Published in final edited form as:

Brain Res. 2007 May 4; 1144: 146–155. doi:10.1016/j.brainres.2007.01.113.

δPKC mediates microcerebrovascular dysfunction in acute ischemia and in chronic hypertensive stress in vivo

Rachel Bright^a, Gary K. Steinberg^{b,c}, and Daria Mochly-Rosen^{a,b,*}

Daria Mochly-Rosen: mochly@stanford.edu

^aDepartment of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA, USA

^bDepartment of Neurosurgery, Stanford University School of Medicine, Stanford, CA, USA

^cStanford Stroke Center, Stanford University School of Medicine, Stanford, CA, USA

Abstract

Maintaining cerebrovascular function is a priority for reducing damage following acute ischemic events such as stroke, and under chronic stress in diseases such as hypertension. Ischemic episodes lead to endothelial cell damage, deleterious inflammatory responses, and altered neuronal and astrocyte regulation of vascular function. These, in turn, can lead to impaired cerebral blood flow and compromised blood–brain barrier function, promoting microvascular collapse, edema, hemorrhagic transformation, and worsened neurological recovery. Multiple studies demonstrate that protein kinase C (PKC), a widely expressed serine/threonine kinase, is involved in mediating arterial tone and microvascular function. However, there is no clear understanding about the role of individual PKC isozymes. We show that intraperitoneal injection of δ V1-1-TAT_{47–57} (0.2 mg/kg in 1 mL), an isozymespecific peptide inhibitor of δ PKC, improved microvascular pathology, increased the number of patent microvessels by 92% compared to control-treated animals, and increased cerebral blood flow by 26% following acute focal ischemia induced by middle cerebral artery occlusion in normotensive rats. In addition, acute delivery of δ V1-1-TAT_{47–57} in hypertensive Dahl rats increased cerebral blood flow by 12%, and sustained delivery δ V1-1-TAT_{47–57} (5 uL/h, 1 mM), reduced infarct size by 25% following an acute stroke induced by MCA occlusion for 90 min. Together, these findings demonstrate that δ PKC is an important therapeutic target for protection of microvascular structure and function under both acute and chronic conditions of cerebrovascular stress.

Keywords

Cerebral blood flow; Hypertension; Microvasculature; Protein kinase C; Stroke; Vasculature

Introduction

The preservation of vascular function is critical for limiting damage in cerebrovascular diseases such as stroke and hypertension (Takahashi and Macdonald, 2004). Following cerebral ischemic/reperfusion injury or other cerebrovascular event, endothelial dysfunction

© 2007 Elsevier B.V. All rights reserved.

*Corresponding author. Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA 94305-5174, USA. Fax: +1 650 723 2253.

Conflict of interest: 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.

and recruitment of inflammatory cells, coupled with loss of regulation by local intraparenchymal cells contributes to compromised vascular responsiveness, reduced blood flow, impaired blood–brain barrier integrity and microvascular collapse (Yang and Betz, 1994; del Zoppo and Mabuchi, 2003; Takahashi and Macdonald, 2004; Zheng and Yenari, 2004). In addition to direct protection of CNS parenchymal neurons and glia with neuroreceptor and ion channel modulators, agents that protect microvascular function are critical adjunct treatments (Faraci, 2003). To our knowledge, there are no agents that acutely improve microvascular pathology and function, increase flow to at-risk tissue under conditions of vascular stress, and protect intraparenchymal cells following an ischemic event.

In patients with chronic arterial hypertension, oxygen consumption and cerebral blood flow (CBF) are unimpaired in the resting state. However, alterations in vascular autoregulation contribute to reduced tolerance to changes in arterial blood pressure, increased susceptibility to vasoconstrictive agents produced under conditions of cerebral ischemia, and impaired endothelium-dependent dilation (Strandgaard, 1978). Hypertension thereby results in increased susceptibility to ischemic and hemorrhagic stroke, and worsened outcome following such an event (Arboix et al., 2004; Rasool et al., 2004). Although multiple anti-hypertensive drugs exist, agents that maintain cerebral microvascular function and confer neuroprotection following an ischemic event have not been identified.

Protein kinase C (PKC) has been shown to regulate cerebrovascular tone (Murray et al., 1992; Uhl et al., 1993; Laher and Zhang, 2001); PKC activators, phorbol esters, induce vasoconstriction in multiple ex vivo and in vivo systems (Murray et al., 1992; Uhl et al., 1993; Akopov et al., 1994; Jin et al., 1994), and pan-specific PKC inhibitors, including staurosporine, calphostin C and H-7, promote vasodilation or block vasoconstriction in isolated arteries (Murray et al., 1992; Henrion and Laher, 1993). The exact mechanism by which PKC inhibition elicits increased blood flow is unclear, in part, due to a lack of understanding about the role of individual PKC isozymes.

PKC isozymes mediate unique cellular functions in response to cellular stresses such as ischemia. δ PKC, in particular, is involved in cell death pathways following ischemic/reperfusion damage in multiple organs (La Porta and Comolli, 1993; Inagaki et al., 2003; Bright et al., 2004). For example, δ PKC is involved in caspase activation (Brodie and Blumberg, 2003), Bcl-2 family activity (Murriel et al., 2004), mitochondrial dysfunction and reactive oxygen species production (Armstead, 1999; Churchill and Szwedda, 2005), and excitatory amino acid release (Nakane et al., 1998), pathways which contribute to necrotic and apoptotic cell death following stroke. Inhibition or reduction of δ PKC levels during reperfusion leads to marked reduction in cerebral damage (Bright et al., 2004; Chou et al., 2004; Raval et al., 2005). This protection is due to inhibition of deleterious δ PKC activity in multiple cell types including parenchymal and inflammatory cells following stroke (Bright et al., 2004; Chou et al., 2004). In addition, the levels of δ PKC increase in endothelial cells 2–3 days following ischemic/reperfusion injury in vivo (Miettinen et al., 1996). The critical role of δ PKC in multiple cell types of the brain suggests that this enzyme is likely to affect coordinated cellular responses, such as maintenance of the blood–brain barrier and regulation of cerebral blood flow. However, whether δ PKC specifically mediates acute cerebrovascular responses during reperfusion injury, thereby contributing to stroke damage, has not been examined.

