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Activating δ PKC antagonizes the protective effect of ERK1/2 inhibition against stroke in rats

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Abstract

Two pathways that have been shown to mediate cerebral ischemic damage are the MEK/ERK cascade and the pro-apoptotic δ PKC pathway. We investigated the relationship between these pathways in a rat model of focal ischemia by observing and modifying the activation state of each pathway. The ERK1/2 inhibitor, U0126, injected at ischemia onset, attenuated the increase in phosphorylated ERK1/2 (P-ERK1/2) after reperfusion. The δ PKC inhibitor, δ V1-1, delivered at reperfusion, did not significantly change P-ERK1/2 levels. In contrast, the δ PKC activator, $\psi\delta$ RACK, injected at reperfusion, reduced ERK1/2 phosphorylation measured 4 h after reperfusion. Additionally, U0126 pretreatment at ischemia onset reduced infarct size compared with vehicle, but U0126 injected at the onset of reperfusion had no protection. Finally, combination of U0126 injection at ischemia onset plus δ V1-1 injection at reperfusion further reduced infarct size, while combination of U0126 delivered at ischemia onset with $\psi\delta$ RACK injected at reperfusion increased infarct size compared with U0126 alone. In conclusion, we find that inhibiting both the MEK/ERK and the δ PKC pathways offers greater protection than either alone, indicating they likely act independently.

Keywords

Cerebral ischemia; MEK/ERK cascade; δ PKC; ERK1/2

1. Introduction

The mitogen-activated protein kinase (MAPK) and the protein kinase C (PKC) pathways are implicated in neuronal death and survival after stroke [4,5,23]. The best characterized members in the MAPK pathways are extracellular signal-regulated kinase 1 and 2 (ERK1/2), whose activity is regulated by a three tiered kinase module, Raf—MEK—ERK, and is increased when ERK1/2 are phosphorylated [25]. ERK1/2 activity is reported to be involved in ischemic injury: levels of phosphorylated ERK1/2 (P-ERK1/2) increase after stroke [23]. But previous studies have shown that increases in P-ERK1/2 are associated with both neuronal survival and death [16,23], and whether ERK1/2 activity exacerbates or attenuates ischemic injury is controversial [27].

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Conflict of interest Dr. Mochly-Rosen is the founder of KAI Pharmaceuticals, Inc, a company that plans to bring PKC regulators to the clinic. However, none of the work described here is based on or supported by the company.

The PKC family consists of 10 isozymes of serine/threonine kinases with distinct roles for neuronal survival [3]. For instance, δ PKC, one member of the PKC family, is pro-apoptotic [4]. We and others have shown that δ PKC activity increases after stroke [32]; it is cleaved, and translocates to the particulate membrane after stroke [24,32]. In addition, the δ PKC-specific inhibitor peptide, δ V1-1, reduces ischemic injury by blocking δ PKC subcellular translocation [4] and the ψ δ RACK, the δ PKC-selective activator, inhibits protection induced by lowering body temperature after stroke [32].

ERK1/2 interacts with the PKC pathways in non-neuronal systems [1,9,13]. PKC directly activates the MAPK pathway by phosphorylating Raf, a signaling molecule which functions upstream of ERK1/2 [6,17]. In addition, VEGF-induced ERK1/2 activation requires δ PKC activation and translocation [18], and PKC has positive feedback on the MAPK pathway [2]. Conversely, activation of ERK1/2 during apoptosis induced by DNA damage involves δ PKC [1]. Nevertheless, whether ERK1/2 interacts with δ PKC after stroke, and how such interplay affects ischemic injury has not been studied.

In this study, we first examined whether inhibiting ERK1/2 by U0126, a MEK1/2 inhibitor, reduces infarct size after stroke, then clarified how the δ PKC inhibitor, δ V1-1, affects the levels of phosphorylated ERK1/2, and finally determined whether δ PKC and ERK1/2 signaling pathways interact in the response to stroke.

