Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia

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Brain subjected to acute ischemic attack caused by an arterial blockage needs immediate arterial recanalization. However, restoration of cerebral blood flow can cause tissue injury, which is termed reperfusion injury. It is important to inhibit reperfusion injury to achieve greater brain protection. Because oxidative stress has been shown to activate mitogen-activated protein kinases (MAPKs), and because oxidative stress contributes to reperfusion injury, MAPK may be a potential target to inhibit reperfusion injury after brain ischemia. Here, we demonstrate that reperfusion after forebrain ischemia dramatically increases phosphorylation level of extracellular signal-regulated kinase 2 (ERK2) in the gerbil hippocampus. In addition, i.v. administration of U0126 (100–200 mg/kg), a specific inhibitor of MEK (MAPK/ERK kinase), protects the hippocampus against forebrain ischemia. Moreover, treatment with U0126 at 3 h after ischemia significantly reduces infarct volume after transient (3 h) focal cerebral ischemia in mice. This protection is accompanied by reduced phosphorylation level of ERK2, substrates for MEK, in the damaged brain areas. Furthermore, U0126 protects mouse primary cultured cortical neurons against oxygen deprivation for 9 h as well as nitric oxide toxicity. These results provide further evidence for the role of MEK/ERK activation in brain injury resulting from ischemia/reperfusion, and indicate that MEK inhibition may increase the resistance of tissue to ischemic injury.

Cardiac arrest or cerebral arterial occlusion can cause a brain attack. Quickly restoring the cerebral blood flow is needed to stop brain injury. The most exciting new development in the field of stroke research is the recent approval of i.v. injection of tissue plasminogen activator that dissolves the blood clot (1, 2). Restoration of blood flow not only brings oxygen and nutrients into the damaged brain, but also produces free radicals such as reactive oxygen and reactive nitrogen species. These free radicals have been shown to contribute to oxidative injury. The tissue damage by the restoration of blood flow is termed reperfusion injury (3, 4). Thus, inhibition of reperfusion injury may be important to achieve greater brain protection.

Mitogen-activated protein kinase (MAPK) family members, including extracellular signal-regulated kinases (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK), respond to various extracellular stimuli, thereby transmitting extracellular signals into the nucleus. ERK1/2 are activated by MAPK/ERK kinase1/2 (MEK1/2) by phosphorylating these MAPKs (5). The MEK/ERK pathway plays a crucial role in cell growth and differentiation (6, 7). ERK1/2 are constitutively expressed in the adult brain (8); however, little is known about the function of ERK1/2 in postmitotic, terminally differentiated neurons. The MEK/ERK pathway is also activated by reactive oxygen and reactive nitrogen species (9–11). Several *in vivo* studies showed that ERK1/2 are phosphorylated in the damaged brain after ischemia, hypoglycemia, and kainate-induced seizure (12–15). We previously reported that intraventricular administration of MEK1 inhibitor PD98059 (16) decreased infarct volume after focal cerebral ischemia (17). This work suggests that the MEK/ERK pathway plays a crucial role in ischemic brain injury.

Recently, a novel and more potent MEK-specific inhibitor U0126 was developed (18, 19). Whereas U0126 inhibits the enzymatic activity of MEK1/2, PD98059 blocks the phosphorylation of MEK1, but cannot efficiently inhibit the activity of MEK1 once it is phosphorylated (16). In this study, we investigated whether U0126 enhances the neuronal survivability after ischemia and reperfusion in experimental models.

Methods

Ischemia Model. Forebrain ischemia was induced by bilateral carotid artery occlusion (BCAO) in male gerbils (50–70 g) under anesthesia with 1.0% halothane in 70% N_2O and 30% O_2. Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) using silicon-coated 8-0 nylon filament in male ICR or ddY mice (20–22 g; Japan SLC, Hamamatsu, Japan) as described (20). Regional cerebral blood flow was monitored by laser-Doppler flowmetry (FLO-C1, Omegawave, Tokyo, Japan). U0126 (Promega) or vehicle (0.1 M PBS containing 0.4% dimethyl sulfoxide) was injected into the femoral vein. Animal protocols followed the National Cardiovascular Center's guidelines for animal care and experiments.

Evaluation of Hippocampal Injury. Seven days after reperfusion, coronal brain sections of 40 μm thickness were made by using a freezing microtome, and stained with 0.1% cresyl violet. The CA1 pyramidal cells were counted and expressed as cells per millimeter.