Here we report that δ PKC mediates microcerebrovascular dysfunction following acute and chronic vascular stress. These data suggest that inhibition of δ PKC is an important therapeutic strategy for improving outcome following cerebrovascular events in both normotensive and hypertensive patients.

2. Results

2.1. Delivery of δ V1-1-TAT following stroke improves cerebrovascular pathology

The neuroprotective effect of δ PKC inhibition during reperfusion was previously demonstrated in a MCA occlusion (MCAO) model of stroke, using δ V1-1 (Bright et al., 2004). δ V1-1 is a rationally designed peptide that selectively inhibits δ PKC translocation and activity (Chen et al., 2001). For intracellular delivery, δ V1-1 peptide is conjugated via a disulfide bond to TAT₄₇₋₅₇ (TAT), a peptide derived from the Tat human immunodeficiency virus (HIV) protein (Schwarze et al., 1999). The selective activity of δ V1-1-TAT has been demonstrated in multiple in vitro and in vivo models (Chen et al., 2001; Begley et al., 2004; Bright et al., 2004). To determine whether reperfusion-induced microvascular dysfunction was also affected by δ PKC inhibition, we first assessed microvascular morphology following stroke in δ V1-1-TAT-, or TAT-treated stroked rats (Fig. 1A; boxed area in diagram). Sham control animals were similarly treated, however they did not undergo ischemia or peptide treatment. Defined regions of the cerebral cortex corresponding to the ischemic penumbra were examined (Fig. 1A; boxed area). Lower magnification TEM images (1200 $\times$) were first used to quantitate microvessel number (Figs. 1C,G,K). Vessels were counted if a recognizable microvascular lumen and surrounding endothelial lining was identified (arrows). Vessels with in-plane axes were therefore not counted. In ischemic animals treated with TAT peptide, a significant reduction in the number of vessels was observed compared with δ V1-1-TAT-treated tissue (TAT, 1.6 ± 0.6 vessels/field; δ V1-1-TAT, 3.1 ± 0.6 vessels/field; $p < 0.05$; $n = 4$; Figs. 1B and G vs. K). Importantly, the number of microvessels in animals subjected to ischemia followed with δ V1-1-TAT-treatment at reperfusion was similar to that in sham animals (sham, 3.8 ± 0.4 vessels/field; n.s.; $n = 4$; Figs. 1B and K vs. C).

Features of healthy microvascular structure were observed using higher magnification TEM in cortical tissue from sham control animals. These included intact basal lamina, clear and patent vessel lumens and lack of abnormal perivascular spaces (Figs. 1D-F). Consistent with previous studies of cerebral microvascular morphology following stroke (Melgar et al., 2005), we found evidence of ultrastructural damage in brain tissue from ischemic animals (Figs. 1H-J), including signs of vascular edema, swollen astrocyte end-foot processes, enlarged endothelial nuclei, breakdown of the basal lamina, compression of vascular lumen, and luminal surface irregularities. However, in ischemic animals treated with δ V1-1-TAT (0.2 mg/kg) at reperfusion only, microvascular structure appeared significantly less damaged (Figs. 1L-N); some astrocyte end-foot swelling was observed in a small number of vessels (Fig. 1L, asterisks), however most vessels appeared normal (Figs. 1M,N).

2.2. Acute delivery of δ V1-1-TAT increases cerebral blood flow during reperfusion

We determined whether delivery of δ V1-1-TAT affected microvascular function by measuring CBF following MCAO. Baseline blood-flow measurements were taken for a period of 20–25 min using laser Doppler flowmetry on the ipsilateral cortical surface (Figs. 2A,B). δ V1-1-TAT (0.2 mg/kg in 1 mL) was then injected intraperitoneally, and CBF measurements were taken for an additional 20–60 min (Fig. 2A). Treatment with δ V1-1-TAT did not alter CBF in sham-treated animals; flow decreased by $7 \pm 6\%$ (n.s.; $n = 4$; Figs. 2C,E). Delivery of δ V1-1-TAT increased CBF following transient ischemia by $26 \pm 7\%$ ($n = 9$; $p < 0.05$; Figs. 2D,E). TAT peptide had no observable effect on flow in stroked animals ($0 \pm 3\%$; n.s.; $n = 4$; Figs. 2D,E).

2.3. Delivery of δ V1-1-TAT increases cerebral blood flow in chronic model of hypertension

The Dahl salt-sensitive rat model is a well established model of hypertension (Iwai and Heine, 1986). Fed an 8% high-salt diet starting at 6 weeks of age, these rats rapidly develop

hypertension, and by 11 weeks their blood pressure stabilized at 233 ± 4 mm Hg, as compared to 156 ± 4 mm Hg in animals fed on a 0.3% low-salt diet (Inagaki et al., 2002). We assessed the effects of $\delta V1-1$ -TAT treatment on CBF in Dahl hypertensive rats at age 11 weeks, following 5 weeks of high-salt diet. Acute delivery of $\delta V1-1$ -TAT induced a $12 \pm 4\%$ increase in CBF ($n=15$; $p<0.05$; Figs. 3A,B), whereas TAT treatment caused no significant change in flow ($-5 \pm 2\%$; $n=5$; n.s.; Fig. 3B). Arterial blood pressure did not change significantly in either treatment group, even after 2 weeks of sustained peptide delivery (at 13 weeks old, following 2 weeks of delivery the blood pressure for $\delta V1-1$ -TAT-treated rats was 247 ± 8 mm Hg, and for TAT-treated rats was 246 ± 5 mm Hg; n.s.).

