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Differential Effects of Delta and Epsilon Protein Kinase C in Modulation of Postischemic Cerebral Blood Flow

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Abstract

Cerebral ischemia causes cerebral blood flow (CBF) derangements resulting in neuronal damage by enhanced protein kinase C delta (δPKC) levels leading to hippocampal and cortical neuronal death after ischemia. Contrarily, activation of εPKC mediates ischemic tolerance by decreasing vascular tone providing neuroprotection. However, whether part of this protection is due to the role of differential isozymes of PKCs on CBF following cerebral ischemia remains poorly understood. Rats pretreated with a δPKC specific inhibitor (δV1-1, 0.5 mg/kg) exhibited attenuation of hyperemia and latent hypoperfusion characterized by vasoconstriction followed by vasodilation of microvessels after two-vessel occlusion plus hypotension. In an asphyxial cardiac arrest (ACA) model, rats treated with δ V1-1 (pre- and postischemia) exhibited improved perfusion after 24 h and less hippocampal CA1 and cortical neuronal death 7 days after ACA. On the contrary, εPKC-selective peptide activator, conferred neuroprotection in the CA1 region of the rat hippocampus 30 min before induction of global cerebral ischemia and decreased regional CBF during the reperfusion phase. These opposing effects of δ v. εPKC suggest a possible therapeutic potential by modulating CBF preventing neuronal damage after cerebral ischemia.

1 Introduction

Cerebral ischemia is defined by little or no blood flow in cerebral circulation characterized by low oxygen, glucose, and accumulation of metabolic products [1]. Upon reperfusion, hyperemia (increased blood flow) and subsequent hypoperfusion (decreased blood flow) of cerebral blood vessels [1] leads to enhanced superoxide generation [2]. The hyperemia phase leads to ischemia-induced cell death to different areas of the brain [2] as well as decreased blood flow (hypoperfusion) [3] resulting in yet another possible ischemic/hypoxic condition.

Protein kinase C (PKC) isozyme, namely δPKC, plays an important role in mediating cerebral reperfusion injury after ischemia [4]. Previously, we demonstrated that δPKC played a key role in CA1 rat hippocampal histopathology following asphyxial cardiac arrest (ACA) [5]. δPKC also modulates micro-cerebrovascular function in acute ischemia by mediating vascular tone, suggesting that δPKC may be involved in microvascular dynamics in the brain [6], which is functionally important and not well characterized due to previous technological limitations. Thus, the neuroprotection afforded by the specific δPKC inhibitor in our previous study after ACA [5] may also be due to CBF modulation.

εPKC is another novel PKC isozyme that has also been implicated in CBF dysfunction and neuronal viability during ischemia. Activation of εPKC during ischemia is thought to be beneficial, providing neuroprotection [7] and appropriate modulation of CBF after ischemia. The neuroprotective properties of e PKC in preconditioning have led to the testing of

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agonists of εPKC [ψε receptors for activated C kinase (RACK)] as possible therapy in cerebral ischemia. We present the detrimental effects (CBF derangement and neuronal damage) of δPKC activation in the brain after global cerebral ischemia [8], while activation of εPKC can reverse these pathologies caused by ischemia [9].

2 Methods

2.1 Chemicals

δPKC inhibitor (δV1-1), εPKC agonist (ψεRACK) [εPKC activator, amino acids 85-92 (HDAPIGYD)], and tat carrier peptide (control) were dissolved in sterile saline (0.9%) (KAI Pharmaceuticals Inc., San Francisco, CA, USA). A final volume of 700 µl (Tat peptide or δV1-1) was injected intravenously (IV) 30 min before induction of two-vessel occlusion with hypotension (2-VO) or ACA. Fluorescein isothiocyanate (FITC)-dextran (MW, 2,000,000) (0.2 mg/ml) was injected IV every 30 min to visualize blood flow and microvessels.