Evaluation of Brain Infarction. Brains were cut into coronal slices, and incubated with 2% 2,3,5-triphenyltetrazolium chloride, as described (21). The infarcted areas were measured on each section by an image analysis system (Olympus, Tokyo, Japan), and infarction volume was calculated by summing the infarct areas. Evaluation of brain atrophy volume was done by calculating using the following formula: (contralateral volume − ipsilateral volume) × 100/contralateral volume.

Primary Culture. Mixed cortical cell cultures containing both neurons and glia were prepared from ICR mouse embryos at 15

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Abbreviations: BCAO, bilateral carotid artery occlusion; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; MEK, MAPK/ERK kinase; MCAO, middle cerebral artery occlusion; NMDA, *N*-methyl-D-aspartic acid; NO, nitric oxide; SNP, sodium nitropruside; CS, control solution.

See commentary on page 10989.

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days of gestation as described (22). The cultures were used at 15 days *in vitro*. For oxygen deprivation, the cultures were transferred to an anaerobic chamber (Forma Scientific, Marietta, OH) containing a gas mixture of 5% CO_2, 10% H_2, and 85% N_2. The culture media were replaced with deoxygenated, glucose free or glucose (20 mM) containing Earle's balanced salt solution (BSS), and the cultures were placed in the chamber at 37°C. Oxygen deprivation was terminated by replacing the medium with oxygenated BSS containing 20 mM glucose, and cultures were returned to the normoxic incubator. For toxicity experiments, the culture media were replaced with Hepes-buffered control solution (CS, pH 7.4) containing toxin. For glutamate, *N*-methyl-D-aspartic acid (NMDA) and sodium nitropruside (SNP) toxicity experiments, the culture was exposed to Hepes-buffered CS containing 300 μM of these compounds for 5 min, and then the culture media were replaced with CS for 24 h. For kainic acid toxicity, the culture was exposed to CS containing 30 μM of kainic acid for 24 h.

Evaluation of Cell Death. Cell death was determined by measuring propidium iodide (PI) fluorescence by using a multiwell plate fluorescence scanner (Cytofluor, Series 4000, PerSeptive Biosystems, Framingham, MA; ref. 23). Data were expressed as percentage of PI fluorescence in sister cultures exposed to 1 mM NMDA for 24 h.

Western Blot Analysis. Western blotting was performed as described previously (17) by using phospho-specific antibodies for the protein kinases (1:1,000; New England Biolabs). To analyze protein levels of ERK1/2, the immunoblots were stripped and reprobed with anti-ERK1 and anti-ERK2 antibodies (1:2,000, respectively; Santa Cruz Biotechnology).

Immunostaining. Immunostaining was done as described previously (24). The dilution of primary antibody was 1:100 for phospho-ERK1/2 and 1:500 for total-ERK2, respectively. For double immunostaining, the sections were also treated with NeuN monoclonal antibody (1:1,000; Chemicon), and then phospho-ERK1/2 and NeuN immunoreactivities were respectively labeled with Alexa Fluor 546 and Alexa Fluor 488 (Molecular Probes). The sections were observed by using an Olympus confocal laser microscope (FLUOVIEW, Tokyo, Japan).

Statistics. Data are presented as mean ± SEM. Data were analyzed by Student *t* test or one-way ANOVA followed by Bonferroni's post hoc test. For neurological score, Mann–Whitney *U* test was used. $P < 0.05$ was considered statistically significant.

Results

Reperfusion After Forebrain Ischemia Phosphorylates ERK1/2 in the Hippocampus. First we asked whether ERK1/2 are involved in hippocampal injury after forebrain ischemia in the gerbil. We examined the changes in phosphorylation of ERK1/2 in the hippocampus by using a phospho-ERK1/2 antibody. ERK1/2 were dephosphorylated by BCAO. The longer duration of BCAO resulted in the greater dephosphorylation of ERK1/2 (Fig. 1*A Top*). This may be a result of decreased ATP production during ischemia. Significant increase in phosphorylation of ERK1/2 was observed by 5 min after reperfusion after 3.5 min of BCAO, and persisted until 1 h, compared with sham control (Fig. 1*A Middle*). However, the total ERK1/2 protein levels did not change during reperfusion period (Fig. 1*A Bottom*). These results indicate that ERK1/2 are activated by reperfusion after BCAO. We next examined changes in phosphorylation levels of other protein kinases. We found a very weak increase in phospho-p38 MAPK at 1 to 5 min after reperfusion; however, we found no significant increase in phospho-JNK and phospho-p70^{S6} kinase over sham (Fig. 1*B*). These results suggest that MEK/ERK pathway is a major MAPK cascade activated in the hippocampus after forebrain ischemia and reperfusion.