2.4. Sustained delivery of $\delta V1-1$ -TAT inhibitor reduces δPKC activity in the brain of hypertensive rats

Previous reports have demonstrated that delivery of $\delta V1-1$ -TAT inhibits δPKC activity in the brain of normotensive animals (Begley et al., 2004). However, whether $\delta V1-1$ -TAT has similar activity in a hypertensive animal model has not been addressed. We therefore determined whether chronic delivery of $\delta V1-1$ -TAT using a subcutaneous Alzet osmotic pump would affect δPKC activity in the brain. Dahl rats were implanted with pumps delivering $\delta V1-1$ -TAT or TAT control peptide ($5 \mu\text{L/h}$, 1 mM) for 4–6 weeks. δPKC activity in brain samples was assessed by determining the translocation of the enzyme from the soluble, cytosolic fraction, to the membrane-bound fraction, a standard marker for PKC activation (Kraft and Anderson, 1983). In animals that received sustained $\delta V1-1$ -TAT delivery, there was a 30% reduction of δPKC translocation (activity) in whole brain samples, compared to animals chronically treated with TAT control peptide ($\delta V1-1$ -TAT, $40 \pm 3\%$ translocation; TAT control, $64 \pm 5\%$ translocation; $p<0.05$; $n=3$; Fig. 4A). The activity of ϵPKC , another PKC isozyme, was unchanged by sustained delivery of the $\delta V1-1$ -TAT peptide ($\delta V1-1$ -TAT-treated, $40 \pm 4\%$ translocation; TAT, $48 \pm 3\%$ translocation; n.s.; Fig. 4B).

2.5. Sustained delivery of $\delta V1-1$ -TAT reduces acute stroke damage in hypertensive rats

Impaired vessel responsiveness in hypertensive patients contributes to a worsened outcome following a cerebrovascular event (Arboix et al., 2004). We therefore next determined whether the observed increase in CBF following $\delta V1-1$ -TAT-treatment correlated with protection in hypertensive rats against transient ischemic damage. Dahl hypertensive rats (11–12 weeks) were implanted with subcutaneous pumps delivering $\delta V1-1$ -TAT or TAT control peptides ($5 \mu\text{L/h}$, 1 mM ; Inagaki et al., 2005). Following 5–6 days of peptide delivery, we induced transient focal ischemia using MCAO. Mortality or development of hemorrhagic transformation was too high using a 120 min occlusion for use as an effective model (80% of animals either died within 24 h of MCAO, or suffered hemorrhage at the MCA/ICA bifurcation; there was no difference between treatment groups). The occlusion period was therefore reduced to 90 min (Fig. 5A). Approximately 40% of animals suffered hemorrhage in either the $\delta V1-1$ -TAT or the TAT-treated animals, and were thus excluded from the study. Following 24 h of reperfusion, infarct size was assessed by TTC staining (Fig. 5B; infarct outlined). In animals without hemorrhage, $\delta V1-1$ -TAT-treatment reduced infarct size by 25%, as compared with TAT controls ($\delta V1-1$ -TAT, $30 \pm 4\%$; TAT, $39 \pm 3\%$; $n=6-7$ per group, $p<0.05$; Figs. 5B,C).

3. Discussion

The central goal of acute stroke treatment is to save vulnerable tissue in the ischemic penumbra. This demands both protection of the brain parenchyma and preservation of microvascular structure and function, as the size and growth of the ischemic penumbra is dependent, at least in part, on the extent of compromised collateral flow. Loss of

microvascular integrity also contributes to edema, hemorrhagic transformation and additional ischemic episodes (Takahashi and Macdonald, 2004). Therefore, agents that preserve microvascular integrity and function under conditions of vascular stress are of great therapeutic importance. We show here that δ PKC is an important target for preserving microvascular function under conditions of both acute and chronic cerebrovascular stress.

3.1. δ PKC and microvascular dysfunction following acute ischemic stroke

Tight control of cerebral perfusion pressure involves complex regulation by local microvascular inputs from endothelial and smooth muscle cells, blood factors and inflammatory cells including platelets and leukocytes, and intraparenchymal cells (del Zoppo and Mabuchi, 2003). Following cerebral ischemic–reperfusion injury, PKCs, and in particular δ PKC, are activated in many cell types (Miettinen et al., 1996; Bright et al., 2004). Multiple studies suggest that PKCs are responsive to increases in lipid peroxidation, excitatory amino acid release, generation of free radicals, and caspase activation that occurs during reperfusion injury (Fukunaga et al., 1992; Kaasinen et al., 2002; Majumder et al., 2001; Raval et al., 2005; Vaccarino et al., 1991; Yoshida et al., 1999). Reperfusion injury may also affect interaction of δ PKC with annexin V, a required step in the activation of this isozyme (Kheifets et al., 2006). The reperfusion-induced production of vasoconstrictive stimuli, such as endothelin, has also been shown to increase δ PKC activity in endothelial cells (Ramzy et al., 2006; Rosendorff, 1997). δ PKC activity is deleterious in multiple cell types, including neurons (Bright et al., 2004), inflammatory cells (Chou et al., 2004) and endothelial cells; in a model of cardiac ischemia and reperfusion injury, inhibition of δ PKC correlates with reduced cardiac endothelial cell apoptosis (Ikeno et al., unpublished data). This suggests that inhibition of δ PKC activity may improve coordinated cellular activities including preservation of microvascular structure and function following stroke.

We show here that intraperitoneal delivery of δ V1-1–TAT, following MCA occlusion, increased the number of patent microvessels and improved microvascular pathology in the ischemic penumbra following stroke. Using TEM, astrocyte swelling, basal lamina breakdown, enlarged endothelial nuclei, and collapsed vessel lumens were observed. In animals treated with δ V1-1–TAT, these features were found in a reduced number of vessels as compared with TAT-treated stroked animals (Fig. 1). The improved structural preservation induced by δ V1-1–TAT treatment correlated with increased cortical microvascular blood-flow; there was a 26% increase in flow within 30 min of δ V1-1–TAT delivery (Fig. 2). This effect was sustained over the course of the experiment (up to 60 min following a single injection of the peptide). As δ V1-1–TAT-delivery has no effect on arterial blood pressure, arterial blood gases and body temperature in this model (Bright et al., 2004), it is unlikely that δ V1-1–TAT increases CBF via effects on arterial flow.