2. Results

The effects of the ERK1/2 inhibitor (U0126), the δ PKC inhibitor (δ V1-1), and the δ PKC activator (ψ δ RACK) on the levels of phosphorylated ERK1/2 (P-ERK1/2) were evaluated/determined. The results of Western blot analysis showed that P-ERK1/2 levels transiently increased from 1 to 4 h after reperfusion in the ischemic cortex (Fig. 1), which is consistent with previous reports [23]. As expected, the ERK1/2 inhibitor, U0126, injected at ischemia onset, attenuated increases in P-ERK1/2 levels at 4 h (Fig. 2). Interestingly, the δ PKC-selective inhibitor, δ V1-1, did not change P-ERK1/2 levels compared with the vehicle. In contrast, the δ PKC-selective activator, ψ δ RACK, reduced ERK1/2 phosphorylation when measured 4 h after reperfusion (Fig. 2). Total levels of ERK did not change (data not shown).

Then we examined the effect of U0126, δ V1-1 and ψ δ RACK, as well as some combinations of these drugs on infarct size. U0126 pretreatment at ischemia onset reduced infarct size compared with vehicle, but U0126 delivered at the onset of reperfusion had no protection (Fig. 3). We have previously reported that delivery of δ V1-1 at reperfusion reduced infarct size in the same model used in this study [4]. In the current study, we found that combination treatment with the δ PKC inhibitor δ V1-1 (delivered at the onset of reperfusion) together with U0126 at the onset of ischemia tended to decrease infarct size further, but it did not reach significant difference. Interestingly, combination treatment with the δ PKC activator ψ δ RACK (delivered at the onset of reperfusion) together with U0126 (delivered at ischemia onset) inhibited the protective effect of U0126; the infarct size with both drugs was larger as compared with U0126 alone ($P < 0.001$) (Fig. 3).

3. Discussion

In this study we confirmed that P-ERK1/2 levels transiently increase after reperfusion [23], and the ERK1/2 inhibitor, U0126, given at the onset of stroke, reduces infarction by decreasing P-ERK1/2 levels [12,29], suggesting a detrimental role of ERK1/2 activity in stroke. In addition, we found that the δ PKC inhibitor, δ V1-1, delivered at the onset of reperfusion tended to enhance the protective effect of U0126, although it increased P-ERK1/2 levels. Thus, the protective effect of δ V1-1 was not achieved by reducing P-ERK1/2 levels. Conversely,

treatment with the δ PKC activator, $\psi\delta$ RACK, which decreased ERK phosphorylation, partly abolished the protection induced by U0126, a MEK inhibitor. These data suggest that the detrimental effect of δ PKC activation by $\psi\delta$ RACK is independent of the ERK1/2 activity and phosphorylation.

P-ERK1/2 is implicated in ischemic damage or neuronal survival after stroke. Previous studies generally agree that P-ERK1/2 transiently increases after reperfusion in both global and focal ischemia [10,11]. Our current study is consistent with these previous reports [11]; we showed that P-ERK1/2 levels transiently increased from 1 to 4 h after reperfusion, and then returned to baseline at 24 and 48 h.

Whether increases in P-ERK1/2 levels are detrimental or protective is controversial, as an increase in P-ERK1/2 after stroke is linked with both detrimental [12] and protective effects [22]. Several lines of evidence support the protective effects of P-ERK1/2. First, P-ERK1/2 is expressed in the ischemic penumbra after focal ischemia [8,14]. Second, P-ERK1/2 is expressed in the ischemic resistant region of dentate gyrus in the hippocampus after transient global ischemia [10,30]. Third, P-ERK1/2 is enhanced by a number of neuro-protectants, including various growth factors; in addition, inhibition of the blood brain barrier permeability augments P-ERK1/2 [16]. Alternatively, some previous studies also strongly support the detrimental effects of ERK1/2 activity. For instance, ERK1/2 inhibition reduces ischemic damage [12] and P-ERK1/2 levels are increased by free radical products [23], inflammatory response [20,26] and hyperglycemia [7], which exacerbate ischemic damage.

Nevertheless, in the current study we found that injection of the ERK1/2 inhibitor, U0126, at ischemia onset robustly reduced infarct size while it had no effect when injected at reperfusion, suggesting a limited therapeutic time window. The underlying mechanisms responsible for such difference are not known, but our pilot study showed that ERK1/2 phosphorylation was increased as early as 10 min after ischemia onset (data not shown), indicating that ERK activity might have been triggered before reperfusion. U0126 delivered at reperfusion, thus, might not be able to attenuate infarct size as long as ERK activity started, which, however, needs further study.