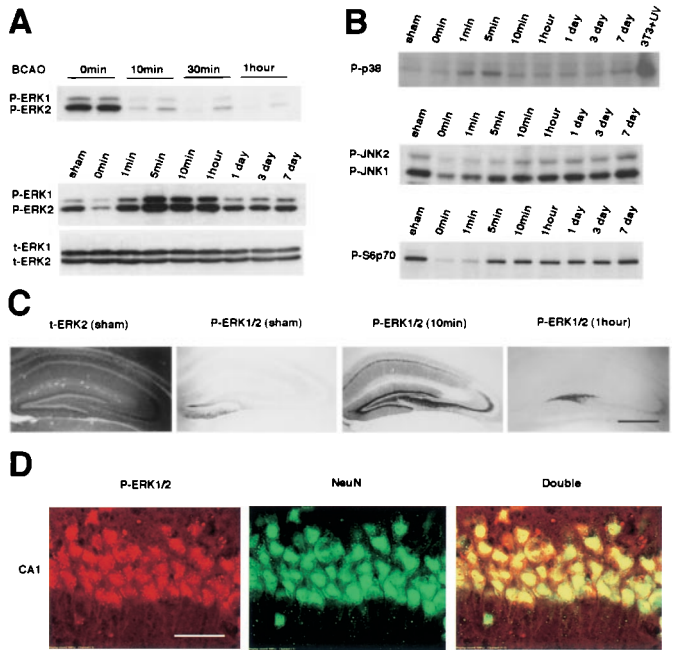

Fig. 1. Reperfusion after forebrain ischemia increases phosphorylation of ERK1/2 in hippocampus. (*A*) Ischemia alone by BCAO dephosphorylates ERK1/2 (*Top*), whereas reperfusion after 3.5 min BCAO increases phosphorylation levels of ERK1/2 (*Middle*). Total protein levels of ERK1/2 are not affected by 3.5 min BCAO and reperfusion (*Bottom*). Data were reproduced by five independent experiments. (*B*) Time-dependent changes in phospho-p38 MAPK (*Top*), phospho-JNK (*Middle*), and phospho-p70^{S6} kinase (*Bottom*) in the hippocampus after 3.5 min BCAO. UV-treated NIH 3T3 cell lysate (3T3 + UV) was used as a control for p38 MAPK phosphorylation. Data were reproduced by five independent experiments. (*C*) Immunostaining of sham-control or ischemic brains after 10 min or 1 h of reperfusion by using total ERK2 (t-ERK2) or phospho-ERK1/2 (P-ERK1/2) antibodies. Scale bar = 1 mm. (*D*) Confocal microscopic images document the localization of phospho-ERK1/2 immunoreactivity (red) and NeuN (CNS neuronal specific marker) immunoreactivity (green) in single tissue section from subfield CA1 after 10 min reperfusion after 3.5 min BCAO. Scale bar = 100 μm.

To determine the cell type in which ERK1/2 are phosphorylated, we did immunostaining using phospho-ERK1/2 antibody on brain sections. Total-ERK2 immunostained cells were found throughout the hippocampus, whereas little phospho-ERK1/2 immunoreactivity was detected in the sham-operated animals. After 10 min of reperfusion, intense phospho-ERK1/2 immunostaining was detected in the CA1, dentate gyrus, and mossy fibers, but not in the pyramidal cell layer of CA3 that survives 3.5 min BCAO (Fig. 1*C*). Closer analysis of CA1 pyramidal cells revealed phospho-ERK1/2 immunostaining in both cytoplasm and nucleus (Fig. 1*D*). Phospho-ERK1/2 immunostaining returned to the basal levels in these areas by 1 h after reperfusion, with some phospho-ERK1/2 immunostaining persisting in neurons in the hilar region (Fig. 1*C*).