Importantly, we demonstrate that δ PKC induces micro-vascular dysfunction only under conditions of vascular stress, and has no effect in the healthy cerebrovascular bed (Fig. 2). This is consistent with the unique roles of δ PKC in basal cell-signaling verses stress-induced cellular responses. Vascular stress conditions such as ischemia and reperfusion initiate cell-signaling cascades different from basal conditions. For example, production of free radicals in microvessels and adjoining astrocyte end-feet contributes to the observed microvessel dysfunction following stroke (Gursoy-Ozdemir et al., 2004). δ PKC is known to mediate free radical production during reperfusion (Vanden Hoek et al., 2000; Churchill and Szweda, 2005), suggesting a role for δ PKC in the microvascular stress response. Indeed, a recent article by Beg et al., suggests that δ PKC plays a role in mediating cerebral flow. Here they observed the deleterious role of δ PKC in increasing cerebral endothelin-B- and 5-hydroxytryptamine-receptor expression and induced vasoconstriction following subarachnoid hemorrhage (Beg et al., 2007), which, similar to cerebral ischemic–reperfusion injury, causes vascular dysregulation.

3.2. Inhibition of δ PKC increases CBF in hypertensive rats

Hypertension is a cause of chronic vascular stress, leading to loss of autoregulation, sensitivity to vasoconstrictive agents, and a reduced ability to respond to changes in arterial pressure. Weakening and loss of microvascular structure and function in hypertension translates to worsened outcome following cerebrovascular events. Previous reports have demonstrated that Dahl salt-sensitive rats display alterations in cerebrovascular autoregulation including loss of pressure-induced vasoconstriction, and that these rats are at risk for ischemic/hemorrhagic events (Smeda and Payne, 2003). We found that δ V1-1-TAT-delivery significantly increased CBF by 12% in the hypertensive animals, in the absence of any additional vascular stresses (Fig. 3). Importantly, this increase in flow was not due to changes in arterial blood flow; neither acute (Bright et al., 2004), nor chronic delivery of δ V1-1-TAT (reported here) altered arterial blood pressure in normotensive and Dahl salt-sensitive hypertensive rats, respectively. Some heterogeneity was observed in the degree of CBF response between individual animals to treatment with δ V1-1-TAT (Figs. 2 and 3), which may be due to differences in the extent of vascular stress in each of these *in vivo* models.

3.3. Chronic delivery of δ V1-1-TAT peptide reduces stroke damage in hypertensive animals

Hypertensive patients frequently undergo a prolonged drug-treatment regimen (e.g. statins, aspirin, and β -blockers) to reduce incidence and damage from cerebrovascular events. We therefore mimicked sustained drug-treatment using a subcutaneous Alzet osmotic pump. We first demonstrated that chronic pump-delivery of δ V1-1-TAT inhibits δ PKC activity in the brain, indicating the efficacy of this method for delivering PKC-regulating peptides in hypertensive Dahl rats (Fig. 4). We found that sustained δ V1-1-TAT-treatment reduced infarct size in hypertensive rats following an induced transient ischemia by 25% as compared with TAT peptide (Fig. 5). The improved stroke outcome seen in δ V1-1-TAT-treated hypertensive rats is due, at least in part, to maintenance of microvascular structure and patency, supplying improved blood-flow to the ischemic penumbra following an ischemic event. Importantly, these data also demonstrate for the first time that chronic treatment with δ V1-1-TAT peptide treatment does not cause desensitization. Previous work has demonstrated that δ V1-1-TAT causes rapid inhibition of δ PKC activity in the brain, within 10 min of systemic delivery (Begley et al., 2004). Here we observed the sustained inhibition of δ PKC activity following 4–6 weeks of continuous δ V1-1-TAT delivery. Indeed, the neuroprotective effect of δ PKC inhibition was observed following 4–5 days of chronic δ V1-1-TAT-treatment.

We also report new pathological observations in the Dahl hypertensive-rat model, which appear relevant to the study of hypertension. Dahl hypertensive rats were prone to intracranial bleeding following suture-induced mechanical strain of cerebral arteries; ~80% of hypertensive rats suffered from hemorrhage following 120 min MCAO (40% following a 90 min MCAO), as compared with normotensive rats, in which the incidence of hemorrhage is less than ~20% in this protocol. The weakened arterial vasculature and increased susceptibility to hemorrhagic events may mimic the pathology observed clinically in hypertensive patients. In addition, Dahl hypertensive rats exhibit higher sensitivity to ischemic damage following stroke; the average infarct size in hypertensive rats following a 90 min MCA occlusion is 30% larger than the average infarct size produced by a 120 min MCA occlusion in normotensive rats, as we previously reported. These findings closely parallel observations in patients with chronic vascular stress, including diabetes and hypertension (Ay et al., 2005), and indicate that the Dahl hypertensive rat is an important model for studying multiple aspects of cerebrovascular disease.

Preservation of microvascular structure and function is critical for saving the ischemic penumbra following cerebrovascular events. We demonstrate here that inhibition of δ PKC improves microvascular pathology and function in two models of vascular stress: transient focal ischemia in normotensive animals and chronic hypertension. These beneficial effects on the microvasculature may contribute to the observed reduction in ischemic damage following an ischemic event in these models. Although the molecular basis of δ PKC activity and its cell-type specific function remains to be determined, our studies demonstrate that δ PKC is an important therapeutic target for the preservation of microcerebrovascular function following stroke, and may be very important for reducing stroke risk and damage in hypertensive patients.