Our current study using the δ PKC inhibitor and agonist reinforces the complicated role of ERK1/2 after stroke. The interplay between δ PKC and ERK1/2 has been studied in the non-neuronal systems, in which δ PKC activity increases P-ERK1/2 [1,9,13]. Thus, one would expect that activating δ PKC would increase P-ERK1/2 and inhibiting δ PKC would decrease P-ERK1/2. Surprisingly, the δ PKC activator, $\psi\delta$ RACK, which increases δ PKC activity, actually attenuated protein levels of P-ERK1/2, and inhibiting δ PKC did not block increases in P-ERK1/2 in our study. Thus, our results are not consistent with previous reports regarding interaction between δ PKC activity and P-ERK1/2 levels [18]. The ERK1/2 inhibitor, U0126, reduced infarct size and protein levels of P-ERK1/2, suggesting that reduction in P-ERK1/2 may be necessary for the protective effect of U0126. However, the δ PKC inhibitor δ V1-1, which we previously reported to block ischemic damage [4,5], did not reduce P-ERK1/2 levels. Furthermore, the δ PKC activator, $\psi\delta$ RACK, inhibited P-ERK1/2 levels, yet it abolished the protective effect of the ERK1/2 inhibitor, U0126. Thus, protein levels of P-ERK1/2 itself may not be as critical for determining the protective or detrimental effect of ERK1/2; some other factors may be responsible for the effects of ERK1/2. It is well known that stroke causes changes in a myriad of genes and proteins [15]. Although U0126 is an ERK1/2 inhibitor, it may also affect many other proteins simultaneously. Likewise, P-ERK1/2 is not the only protein altered by the δ PKC activator or inhibitor. Thus, P-ERK1/2 appears to have a complex role in mediating injury or survival of brain after stroke.

In conclusion, we demonstrated for the first time that inhibition of both ERK1/2 and the δ PKC pathways offers greater protection than does inhibition of either alone, suggesting they act in parallel. Our results imply that the protein level of P-ERK1/2 is not a simple marker for cerebral neuroprotection or worsened injury after stroke.

4. Experimental procedures

Procedures using laboratory animals were approved by the Stanford University Administrative Panel on Laboratory Animal Care.

4.1. Focal ischemia model

Focal ischemia was induced by occluding the left middle cerebral artery (MCA) in male Sprague Dawley rats (250–300 g) as described previously [19,34]. Briefly, anesthesia was induced by a dose of 5% isoflurane and maintained by 2% isoflurane through a face mask during all surgical procedures. Physiological parameters, including respiration rate, were monitored. Body temperature was maintained at 37 °C using a heating blanket. The left common carotid artery was exposed, and incised. An uncoated 30 mm long segment of 3-0 nylon monofilament suture was inserted to occlude the ostium of the MCA. The suture was left in place for 2 h during which the rat was removed from the isoflurane. At the end of the ischemic period, the rat was re-anesthetized, the suture was removed, and the rat was allowed to recover.

4.2. Drug injection

The MEK-1 inhibitor, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) (Cell Signaling Technology, Danvers, MA) was dissolved in 2 ml DMSO (100%), and 1 ml of U0126 (10 mg/kg) was injected intraperitoneal right before ischemia onset or at onset of reperfusion. The same volume of vehicle (1 ml 100% DMSO) was injected at the onset of ischemia. The dosage was chosen based on the past studies that have shown that doses from 0.5 mg/kg [28] to 200 mg/kg [21] of U0126 reduced infarct size after focal ischemia. The δ PKC-specific activator, $\psi\delta$ RACK (0.2 mg/kg), and the δ PKC-specific inhibitor, δ V1-1 (0.2 mg/kg) [4], were injected intraperitoneally at the onset of reperfusion. Peptide regulators of δ PKC were designed and synthesized as described previously [4].