2.2 Animal Model

All experimental procedures were approved by the laboratory animal care and use committee (University of Miami, Miller School of Medicine). Adult male Sprague-Dawley rats (250–350 g) were fasted overnight before surgery. Rats were anesthetized with 4% isoflurane and a 30:70 mixture of oxygen and nitrous oxide followed by endotracheal intubation. Isoflurane was lowered to 1.5–2% for endovascular access. The femoral vein and artery were cannulated using a single-lumen (PE-50) catheter for blood pressure monitoring, blood gas analysis, and intravenous (IV) injection of pharmacological agents.

2.3 Two-Vessel Occlusion with Hypotension

After cannulation, hypotension was induced by withdrawing blood from the femoral artery reducing systemic blood pressure to 45–50 mmHg during ischemia. Next, cerebral ischemia was induced by tightening the carotid ligatures bilaterally for 10 min. To allow postischemic reperfusion, the carotid ligatures were removed, and shed blood re-injected into the artery restoring mean arterial blood pressure to baseline levels (∼130–140 mmHg) [10].

2.4 Asphyxial Cardiac Arrest

To induce ACA, apnea was induced by disconnecting the ventilator from the endotracheal tube. Six minutes after asphyxia, resuscitation was initiated by administering a bolus injection of epinephrine (0.005 mg/kg, IV) and sodium bicarbonate (1 meq/kg, IV) followed by mechanical ventilation. Arterial blood gases were then measured. After ACA, the animal was placed directly on the 2-photon microscopy (2-PM) stage with the stereotaxic device in place for cortical microvessel imaging [8].

2.5 Two-Photon Microscopy

A thin circular area of the skull (∼2 mm in diameter, 1 mm from bregma) was made via micro-drill until the skull was half the thickness. The rat was placed on a custom stereotaxic device on the microscope stage of the 2-PM (Lasersharp2000, BioRad). Fluorescent images were captured at 910 nm with the introduction of FITC-dextran (0.2 mg/kg), IV. Linescans

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for red blood cell (RBC) velocities and blood vessel diameter measurements were analyzed with Image J analysis software [8].

2.6 Laser-Doppler Flowmetry

A 2 mm^2 burr hole was made over the left frontoparietal cortex approximately 5.0 mm posterior and 3.5 mm lateral to bregma. A fiber-optic probe (1 mm) was placed measuring cerebral blood perfusion on a 1 mm^3 tissue region [8].

2.7 Histopathology

Seven days after ACA insults, rats were anesthetized and perfused from the ascending aorta with physiologic saline (1 min), following a mixture of 40% formaldehyde, glacial acetic acid, and methanol (FAM) for 19 min and immersed in FAM at 4°C (24 h). Rat brains were removed from the skull and coronal sections (10 mm thickness) from the brains were paraffin embedded and stained with hematoxylin and eosin. CA1 hippocampal sections were visualized at 40× magnifi cation. Ischemic neurons were counted at 18 fields/section along the medial to lateral extent of the CA1 region of the hippocampus 3.8 mm posterior to the bregma. In the same series of animals, brain sections spanning the subfornical organ (SFO) were analyzed to assess cortical (layers II–IV) neuronal damage 7 days after ACA. This region was determined to be −1.4 to −0.8 mm with respect to bregma, similar to the location where 2-PM imaging of cortical microvessels was analyzed [8].

3 Results and Discussion

Representative images of cortical microvasculature are shown in Fig. 10.1a, b. Linescans of RBC traversing the microvasculature were measured (Fig. 10.1c). Black shadows traveling from left to right indicate RBC flow across the scanned vessel. A less negative slope (rise/run) represents RBC (baseline) traveling at a faster rate whereas a more negative slope represents RBC traveling at a slower rate.