4. Experimental procedure

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

4.1. Peptide preparation

TAT_(47–57) carrier peptide (TAT) (Schwarze et al., 1999) and δ V1-1-TAT_(47–57) (δ PKC inhibitor peptide) were synthesized and conjugated via a Cys S–S bond (Chen et al., 2001). Selectivity of δ V1-1-TAT for δ PKC, as compared with other isozymes, has previously been demonstrated (Chen et al., 2001; Begley et al., 2004).

4.2. MCA occlusion model

Transient focal ischemia was induced in male normotensive Sprague–Dawley rats (Charles River, MA; 290–320 g) or hypertensive Dahl salt-sensitive rats (Brookhaven Labs, New York; 290–320 g) using an occluding intraluminal suture, as previously described (Bright et al., 2004). Briefly, an uncoated 30 mm long segment of 3-0 nylon monofilament suture with the tip rounded by a flame was inserted into the stump of the external carotid artery and advanced into the internal carotid artery approximately 19–20 mm from the bifurcation to occlude the ostium of the middle cerebral artery (MCA). The ischemic period in the Sprague–Dawley rat was 120 min. The ischemic period in the Dahl hypertensive rat was 90 min due to high mortality using a 120 min occlusion period. Following the ischemic period the suture was removed and the animal was allowed to recover. In the hypertensive model, peptides were delivered chronically using an Alzet subcutaneous pump (Alza, CA; 1 mM, 5 μ L/h), implanted dorsally, for a period spanning 4–5 days prior to surgery until sacrifice at 24 h following surgery (Inagaki et al., 2005).

4.3. Transmission electron microscopy

Sprague–Dawley rats underwent 120 min MCA occlusion, followed by 4 h of reperfusion. Rats were treated with either TAT or δ V1-1-TAT peptides at the onset of reperfusion by bolus intraperitoneal injection (0.2 mg/kg in 1 mL, $n=4$ per group). Sham animals were similarly treated, but did not undergo ischemia, and did not receive peptides ($n=4$). Following the reperfusion period, rats were heavily anesthetized using isoflurane, and underwent transcardial perfusion using 0.7% NaCl/PBS followed by 4% formaldehyde in 0.7% NaCl/PBS. Brains were removed and kept at 4 °C overnight in 2% formaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. Brain sections (~ 1 mm³) were then dissected from the cortical surface, at ~ 1 mm posterior/ ~ 6 mm lateral to bregma on the ipsilateral cortex (corresponding to the area measured by laser Doppler flowmetry). Tissue was post-fixed in 1% osmium tetroxide for 1 h, washed with water, and stained in 1% uranyl acetate solution. Tissue was dehydrated using an ethanol gradient followed by 100% propylene oxide, and infiltrated with Epon and Embed 812 medium, placed in molds, and polymerized overnight at 65 °C before slicings. Sections (75 and 90 nm) were picked up on

formvar/Carbon coated Cu grids, stained for 15 s in 1:1 Super-saturated uranyl acetate (7.7%) in acetone followed by staining in 0.2% lead citrate for 3 to 4 min (all reagents from Electron Microscopy Sciences, PA). Tissue was observed in the JEOL 1230 transmission electron microscope (TEM) at 80 kV and photos were taken using a Gatan Multiscan 791 digital camera.

To quantitate number of patent vessels, 10–15 non-overlapping fields (1200× magnification) were photographed from 2–3 slices taken from each animal. Vessels were identified and counted if lumen and endothelial lining was apparent; vessels were confirmed by scanning over these fields at higher magnification. Assessments of vessel count were performed by an observer blinded to treatment group. The number of vessels per field was then averaged for each group of animals ($n=4$ per group). Statistical differences between groups were analyzed using an unpaired *t*-test. Higher magnification images (5000–15000×) were taken to assess microvascular morphology.

4.4. Laser Doppler flowmetry

Cerebral blood flow (CBF) was measured in two rat models of vascular stress; a normotensive model of transient focal ischemia, and a chronic hypertension model.

Acute stroke model—Sprague–Dawley rats underwent 120 min MCA occlusion. Following ischemia, a burr hole was drilled 1 mm posterior and 6 mm lateral to bregma, corresponding to the ischemic territory. A laser Doppler transducer probe (Laserflo) was affixed above the cortex using a stereotaxic frame (Harvard Apparatus, MA), and a baseline CBF was established over a period of 20–25 min (recordings at 1 min intervals). TAT control peptide ($n=4$) or $\delta V1-1$ -TAT peptide ($n=9$) was injected by intraperitoneal injection (0.2 mg/kg in 1 mL), and CBF was monitored for an additional 20–60 min. CBF was also measured in the sham control animals ($n=4$).

Hypertensive rat model—6 week old Dahl salt-sensitive rats were placed on an 8% high-salt diet to induce hypertension. CBF was measured, as described above, at 11 weeks, 4 weeks following high-salt diet onset. Following baseline CBF recording for a period of 20–30 min (1 min intervals), TAT control peptide ($n=5$) or $\delta V1-1$ -TAT peptide ($n=15$) was injected by intraperitoneal injection (0.2 mg/kg in 1 mL) and CBF was monitored for an additional 20–30 min. A tail-cuff blood pressure monitor was used to measure blood pressure in Dahl rats chronically treated with $\delta V1-1$ -TAT or TAT peptide using a subcutaneous osmotic pump weekly for a period of 2 weeks.

Analysis of changes in CBF was performed by comparing the baseline flow-rate at 20 min, to the flow-rate at 25 min post-treatment in all animals. Results were expressed both as a scattergram of percent flow changes in individual animals, and as the mean $\pm$ S.E.M. flow in each group. Statistical analysis between TAT and $\delta V1-1$ -TAT treatment groups was performed using an unpaired *t*-test.

4.5. Assessment of brain infarct size in hypertensive rats

Animals were euthanized following 24 h of reperfusion by isoflurane overdose. Brains were quickly removed and sliced into 3 mm coronal sections, resulting in five slices of the brain. Slices were stained using 3% triphenyl tetrazolium chloride (TTC) and both faces of each slice were photographed for infarct assessment. 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) of the five slices made from each brain (Bright et al., 2004). Results were expressed both as a scattergram of individual animals, and as the mean $\pm$

S.E.M. infarct size of these six faces. Infarct size statistics between TAT and δ V1-1-TAT treated groups were performed using a Student's *t*-test.