U0126 Attenuates Hippocampal Injury After Forebrain Ischemia. To examine whether inhibition of MEK1/2 protects the hippocampus, we tested i.v. administration of U0126 in gerbils subjected to 3.5 min BCAO. U0126 reduced phospho-ERK1/2 immunoreactivity on immunoblots as well as phospho-ERK1/2 immunostaining in the CA1 pyramidal cells 10 min after reperfusion in a dose-dependent manner (Fig. 2 *A* and *B*). Moreover, U0126 pretreatment decreased the loss of CA1 pyramidal cells at 7 days

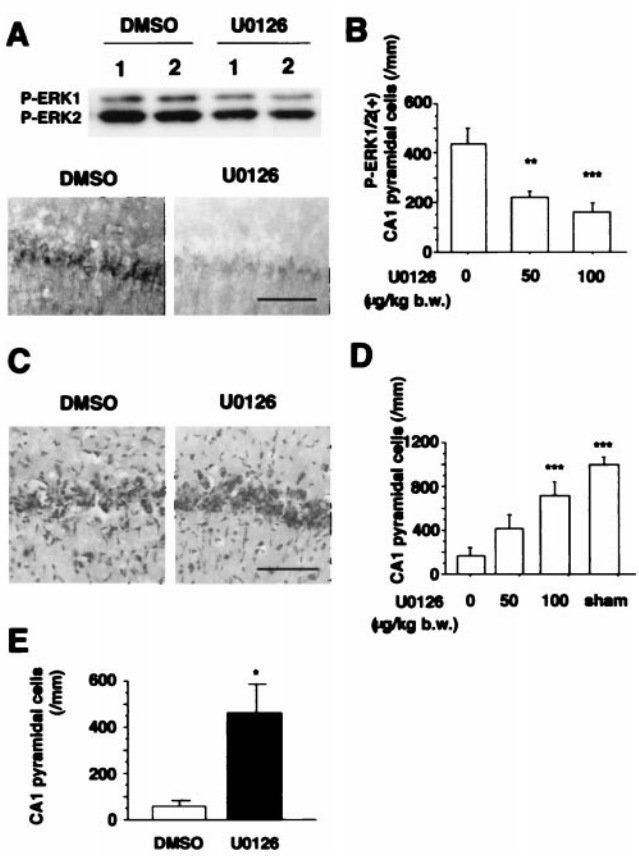

Fig. 2. U0126 reduces phosphorylation of ERK1/2 and subsequent neuronal death in the CA1 after forebrain ischemia. (*A*) Western blot analysis and immunostaining by using phospho-ERK1/2 antibody demonstrating that i.v. injection of U0126 (100 μg/kg) reduces phosphorylation level of ERK1/2 in the hippocampus after 10 min of reperfusion after 3.5 min BCAO. Photomicrographs of phospho-ERK1/2 immunostaining were taken from the subfield CA1. Scale bar = 200 μm. (*B*) The number of phospho-ERK1/2 immunoreactive CA1 pyramidal cells. U0126 was injected i.v. 10 min before 3.5 min BCAO. **, $P < 0.01$; ***, $P < 0.001$, compared with the DMSO-injected group (ANOVA). Data are presented as mean + SEM ($n = 6–8$). (*C*) Photomicrographs of the subfield CA1 stained with 0.1% cresyl violet from DMSO- and U0126-administrated gerbils. DMSO or U0126 was injected i.v. 10 min before 3.5 min BCAO, and the brains were evaluated 7 days after reperfusion. Scale bar = 200 μm. (*D*) Dose-dependent protective effect by injection of U0126 10 min before ischemia. The brains were examined 7 days after reperfusion. ***, $P < 0.001$, compared with the DMSO-injected group (ANOVA). Data are presented as mean + SEM ($n = 5–8$). (*E*) Neuroprotection by i.v. injection of U0126 (100 μg/kg) during ischemia (3 min after BCAO). The brains were examined 7 days after reperfusion. *, $P < 0.05$ (unpaired Student *t* test). Data are presented as mean + SEM ($n = 5$ and 6 in DMSO and U0126 group).

(Fig. 2 *C* and *D*). At the doses used, U0126 did not affect the rectal temperature during ischemia and after reperfusion (Table 1). Mean arterial blood pressure, partial oxygen (pO_2) and carbon dioxide (pCO_2) tensions, blood pH, and glucose levels were not affected by U0126 when compared with vehicle injection (Table 2). Furthermore, injection of U0126 at 3 min after BCAO was protective in the CA1 when evaluated at 7 days (Fig. 2*E*).

U0126 Protects Brain in Stroke Models. We next tested U0126 in mouse ischemic stroke models. Pretreatment with i.v. administration of U0126 dramatically decreased infarct volume 24 h after permanent MCAO. Maximum protection was achieved with 200 μg/kg of U0126, with a 42% ($P < 0.001$) reduction in infarct volume (Fig. 3*A*). Neurological deficits 24 h after ischemia were improved by U0126 (Table 3). U0126 administration 1 h after MCAO was protective, but not when given 3 h after MCAO in the permanent focal ischemia model (Fig. 3*A*). U0126 did not affect regional cerebral blood flow, core temperature, and body weight after MCAO (Table 3).