Rats pretreated with δV1-1 for 30 min significantly attenuated hyperemia (<75% change in flow at 5 and 15 min) and hypoperfusion (<75% change in fl ow at 45 and 60 min) after 2-VO [8]. In the presence of δV1-1, postischemic hyperemia and hypoperfusion suggest cortical microvessel vasoconstriction (<−20% change in blood vessel diameter 5 and 10 min with δV1-1 treated animals) followed by vasodilation (>10% change in blood vessel diameter 45 and 60 min with δV1-1 treated animals) after 2-VO [8]. Moreover, δV1-1 pretreatment significantly attenuated hyperemia 5 min after 2-VO [52% ± 28 increase in flow measured by laser-Doppler flowmetry (LDF)] [8]. Vasoactive factors that influence vasoconstriction/vasodilation during reperfusion have been highly debated over the years. Nonetheless, a recent development in cerebral vascular physiology describes a novel and highly potent vasodilator (palmitic acid methyl ester) that may be involved in PKC regulation during cerebral ischemia [11].

We also measured CBF changes with the 2-PM technique 24 h after ACA in the presence or absence of δV1-1. Rats pretreated with δV1-1 enhanced CBF 24 h after ACA (700% increase in flow as compared to tat peptide) (Fig. 10.1d). Since δV1-1 pretreatment may be protecting the brain parenchyma against the deleterious effects of the ischemic insult, in a

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separate set of experiments, δV1-1 was administered directly after ACA and CBF changes were monitored via 2-PM. CBF was enhanced in the presence of δV1-1 postarrest (311 and 314% increase in CBF 45 and 60 min after ACA, respectively, as compared to tat peptide) (Fig. 10.1e). These results suggest that posttreatment with δV1-1 attenuated ACA-induced hypoperfusion. Additionally, our results also showed that pretreatment with δV1-1 in the rat inhibited δPKC translocation after ACA with a 61% reduction as compared to tat peptide (vehicle) treatment (Fig. 10.2). These results further confirm that δV1-1 inhibits translocation of δPKC to the cellular membrane in the brain, rendering δPKC inactive. These findings can also be extended from CBF dynamics to neuronal cell viability by infl uence via δPKC levels. Our findings suggest that δPKC promotes neuronal cell death following 6 min of ACA. Sham-operated experimental group (1,023 ± 11 normal neurons in CA1 region of the hippocampus) was used as control. Rats pretreated with δV1-1 afforded neuroprotection (813 ± 62 normal neurons) in the CA1 region of the rat hippocampus 7 days after 6 min of ACA, as compared to tat peptide-treated animals (639 ± 20 normal neurons) and control (527 ± 19 normal neurons). Similar results were found in the cortex since cortical CBF was measured throughout this study. Rats pretreated with d V1-1 (3,187 ± 458.0 dead neurons) afforded neuroprotection suggesting a decrease in the number of dead cortical neurons as compared to no drug (8,400 ± 1,621 dead neurons) and tat peptide (6,863 ± 645.5 dead neurons) experimental groups. These results suggest that δPKC is intimately involved in neuronal cell viability in the hippocampus as well as in cortex [8].

It has already been demonstrated that εPKC activation in certain pathological situations can prove beneficial in the heart and brain [7,12,13]. The role of εPKC during ischemia is thought to be benefi cial during and after injury but this has not been well defined. Our histological assessment of the CA1 of the rat hippocampus 7 days after 2-VO suggests that upon pretreatment of ψεRACK, the number of normal neurons (712 ± 109) increased as compared to vehicle (429 ± 90) or tat peptide (429 ± 90) treated groups by 38 and 25%, respectively. These results suggest that pretreatment with ψεRACK (712 ± 109) afforded neuroprotection due to the fact that the number of normal neurons in sham-operated rats was 1,034 ± 58 [9].