4.6. Western blot analysis

Brains from Dahl hypertensive rats that received δ V1-1-TAT or TAT peptide delivery for 4 weeks, starting at 11 weeks of age, were quickly isolated and frozen. Tissue fractionation was performed to collect soluble (cytosolic) and particulate (membrane) fractions, as previously described (Johnson and Mochly-Rosen, 1995). A total of 5 μ g of protein from each fraction was used for western blot analysis using a rabbit anti-phosphoserine 643 δ PKC antibody (Santa Cruz Biotechnology; 1:500) followed with an anti-rabbit secondary antibody (Amersham). Density of δ PKC bands was measured to assess translocation of PKC from the cytosol to membrane fractions, a measure of activation (Kraft and Anderson, 1983). Blots were then reprobbed with a rabbit anti- ϵ PKC antibody (Santa Cruz Biotechnology; 1:500), to assess translocation of the ϵ PKC isozyme. Statistical analysis was performed using Student's *t*-test.

Acknowledgments

The work in the hypertensive rat model was triggered by a preliminary observation by Dr. Koichi Inagaki. The authors gratefully acknowledge the assistance of John Perrino with the transmission electron microscopy study. This work was supported by research grants HL76675 and NS 44350 (DMR), and NIH-NINDS predoctoral fellowship NS45413-01 (RB).

References

- 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]
- Arboix A, Roig H, Rossich R, Martinez EM, Garcia-Eroles L. Differences between hypertensive and non-hypertensive ischemic stroke. *Eur J Neurol.* 2004; 11:687–692. [PubMed: 15469453]
- Armstead WM. Superoxide generation links protein kinase C activation to impaired ATP-sensitive K⁺ channel function after brain injury. *Stroke.* 1999; 30:153–159. [PubMed: 9880404]
- Ay H, Koroshetz WJ, Vangel M, Benner T, Melinosky C, Zhu M, Menezes N, Lopez CJ, Sorensen AG. Conversion of ischemic brain tissue into infarction increases with age. *Stroke.* 2005; 36:2632–2636. [PubMed: 16269639]
- Beg SS, Hansen-Schwartz JA, Vikman PJ, Xu CB, Edvinsson LI. Protein kinase C inhibition prevents upregulation of vascular ET(B) and 5-HT(1B) receptors and reverses cerebral blood flow reduction after subarachnoid haemorrhage in rats. *J Cereb Blood Flow Metab.* 2007; 27:21–32. [PubMed: 16736053]
- 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]
- Bright R, Raval AP, Dembner JM, Perez-Pinzon MA, Steinberg GK, Yenari MA, Mochly-Rosen D. Protein kinase C delta mediates cerebral reperfusion injury in vivo. *J Neurosci.* 2004; 24:6880–6888. [PubMed: 15295022]
- Brodie C, Blumberg PM. Regulation of apoptosis by protein kinase C delta. *Apoptosis.* 2003; 8:19–27. [PubMed: 12510148]
- Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW II, Mochly-Rosen D. Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. *Proc Natl Acad Sci U S A.* 2001; 98:11114–11119. [PubMed: 11553773]
- Chou WH, Choi DS, Zhang H, Mu D, McMahon T, Kharazia VN, Lowell CA, Ferriero DM, Messing RO. Neutrophil protein kinase Cdelta as a mediator of stroke–reperfusion injury. *J Clin Invest.* 2004; 114:49–56. [PubMed: 15232611]

- Churchill EN, Szveda LI. Translocation of deltaPKC to mitochondria during cardiac reperfusion enhances superoxide anion production and induces loss in mitochondrial function. *Arch Biochem Biophys*. 2005; 439:194–199. [PubMed: 15963450]
- del Zoppo GJ, Mabuchi T. Cerebral microvessel responses to focal ischemia. *J Cereb Blood Flow Metab*. 2003; 23:879–894. [PubMed: 12902832]
- Faraci FM. Vascular protection. *Stroke*. 2003; 34:327–329. [PubMed: 12574525]
- Fukunaga K, Soderling TR, Miyamoto E. Activation of Ca²⁺/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons. *J Biol Chem*. 1992; 267:22527–22533. [PubMed: 1358879]
- Gursoy-Ozdemir Y, Can A, Dalkara T. Reperfusion-induced oxidative/nitrative injury to neurovascular unit after focal cerebral ischemia. *Stroke*. 2004; 35:1449–1453. [PubMed: 15073398]
- Henrion D, Laher I. Effects of staurosporine and calphostin C, two structurally unrelated inhibitors of protein kinase C, on vascular tone. *Can J Physiol Pharmacol*. 1993; 71:521–524. [PubMed: 7694783]
- Inagaki K, Iwanaga Y, Sarai N, Onozawa Y, Takenaka H, Mochly-Rosen D, Kihara Y. Tissue angiotensin II during progression of ventricular hypertrophy to heart failure in hypertensive rats; differential effects on PKC epsilon and PKC beta. *J Mol Cell Cardiol*. 2002; 34:1377–1385. [PubMed: 12392998]
- Inagaki K, Chen L, Ikeno F, Lee FH, Imahashi K, Bouley DM, Rezaee M, Yock PG, Murphy E, Mochly-Rosen D. Inhibition of delta-protein kinase C protects against reperfusion injury of the ischemic heart in vivo. *Circulation*. 2003; 108:2304–2307. [PubMed: 14597593]
- 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]
- Iwai J, Heine M. Dahl salt-sensitive rats and human essential hypertension. *J Hypertens Suppl*. 1986; 3:S29–S31. [PubMed: 3465902]
- Jin Y, Sagher O, Thai QA, Kassell NF, Lee KS. The effects of papaverine on phorbol dibutyrate-induced vasoconstriction in brain slice microvessels. *J Neurosurg*. 1994; 81:574–578. [PubMed: 7931591]
- 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]
- Kaasinen SK, Goldsteins G, Alhonen L, Janne J, Koistinaho J. Induction and activation of protein kinase C delta in hippocampus and cortex after kainic acid treatment. *Exp Neurol*. 2002; 176:203–212. [PubMed: 12093097]
- Kheifets V, Bright R, Inagaki K, Schechtman D, Mochly-Rosen D. Protein kinase C delta (deltaPKC)-annexin V interaction: a required step in deltaPKC translocation and function. *J Biol Chem*. 2006; 281:23218–23226. [PubMed: 16785226]
- 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]
- Laher I, Zhang JH. Protein kinase C and cerebral vasospasm. *J Cereb Blood Flow Metab*. 2001; 21:887–906. [PubMed: 11487724]
- La Porta CA, Comolli R. Biochemical and immunological characterization of calcium-dependent and -independent PKC isoenzymes in renal ischemia. *Biochem Biophys Res Commun*. 1993; 191:1124–1130. [PubMed: 8466490]
- Majumder PK, Mishra NC, Sun X, Bharti A, Kharbanda S, Saxena S, Kufe D. Targeting of protein kinase C delta to mitochondria in the oxidative stress response. *Cell Growth Differ*. 2001; 12:465–470. [PubMed: 11571229]
- Melgar MA, Rafols J, Gloss D, Diaz FG. Postischemic reperfusion: ultrastructural blood–brain barrier and hemodynamic correlative changes in an awake model of transient forebrain ischemia. *Neurosurgery*. 2005; 56:571–581. [PubMed: 15730583]