4.3. Assessment of brain infarct size

Rats were sacrificed after 24 h of reperfusion by overdoses of Isoflurane. Brains were quickly removed and sliced into 3 mm coronal sections, resulting in five slices. The slices were stained using 2% triphenyl tetrazolium chloride (TTC), and both faces of each slice were digitally scanned (EPSON®). Stroke regions of the five slices were measured and normalized to the contralateral hemisphere and expressed as percentages [31].

4.4. Western blot analysis [32,33]

Rat brains were harvested at various time points after ischemia for Western blot. The brains were quickly sliced. Since ERK1/2 phosphorylation was reported to occur in the ischemic cortex [23], the ischemic and non-ischemic cortex from each central slice were dissected and frozen until homogenization for detecting ERK1/2 phosphorylation. The tissues were added with a 7 times volume of 1x cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing a protease inhibitor (Sigma) and phosphatase inhibitor cocktail I and II (Sigma), and homogenized by 35 strokes in 1 ml vessels then incubated in ice for 5 min and centrifuged at 14,000 $\times g$ for 10 min at 4 °C, as described. Protein concentration was assessed using Bradford reagent (BioRad). In each lane 25 μ g protein was subjected to sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) using 4–15% Ready Gel (Cat#

L050505A2, BIO-RAD Laboratories, USA) for 1.5 h. Proteins were transferred for 60 min to a nitrocellulose membrane (Amersham). The membrane was blocked in PBS solution containing 3% milk and 0.1% Tween 20 (Sigma), and then incubated in the solution containing a rabbit anti-phospho-ERK1/2(Thr202/Tyr204) antibody (1:1000; Cell Signaling Technology, Danvers, MA) overnight at 4 °C. Then the membrane was incubated in the solution containing a secondary anti-rabbit IgG HRP-linked antibody (1:2000, Cell Signaling Technology, Danvers, MA) for 1 h. Protein bands were detected using an enhanced chemiluminescence system (Amersham Biosciences, Arlington heights, IL) and exposed to hyperfilm. To determine even-loading of proteins, membranes were stripped and re-probed with a β -actin antibody (1:10,000; Cat#A3854 Sigma). Densities of protein bands from Western blot were measured by NIH Image J program.

Statistics—Data were expressed as the mean $\pm$ SEM. All statistical analyses were performed using ANOVA followed by Bonferroni's *post hoc* test.

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Fig. 1. P-ERK1/2 levels transiently increased in the ischemic penumbra after reperfusion. (A) Representative Western blot analysis with anti-P-ERK1/2 and anti-ERK1/2 of samples from the ischemic cortex (penumbra) at 0 min, 15 min, 1 h, 2 h, 4 h, 24 h and 48 h after reperfusion. β-actin was used to normalize protein loading. (B) Bar graphs show relative optical densities of P-ERK1/2 bands normalized to that of the sham. Total ERK1/2 protein did not change after stroke (data not shown). * $P < 0.05$, vs. sham. $n = 7$ per group.

Fig. 2.

The effect of $\delta V1-1$, U0126 and $\psi\delta RACK$ on ERK1/2 phosphorylation at 4 h after reperfusion. U0126 was delivered at ischemia onset while $\delta V1-1$ and $\psi\delta RACK$ were injected at reperfusion. Sham rats received sham surgery without ischemia; vehicle, rats received ischemia, but were treated with the DMSO only. (A) Representative Western blot of P-ERK1/2. (B) Bar graphs showing relative optical densities of P-ERK1/2 bands normalized to that of the sham in the ischemic cortex. * $P < 0.001$ vs sham, # $P < 0.05$ vs. vehicle. $n = 5$ per group.

Fig. 3.

The effects of ERK1/2 inhibitor and δPKC inhibitor or activator on infarct size. U0126 at ischemia, U0126 was delivered at ischemia onset; U0126 at reperfusion, U0126 was injected at reperfusion; U0126+δV1-1, U0126 (injected at ischemia onset) plus δV1-1 (injected at reperfusion); U0126+ψδRACK, U0126 (injected at ischemia onset) plus ψδRACK (injected at reperfusion). *** $P < 0.001$ vs. control; ## $P < 0.001$, ### $P < 0.001$ vs. U0126 at reperfusion; ††† $P < 0.001$ vs U0126 at ischemia+ψδRACK at reperfusion ($n = 7$ per group).