Table 1. Changes in rectal temperature (°C) after 3.5 min BCAO in the gerbil

	U0126, μg/kg		
	0 ($n = 6$)	50 ($n = 7$)	100 ($n = 7$)
BCAO	37.4 ± 0.1	37.4 ± 0.2	37.5 ± 0.1
Reperfusion	37.8 ± 0.2	38.0 ± 0.2	38.0 ± 0.3
1 hr	38.6 ± 0.2	39.0 ± 0.3	39.1 ± 0.5
3 hr	37.9 ± 0.2	37.6 ± 0.3	38.3 ± 0.4
6 hr	38.4 ± 0.3	38.4 ± 0.2	38.5 ± 0.1

We next examined whether delayed injection of U0126 during ischemia protects brain against 3 h reversible MCAO. U0126 (200 μg/kg) injected 10 min before reperfusion significantly decreased infarct volume by 40% ($P < 0.01$) at 24 h after reperfusion (Fig. 3*B*). Densitometric analysis of phospho-ERK2 immunoblots demonstrated that the level of phosphorylation after U0126 was significantly reduced by 27% in the ischemic hemisphere ($P = 0.026$, $n = 3$; Fig. 3*C*). Phospho-ERK1/2 was also reduced in the damaged brain areas in U0126-injected mice by immunostaining (Fig. 3*D*). This brain protection was sustained for 35 days. In U0126-injected animals, there was a 41% decrease in brain atrophy compared with vehicle-injected animals ($P < 0.05$; Fig. 3*E*). U0126 did not affect regional cerebral blood flow and core temperature during and after MCAO (data not shown). Thus, these data suggest that administration of U0126 after onset of ischemic stroke inhibits brain injury.

U0126 Protects Cortical Cultures Against Oxygen Deprivation as Well as Nitric Oxide (NO) Toxicity. Finally, to study the protective effect of U0126 at the cellular level, we examined the drug effect in mouse primary cultured cortical neurons. First, we tested U0126 in cortical neurons subjected to hypoxia. Nine hours of oxygen deprivation followed by 24 h reoxygenation caused death in 80–90% of neuronal cells, which could be inhibited by U0126. NMDA antagonist dizocilpine (MK-801; 10 μM) was also protective as reported previously (ref. 25; Fig. 4*A*). The protection

Table 2. Effects of i.v. injection of U0126 on physiological parameters in the gerbil

	DMSO	U0126 (100 μg/kg)
MABP, mmHg		
Before	74.8 ± 7.5 ($n = 5$)	74.6 ± 8.5 ($n = 5$)
15 min	81.2 ± 5.1 ($n = 5$)	75.0 ± 5.6 ($n = 5$)
pH		
Before	7.38 ± 0.01 ($n = 10$)	7.35 ± 0.02 ($n = 10$)
15 min	7.39 ± 0.02 ($n = 10$)	7.35 ± 0.02 ($n = 10$)
pCO_2, mmHg		
Before	42.6 ± 2.2 ($n = 10$)	40.6 ± 1.8 ($n = 10$)
15 min	40.8 ± 1.6 ($n = 10$)	38.0 ± 2.0 ($n = 10$)
pO_2, mmHg		
Before	147.2 ± 4.8 ($n = 10$)	145.9 ± 8.1 ($n = 10$)
15 min	156.5 ± 5.5 ($n = 10$)	152.2 ± 6.2 ($n = 10$)
Glucose, mg/dl		
Before	97.5 ± 4.9 ($n = 10$)	108.3 ± 6.8 ($n = 10$)
15 min	111.1 ± 7.9 ($n = 10$)	115.4 ± 15.6 ($n = 10$)
30 min	116.2 ± 5.0 ($n = 5$)	105.0 ± 12.6 ($n = 5$)

MABP, mean arterial blood pressure. Results are mean ± SEM.