- Miettinen S, Roivainen R, Keinänen R, Hokfelt T, Koistinaho J. Specific induction of protein kinase C delta subspecies after transient middle cerebral artery occlusion in the rat brain: inhibition by MK-801. *J Neurosci*. 1996; 16:6236–6245. [PubMed: 8815904]
- Murray MA, Faraci FM, Heistad DD. Role of protein kinase C in constrictor responses of the rat basilar artery in vivo. *J Physiol*. 1992; 445:169–179. [PubMed: 1501132]
- Murriel CL, Churchill E, Inagaki K, Szweda LI, Mochly-Rosen D. Protein kinase Cdelta activation induces apoptosis in response to cardiac ischemia and reperfusion damage: a mechanism involving BAD and the mitochondria. *J Biol Chem*. 2004; 279:47985–47992. [PubMed: 15339931]
- Nakane H, Yao H, Ibayashi S, Kitazono T, Ooboshi H, Uchimura H, Fujishima M. Protein kinase C modulates ischemia-induced amino acids release in the striatum of hypertensive rats. *Brain Res*. 1998; 782:290–296. [PubMed: 9519275]
- Rasool AH, Rahman AR, Choudhury SR, Singh RB. Blood pressure in acute intracerebral haemorrhage. *J Hum Hypertens*. 2004; 18:187–192. [PubMed: 14973513]
- Raval AP, Dave KR, Prado R, Katz LM, Busto R, Sick TJ, Ginsberg MD, Mochly-Rosen D, Perez-Pinzon MA. Protein kinase C delta cleavage initiates an aberrant signal transduction pathway after cardiac arrest and oxygen glucose deprivation. *J Cereb Blood Flow Metab*. 2005; 25:730–741. [PubMed: 15716854]
- Ramzy D, Rao V, Tumiati LC, Xu N, Sheshgiri R, Miriuka S, Delgado DH, Ross HJ. Elevated endothelin-1 levels impair nitric oxide homeostasis through a PKC-dependent pathway. *Circulation*. 2006; 114:I319–I326. [PubMed: 16820593]
- Rosendorff C. Endothelin, vascular hypertrophy and hypertension. *Cardiovasc Drugs Ther*. 1997; 10:795–802. [PubMed: 9110124]
- Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science*. 1999; 285:1569–1572. [PubMed: 10477521]
- Smeda JS, Payne GW. Alterations in autoregulatory and myogenic function in the cerebrovasculature of Dahl salt-sensitive rats. *Stroke*. 2003; 34:1484–1490. [PubMed: 12750541]
- Strandgaard S. Autoregulation of cerebral circulation in hypertension. *Acta Neurol Scand Suppl*. 1978; 66:1–82. [PubMed: 96654]
- Takahashi M, Macdonald RL. Vascular aspects of neuroprotection. *Neurol Res*. 2004; 26:862–869. [PubMed: 15727270]
- Uhl MW, Kochanek PM, Schiding JK, Nemoto EM. Effect of phorbol myristate acetate on cerebral blood flow in normal and neutrophil-depleted rats. *Stroke*. 1993; 24:1977–1982. [PubMed: 8248980]
- Vaccarino FM, Liljequist S, Tallman JF. Modulation of protein kinase C translocation by excitatory and inhibitory amino acids in primary cultures of neurons. *J Neurochem*. 1991; 57:391–396. [PubMed: 1649249]
- Vanden Hoek T, Becker LB, Shhao ZH, Li CQ, Schumacker PT. Preconditioning in cardiomyocytes protects by attenuating oxidant stress at reperfusion. *Circ Res*. 2000; 86:541–548. [PubMed: 10720416]
- Yang GY, Betz AL. Reperfusion-induced injury to the blood–brain barrier after middle cerebral artery occlusion in rats. *Stroke*. 1994; 25:1658–1664. [PubMed: 8042219]
- Yoshida K, Mizukami Y, Kitakaze M. Nitric oxide mediates protein kinase C isoform translocation in rat heart during postischemic reperfusion. *Biochem Biophys Acta*. 1999; 1453:230–238. [PubMed: 10036321]
- Zheng Z, Yenari MA. Post-ischemic inflammation: molecular mechanisms and therapeutic implications. *Neurol Res*. 2004; 26:884–892. [PubMed: 15727272]

Abbreviations

PKC	Protein kinase C
CBF	Cerebral blood flow

Fig. 1.