Similar to δPKC, we suspect that the neuroprotective effect of εPKC is directly related to CBF after 2-VO. Therefore, we measured regional CBF with LDF 30 min before induction of ischemia and up to 1 h following reperfusion. There was a 30% reduction in regional CBF in ψεRACK-treated groups during the fi rst 25 min of reperfusion after ischemia as compared to tat peptide groups (Fig. 10.3) [9]. These results suggest that εPKC activation may be important in regulating hyperemia after ischemia. The differential actions of δ and εPKCs can be further emphasized by the fact that inhibition of δPKC can attenuate hyperemia after 2-VO [8].

Our data suggest that δ and εPKCs are involved in mediating hyperemia and during reperfusion after global cerebral ischemia. Currently, only δPKC is thought to mediate both states of hyperemia and hypoperfusion during cerebral ischemia. In addition, δV1-1 can modulate postischemic blood flow/vessel dynamics that may be beneficial in the treatment of cerebral ischemia. Using in vivo models of cerebral ischemia, we have demonstrated that δPKC is activated following cardiac arrest. When activation of δPKC is attenuated with a

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peptide inhibitor or enhanced with εPKC agonist, the brain is protected from ischemic damage [8,9] through possible revival and/or stabilization of cerebral circulation. The therapeutic potential by inhibiting δPKC and/or activating εPKC may prove beneficial in the treatment of cerebral ischemia.

Acknowledgments

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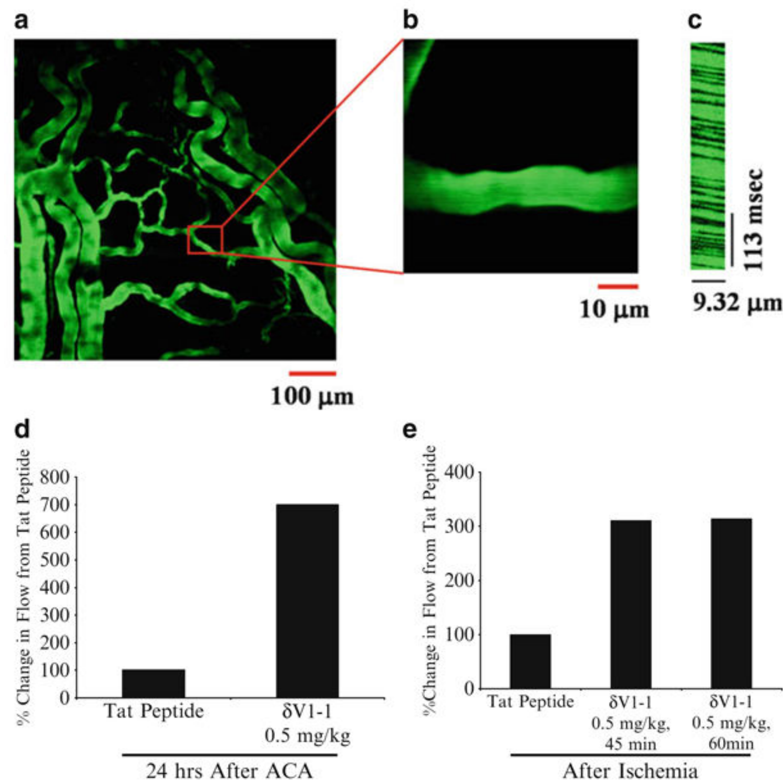

Fig. 10.1.

In vivo imaging of cerebral blood vessels using 2-photon microscopy (2-PM). Rats were injected with fluorescein isothiocyanate (FITC)-dextran (0.2 mg/kg) shown in *green* (**a**, **b**). Images were captured at 20× (**a**) and 200× (**b**) of a particular cortical blood vessel. Blood flow measurements using linescans from single vessels (linescans at 512 Hz) were used to determine red blood cell (RBC) flow (**c**). RBC flow values were calculated based on the slope of the shadows (measured using NIH Image J) produced by RBCs when traversed through the blood vessel. Pretreatment with δV1-1-induced attenuation of hypoperfusion 24 h after ACA. Rats pretreated (30 min) with δV1-1 after 24 h of ACA signifi cantly enhanced CBF by 700% as compared to tat peptide (vehicle) (**d**). In a separate set of experiments, δV1-1 was administered directly after 6 min of ACA and CBF changes were monitored. CBF was enhanced 45 and 60 min after ACA by 311 and 314%, respectively, as compared to tat peptide (**e**). For more details, please see Lin et al. [8]