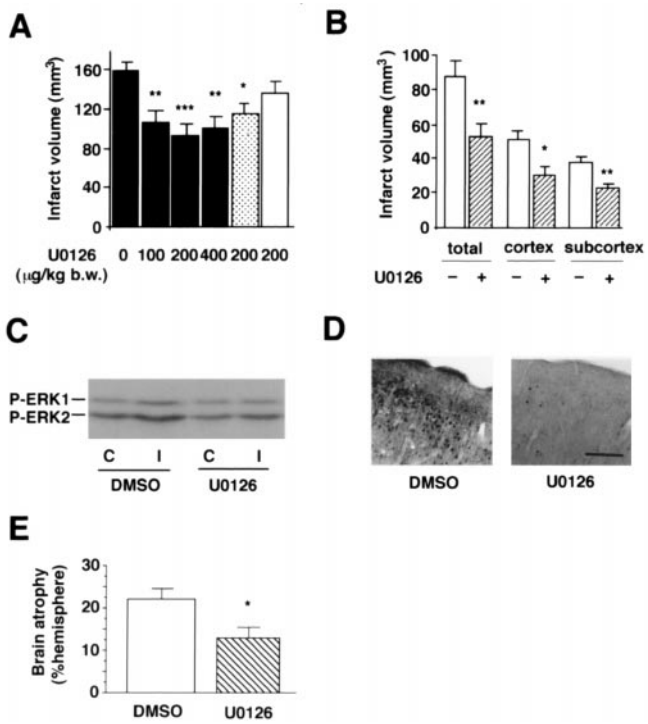

Fig. 3. U0126 protects brain against damage resulting from ischemic stroke in mice. (*A*) Infarct volume after 24 h of MCAO. U0126 was injected 10 min before (filled columns), 1 h (dotted column), or 3 h (open column) after MCAO. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with the vehicle-injected group (ANOVA). Data are presented as mean + SEM ($n = 8–11$). (*B*) Infarct volume 24 h after reperfusion after 3 h of reversible MCAO. DMSO (open columns) or U0126 (200 μg/kg; hatched columns) was injected i.v. at 2 h and 50 min after induction of MCAO (i.e., 10 min before reperfusion). Data are presented as mean + SEM ($n = 12$ in each group). *, $P < 0.05$; **, $P < 0.01$ (unpaired Student *t* test). (*C* and *D*) Immunoblotting (*C*) and immunostaining (*D*) using phospho-ERK1/2 antibody show that i.v. injection of U0126 (200 μg/kg) during ischemia diminishes phospho-ERK1/2 immunoreactivity in the damaged brain 5 min after reperfusion after 3 h of MCAO. C, contralateral; I, ipsilateral. Scale bar = 200 μm. (*E*) Brain atrophy volume 35 days after reperfusion after 3 h of MCAO. Single injection of DMSO or U0126 (200 μg/kg) was given 10 min before reperfusion. Data are presented as mean + SEM ($n =$ 8 and 10 in DMSO and U0126 group, respectively). *, $P < 0.05$ (unpaired Student *t* test).

by U0126 was dose dependent (Fig. 4*B*). Ten micromolar of U0126 afforded a 40% reduction in cell death. We also tested U0126 in cortical neurons subjected to combined oxygen and glucose deprivation. Although MK-801 protected against oxygen/glucose deprivation consistently with previous reports (26), U0126 was not effective (Fig. 4*C*).

We next examined the phosphorylation levels of ERK1/2 in cortical neurons subjected to oxygen deprivation. Nine hours of oxygen deprivation resulted in a concomitant increase and decrease in phosphorylated ERK1 and ERK2, respectively (Fig. 4*D Top*). At 2 h after reoxygenation, the levels of phosphorylation in both ERK1 and ERK2 were increased, and sustained until 3 h. U0126 inhibited phosphorylation of ERK2 in a dose-dependent manner, but interestingly did not affect the phosphorylation level of ERK1. Total protein levels of ERK1/2 did not change after oxygen deprivation and reoxygenation (Fig. 4*D Middle*). We also studied the phosphorylation levels of MEK1/2, direct activators of ERK1/2. U0126 blocks activity but not phosphorylation of MEK1/2 (18). The phosphorylation of MEK1/2 was greater in U0126 treated cultures at all time points (Fig. 4*D Bottom*). This finding may result from inhibition of MEK1/2 turnover, or the lack of activation of downstream molecules, such as phosphatases, that ordinarily play a negative feedback role. To further verify the specificity of U0126, we examined the effects of U0126 on phosphorylation of other protein kinases. U0126, at concentrations up to 10 μM, did not affect phosphorylation levels in p38 MAPK, JNK, and $p70^{S6}$ kinase (Fig. 4*E*).