Delivery of $\delta V1-1$ -TAT decreases microvascular pathology following ischemic reperfusion injury. (A) Ischemia was induced for 2 h using intraluminal suture occlusion model (MCA occlusion) followed by 4 h of reperfusion. $\delta V1-1$ -TAT or TAT control peptides were delivered by single intraperitoneal dose (0.2 mg/kg) at the onset of reperfusion. Sham animals were treated similarly but they did not receive ischemia or peptide treatments. The defined region (boxed gray area) of the ipsilateral cerebral cortex that was prepared for TEM analysis is shown schematically. (B) Low magnification transmission electron micrographs were taken of cerebral microvasculature, and vessels in 10–15 non-overlapping fields were counted in sham, and ischemic animals treated with TAT control, or $\delta V1-1$ -TAT peptide. Following stroke, there was a 58% decrease of discernable microvessels compared to sham controls, which was markedly prevented in animals treated with $\delta V1-1$ -TAT following ischemia (* $p < 0.05$ compared with TAT control; scored by blinded observer); there was no significant reductions in microvessel number compared to sham controls. (C,G,K) Low magnification images (1200×) demonstrate reduction in number of microvessels (arrows) in animals subject to ischemia treated with TAT control (G) and $\delta V1-1$ -TAT peptide (K), compared to sham controls (C). High magnification images (5000–15,000×) demonstrate vascular pathology in brains from sham animals (D–F), and animals following ischemia/reperfusion damage (H–J). Vascular damage, including astrocyte end-foot edema (asterisk in H,L), enlarged nuclei (arrow in I), basal lamina breakdown and loss of lumen patency (I) were apparent in animals treated with TAT control peptide following ischemic/reperfusion injury. Animals treated with $\delta V1-1$ -TAT had reduced signs of pathology (L–N).

Fig. 2.

Delivery of δ V1-1-TAT increases cerebral blood flow during ischemic reperfusion injury. (A) Protocol for ischemic reperfusion injury and measurement of cerebral blood flow. Ischemia was induced for 2 h using intraluminal suture occlusion model (MCA occlusion). Following removal of the suture, cerebral blood flow was monitored using laser Doppler for 20–25 min to establish baseline flow. δ V1-1-TAT or TAT control peptides were delivered by single intraperitoneal dose (0.2 mg/kg), and laser Doppler measurements were taken for an additional 20–60 min. (B) Location of laser Doppler flow probe on cortical surface (1 mm posterior, 6 mm lateral to bregma), consistent with cortical ischemic penumbra is shown schematically. (C) Representative CBF trace showing no effect of TAT delivery and δ V1-1-TAT delivery in sham animals. Flow values are shown as a percentage of initial baseline reading. (D) Representative CBF trace showing no change in flow with TAT control peptide delivery, and increase in flow with δ V1-1-TAT peptide delivery after stroke. Flow values are shown as a percentage of initial baseline reading. (E) Scattergram of all animals: sham animals treated with δ V1-1-TAT peptide ($-7 \pm 6\%$; $n=4$), and ischemic animals treated with TAT ($0 \pm 3\%$; $n=4$) or δ V1-1-TAT peptide ($26 \pm 7\%$; $n=9$). Individual animals (triangles), and average increase in flow of animals in group (circles) are given as percent change in CBF from baseline following peptide delivery (* $p < 0.05$ compared with baseline).

Fig. 3.

Delivery of $\delta V1-1-TAT$ increases cerebral blood flow in chronic hypertension model. (A) Representative trace of increase in cerebral blood flow following intraperitoneal delivery of $\delta V1-1-TAT$ peptide (0.2 mg/kg). Flow values are shown as a percentage of initial baseline reading. (B) Scattergram of all animals treated with $\delta V1-1-TAT$ peptide, graphed as average percent increase in CBF from baseline following peptide delivery. Individual animals (triangles), and average increase in flow of animals in each group (circles) are given as percent change in CBF from baseline following peptide delivery. Animals treated with $\delta V1-1-TAT$ peptide showed a significant increase in CBF from baseline by $12 \pm 4\%$ ($n=15$, $*p<0.05$). TAT control peptide did not alter CBF from baseline flow ($-5 \pm 2\%$; $n=5$).

Fig. 4.

Sustained delivery of δV1-1-TAT selectively inhibits δPKC activity in the brain of Dahl hypertensive rats. Dahl rats were fed an 8% diet to induce hypertension starting at age 6 weeks. At 11 weeks of age, subcutaneous pumps were implanted for delivery of TAT or δV1-1-TAT peptide (1 mM, 5 μL/h; $n=3$ per group). (A) Sustained delivery of δV1-1-TAT inhibited activity of δPKC enzyme in the brain of hypertensive rats, as assessed by translocation of the enzyme from the cytosol to the membrane bound state (δV1-1-TAT, $40 \pm 5\%$ translocation, TAT $64 \pm 5\%$ translocation, $*p < 0.05$). (B) Chronic delivery of δV1-1-TAT did not affect the activity of εPKC, a related PKC isozyme, demonstrating the isozyme selectivity of the δV1-1-TAT peptide.

Fig. 5.

Sustained delivery of δ V1-1-TAT peptide reduces infarct size following an acute transient focal ischemia in hypertensive rats (A) Protocol for acute ischemia model. MCA occlusion was performed in 10–11 week old animals, 5–6 days following implantation of osmotic pumps continuously delivering TAT or δ V1-1-TAT peptides (1 mM, 5 μ L/h). Animals underwent 90 min MCA occlusion, followed by 24 h of survival. (B) Representative photographs of TTC stained brain slices with cerebral infarction in rats treated with TAT or δ V1-1-TAT peptides. (C) Average infarct size in animals treated with TAT control peptide or δ V1-1-TAT peptide. Animals treated with δ V1-1-TAT showed a 25% reduction in infarct size compared to TAT-control treated animals ($n=6-7$ per group; $*p<0.05$).