemic and reperfusion injury. *Stroke; a journal of cerebral circulation* 2005;36:2781–2790. [PubMed: 16254221]
5. Cavero I, Djellal Y, Guillon JM. Ischemic myocardial cell protection conferred by the opening of ATP-sensitive potassium channels. *Cardiovasc Drugs Ther* 1995;9:245–255. [PubMed: 7647029]
6. Chrissobolis S, Sobey CG. Inhibitory effects of protein kinase C on inwardly rectifying K⁺- and ATP-sensitive K⁺ channel-mediated responses of the basilar artery. *Stroke; a journal of cerebral circulation* 2002;33:1692–1697. [PubMed: 12053013]
7. Dave KR, DeFazio RA, Raval AP, Torraco A, Saul I, Barrientos A, Perez-Pinzon MA. Ischemic preconditioning targets the respiration of synaptic mitochondria via protein kinase Cepsilon. *J Neurosci* 2008;28:4172–4182. [PubMed: 18417696]
8. Dirnagl U, Simon RP, Hallenbeck JM. Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* 2003;26:248–254. [PubMed: 12744841]
9. Harada K, Maekawa T, Abu Shama KM, Yamashima T, Yoshida K. Translocation and down-regulation of protein kinase C-alpha, -beta, and -gamma isoforms during ischemia-reperfusion in rat brain. *J Neurochem* 1999;72:2556–2564. [PubMed: 10349867]
10. Henriksson M, Vikman P, Stenman E, Beg S, Edvinsson L. Inhibition of PKC activity blocks the increase of ETB receptor expression in cerebral arteries. *BMC pharmacology* 2006;6:13. [PubMed: 17129394]
11. Inagaki K, Begley R, Ikeno F, Mochly-Rosen D. Cardioprotection by epsilon-protein kinase C activation from ischemia: continuous delivery and antiarrhythmic effect of an epsilon-protein kinase C-activating peptide. *Circulation* 2005;111:44–50. [PubMed: 15611364]
12. Johnson JA, Mochly-Rosen D. Inhibition of the spontaneous rate of contraction of neonatal cardiac myocytes by protein kinase C isozymes. A putative role for the epsilon isozyme. *Circ Res* 1995;76:654–663. [PubMed: 7895339]
13. Kirino T. Ischemic tolerance. *J Cereb Blood Flow Metab* 2002;22:1283–1296. [PubMed: 12439285]
14. Koponen S, Keinänen R, Roivainen R, Hirvonen T, Narhi M, Chan PH, Koistinaho J. Spreading depression induces expression of calcium-independent protein kinase C subspecies in ischaemia-

sensitive cortical layers: regulation by N-methyl-D-aspartate receptors and glucocorticoids. *Neuroscience* 1999;93:985–993. [PubMed: 10473263]

15. Kraft AS, Anderson WB. Phorbol esters increase the amount of Ca²⁺, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* 1983;301:621–623. [PubMed: 6828143]
16. Lange-Asschenfeldt C, Raval AP, Dave KR, Mochly-Rosen D, Sick TJ, Perez-Pinzon MA. Epsilon protein kinase C mediated ischemic tolerance requires activation of the extracellular regulated kinase pathway in the organotypic hippocampal slice. *J Cereb Blood Flow Metab* 2004;24:636–645. [PubMed: 15181371]
17. Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. *Nat Rev Neurosci* 2003;4:399–415. [PubMed: 12728267]
18. Maier CM, Sun GH, Kunis D, Yenari MA, Steinberg GK. Delayed induction and long-term effects of mild hypothermia in a focal model of transient cerebral ischemia: neurological outcome and infarct size. *J Neurosurg* 2001;94:90–96. [PubMed: 11147904]
19. Mayhan WG, Patel KP. Acute effects of glucose on reactivity of cerebral microcirculation: role of activation of protein kinase C. *Am J Physiol* 1995;269:H1297–H1302. [PubMed: 7485561]
20. McNamara RK, Wees EA, Lenox RH. Differential subcellular redistribution of protein kinase C isozymes in the rat hippocampus induced by kainic acid. *J Neurochem* 1999;72:1735–1743. [PubMed: 10098884]
21. Morikawa E, Moskowitz MA, Huang Z, Yoshida T, Irikura K, Dalkara T. L-arginine infusion promotes nitric oxide-dependent vasodilation, increases regional cerebral blood flow, and reduces infarction volume in the rat. *Stroke; a journal of cerebral circulation* 1994;25:429–435. [PubMed: 7508154]
22. Perez-Pinzon MA, Dave KR, Raval AP. Role of reactive oxygen species and protein kinase C in ischemic tolerance in the brain. *Antioxidants & redox signaling* 2005;7:1150–1157. [PubMed: 16115018]
23. Pong K. Ischaemic preconditioning: therapeutic implications for stroke? *Expert Opin Ther Targets* 2004;8:125–139. [PubMed: 15102554]
24. Pradillo JM, Romera C, Hurtado O, Cardenas A, Moro MA, Leza JC, Davalos A, Castillo J, Lorenzo P, Lizasoain I. TNFR1 upregulation mediates tolerance after brain ischemic preconditioning. *J Cereb Blood Flow Metab* 2005;25:193–203. [PubMed: 15647744]
25. Raval P, Dave KR, DeFazio RA, Perez-Pinzon MA. epsilonPKC phosphorylates the mitochondrial K(+) (ATP) channel during induction of ischemic preconditioning in the rat hippocampus. *Brain research* 2007;1184:345–353. [PubMed: 17988655]
26. Raval AP, Dave KR, Mochly-Rosen D, Sick TJ, Perez-Pinzon MA. Epsilon PKC is required for the induction of tolerance by ischemic and NMDA-mediated preconditioning in the organotypic hippocampal slice. *J Neurosci* 2003;23:384–391. [PubMed: 12533598]
27. Schulz R, Cohen MV, Behrends M, Downey JM, Heusch G. Signal transduction of ischemic preconditioning. *Cardiovasc Res* 2001;52:181–198. [PubMed: 11684066]
28. Selvatici R, Marino S, Piubello C, Rodi D, Beani L, Gandini E, Siniscalchi A. Protein kinase C activity, translocation, and selective isoform subcellular redistribution in the rat cerebral cortex after in vitro ischemia. *J Neurosci Res* 2003;71:64–71. [PubMed: 12478614]
29. Shimohata T, Zhao H, Steinberg GK. Epsilon PKC may contribute to the protective effect of hypothermia in a rat focal cerebral ischemia model. *Stroke; a journal of cerebral circulation* 2007;38:375–380. [PubMed: 17204679]
30. Tauskela JS, chakravarthy BR, Murray CL, Wang Y, Comas T, Hogan M, Hakim A, Morley P. Evidence from cultured rat cortical neurons of differences in the mechanism of ischemic preconditioning of brain and heart. *Brain research* 1999;827:143–151. [PubMed: 10320703]
31. Wang J, Bright R, Mochly-Rosen D, Giffard RG. Cell-specific role for epsilon- and betaI-protein kinase C isozymes in protecting cortical neurons and astrocytes from ischemia-like injury. *Neuropharmacology* 2004;47:136–145. [PubMed: 15165841]
32. Yoshida M, Nakakimura K, Cui YJ, Matsumoto M, Sakabe T. Adenosine A(1) receptor antagonist and mitochondrial ATP-sensitive potassium channel blocker attenuate the tolerance to focal cerebral ischemia in rats. *J Cereb Blood Flow Metab* 2004;24:771–779. [PubMed: 15241185]