Both excessive excitation of the glutamate receptors and overproduction of free radicals have been implicated in neuronal cell death resulting from ischemia (27, 28). In addition, glutamate receptor stimulation and NO have been shown to phosphorylate ERK2 in cultured cortical neurons (11, 29). We next asked whether U0126 is protective under these specific toxic conditions. We tested U0126 in primary cultured cortical neurons exposed to glutamate, NMDA, or kainic acid. U0126 was not protective against these excitotoxins (Fig. 4*F*). Rather, 10 μM U0126 enhanced cell death by NMDA. In contrast, U0126 dramatically inhibited neuronal cell death by exposure for 5 min to the NO donor, SNP, in a dose-dependent manner, with a maximum inhibition at 10 μM (Fig. 4*G*). Another MEK1 inhibitor, PD98059 (25 μM), also attenuated neuronal cell death by SNP at 12 h; however, this protection was not found at 24 h (data not shown). p38 MAPK inhibitor SB203580 (10 μM) was not protective against SNP (Fig. 4*H*).

Table 3. Physiological parameters and neurological deficit score after permanent MCAO

	U0126, μg/kg			
	0 ($n = 8$)	100 ($n = 9$)	200 ($n = 9$)	400 ($n = 11$)
Core temp., °C	37.0 ± 0.1	37.0 ± 0.0	36.9 ± 0.1	37.0 ± 0.1
TM temp., °C	37.3 ± 0.2	37.1 ± 0.1	37.5 ± 0.2	37.3 ± 0.1
rCBF, %	15.0 ± 2.0	13.5 ± 1.1	17.0 ± 1.4	17.4 ± 0.7
Neurological score	1.5 ± 0.3	0.6 ± 0.2*	0.6 ± 0.2*	0.5 ± 0.2*
BW loss, %	21.8 ± 1.6	18.2 ± 0.9	20.8 ± 0.9	14.5 ± 1.7

Results are mean ± SEM. Temp., temperature; TM, Temporal muscle; BW, body weight. Data for temperatures and rCBF were collected at 30 min after induction of MCAO. rCBF is expressed as a percentage of the value before MCAO. Neurological scoring was determined according to Hara *et al.* (20) and done at 24 hr after MCAO. Body weight loss (%) was calculated as 100 × [body weight (before) − body weight (24 h)]/body weight (before). *, $P < 0.05$ by Mann–Whitney *U* test, compared with 0 μg/kg group.

Discussion

We demonstrated that i.v. administration of U0126, a MEK1/2 inhibitor, protects brain against damage after forebrain as well as focal cerebral ischemia. In addition, we clearly demonstrated that U0126 provides protection in primary cultured cortical neurons against SNP, as well as oxygen deprivation.

Ischemia and reperfusion enhanced phospho-ERK1/2 immunostaining not only in the CA1 pyramidal cells but also in dentate granule cells that escape damage under the current ischemic condition. These results indicate that MEK/ERK activation results in damage or survival in a cell type-specific manner. Different outcomes after MEK/ERK activation in these two neuronal cell types may be explained by other signaling pathways, such as phosphatidylinositol 3-kinase (PI3-kinase)/Akt. PI3-kinase/Akt have been implicated in cell survival and are differentially regulated and/or activated in a neuronal cell type-specific manner. We demonstrated that Akt activation is more dramatic and prolonged in the dentate gyrus than CA1 after forebrain ischemia in the gerbil (30). Hence, these signaling pathways need to be investigated in specific cell types in any given experimental model.

The mechanisms by which ERK activation leads to neuronal cell death remain unclear. However, the evidence that ERK activation contributes to cell death is accumulating. A critical role for ERK in hydrogen peroxide-induced cell death was