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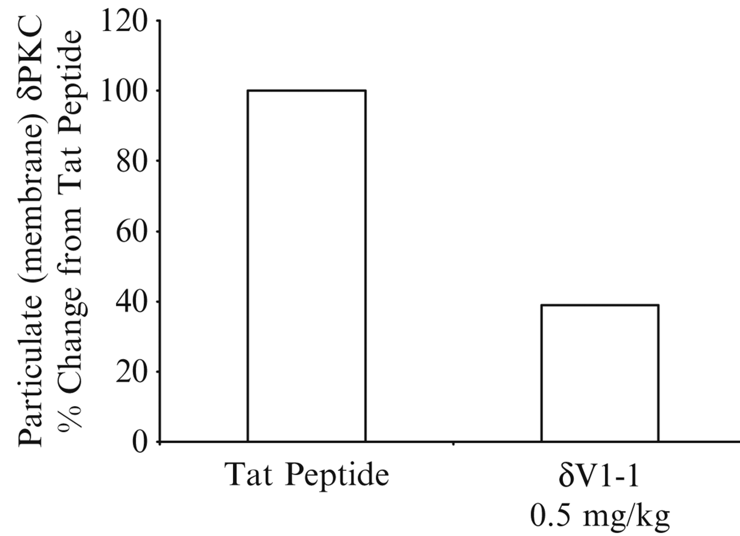

Fig. 10.2.
δV1-1 inhibits δPKC translocation in cortical lysates. Pretreatment with δV1-1 in the rat inhibited δPKC translocation after 6 min of ACA with a 61% reduction in protein as compared to tat peptide (vehicle) detected by Western blot analysis. For more details, please see Lin et al. [8]

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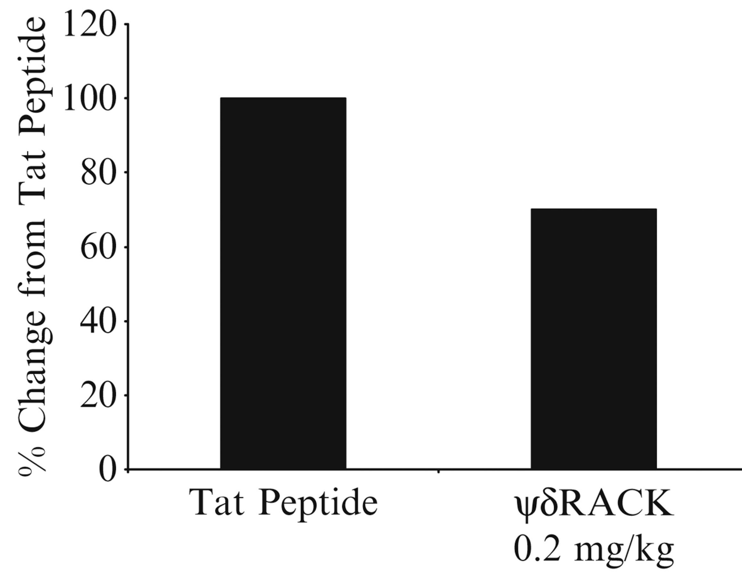

Fig. 10.3.

Rats pretreated with ψεRACK reduced cerebral reperfusion after 2-VO. A bolus IV injection of ψεRACK 30 min before ischemia reduced postischemic hyperemia by 30% as compared to tat peptide 25 min after 2-VO detected via laser-Doppler flowmetry. For more details, please see Della-Morte et al. [9]