33. Zhang F, White JG, Iadecola C. Nitric oxide donors increase blood flow and reduce brain damage in focal ischemia: evidence that nitric oxide is beneficial in the early stages of cerebral ischemia. *J Cereb Blood Flow Metab* 1994;14:217–226. [PubMed: 8113318]

Figure 1. ϵ PKC is activated during cerebral ischemia

ϵ PKC activation was measured by translocation of the enzyme from the cytosol (C) to the membrane-enriched particulate (membrane) fraction (M), and is represented as percent translocation. ϵ PKC activity increased in the ischemic striatum within 10 minutes of MCA occlusion, and activity was maintained at 30 minutes of ischemia. By 120 minutes of ischemia, ϵ PKC activity was reduced to pre-ischemic levels. A) representative western blots, B) quantitation of ϵ PKC translocation, bars represent fraction of membrane- translocated ϵ PKC (n=3–5 per group). Statistical analysis by ANOVA followed by Fisher's post-hoc tests.

Figure 2. Delivery of $\psi\epsilon$ RACK peptide reduces cerebral damage when delivered prior to transient focal ischemia

A) Protocols for delivery of peptides; $\psi\epsilon$ RACK or Tat control peptides were delivered at time points; I. 30 mins prior to ischemia and 3 mins intra-ischemia (n=9), II. 30 mins prior to ischemia (n=14), III. 3 mins intra-ischemia (n=11), IV. 24 hrs prior to ischemia (n=9), or V. 3 mins post- ischemia (n=8). Animals were sacrificed and infarct size quantitated after 24 hrs of reperfusion. B) Treatment with $\psi\epsilon$ RACK (30 mins prior to ischemia and 3 mins intra-ischemia) reduces infarct size. Representative photographs of coronal sections of rat brain following infarction stained with triphenyl-tetrazolium chloride. Red area is live tissue, white area is dead or dying tissue. C) Infarct size assessment; represented as percent infarct with respect to

contralateral hemisphere. Treatment with ψ εRACK reduced infarct size significantly (* $p < 0.05$) when delivered at 30 mins prior to ischemia and 3 mins intra-ischemia, and at 30 mins prior to ischemia alone. Treatment with ψ εRACK at 3 mins intra-ischemia alone demonstrated a reduction in infarct size which was not statistically significant (# $p = 0.07$), bars represent average infarct size. Treatment with ψ εRACK at 24 hrs prior to ischemia did not reduce infarct size. D) Delivery of ψ εRACK post-ischemia did not reduce infarct size. Statistical analysis was performed using ANOVA followed by Bonferroni's post-hoc test.

Figure 3. Delivery of $\psi\epsilon$ RACK decreases cerebral blood flow *in vivo*

Blood flow measurements were performed using laser Doppler flowmetry on the cortical surface *via* a cranial burr hole. A) Representative trace of animal treated with $\psi\epsilon$ RACK peptide. Data is represented as percent of initial baseline flow. Flow decreased within minutes of systemic $\psi\epsilon$ RACK delivery. B) Quantitative data showing percent decrease in flow over baseline readings following delivery of $\psi\epsilon$ RACK peptide, point represents mean $\pm$ SEM. Statistical analysis was performed using Student's t-test. (n=10, *p<0.05)