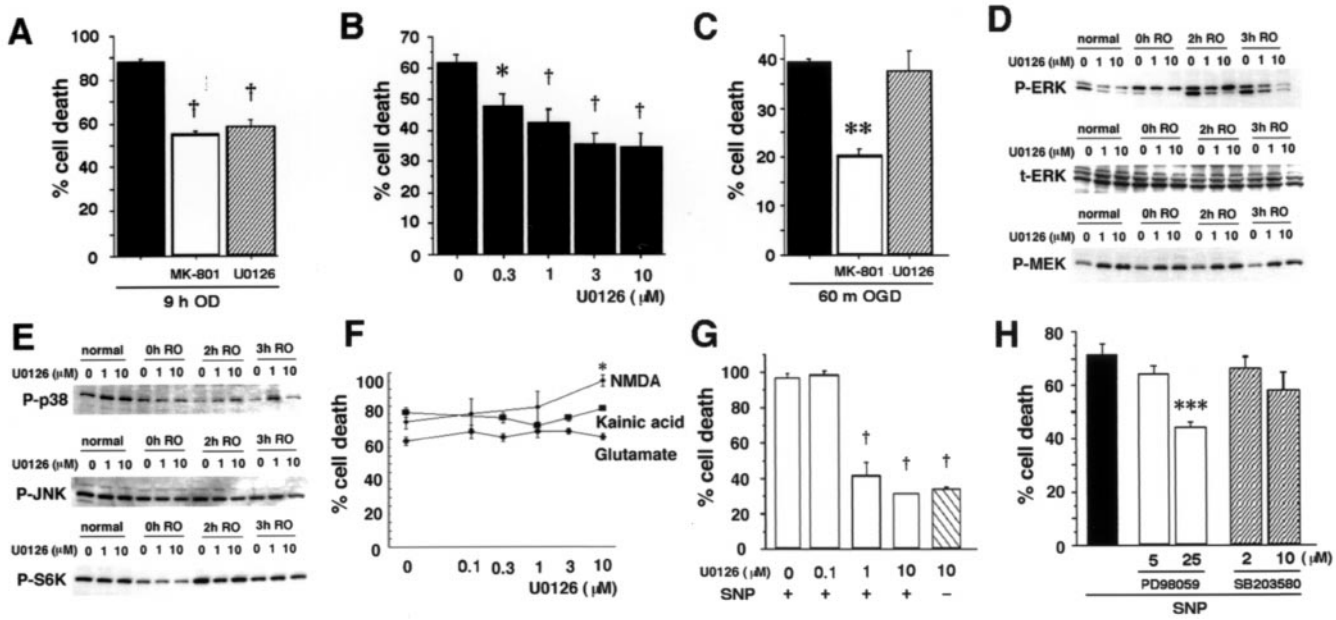

Fig. 4. U0126 protects cortical cultures against oxygen deprivation and SNP. (*A*) Protection by MK-801 (10 μM) and U0126 (10 μM) against neuronal death resulting from 9 h of oxygen deprivation followed by 24 h reoxygenation. †, $P < 0.0001$, compared with oxygen deprivation alone (ANOVA). Data are presented as mean ± SEM ($n = 16$). (*B*) Dose-dependent protective efficacy of U0126 in primary cultured cortical neurons exposed to 9 h of oxygen deprivation followed by 24 h reoxygenation. *, $P < 0.05$; †, $P < 0.0001$, compared with 0 μM (ANOVA). Data are presented as mean ± SEM ($n = 12$). (*C*) U0126 is not protective against 60 min of oxygen/glucose deprivation. **, $P < 0.01$, compared with control (ANOVA). Data are presented as mean ± SEM ($n = 4$). (*D*) Dose-dependent effects of U0126 on phosphorylation of ERK and MEK in primary cultured cortical neurons exposed to 9 h of oxygen deprivation and reoxygenation (RO) for the indicated time. (*E*) U0126 does not affect phosphorylation levels of p38 MAPK, JNK, and $p70^{S6}$ kinase in primary cultured cortical neurons exposed to 9 h of oxygen deprivation and reoxygenation (RO) for the indicated time. (*F*) U0126 does not protect primary cultured cortical neurons exposed for 5 min to 300 μM glutamate (filled circle) or 300 μM NMDA (diamond). U0126 is not protective against exposure to 30 μM kainic acid (square) for 24 h. *, $P < 0.05$, compared with 0 μM (ANOVA). Data are presented as mean ± SEM ($n = 8$). (*G*) Dose-dependent efficacy of U0126 in primary cultured cortical neurons exposed to 300 μM SNP for 5 min. Cell death was determined at 24 h after exposure. †, $P < 0.0001$, compared with 0 μM (ANOVA). Data are presented as mean ± SEM ($n = 8$). (*H*) Another MEK1 inhibitor, PD98059, but not p38 MAPK inhibitor SB203580 attenuates neuronal death by exposure to SNP (300 μM) for 5 min. Cell death was determined at 12 h. Data are presented as mean ± SEM ($n = 8$). *, $P < 0.05$, compared with control (ANOVA).

demonstrated in an oligodendrocyte cell line (31). Another study provides evidence that ERK activation contributes to zinc toxicity in cortical neurons (32). Our laboratory and Stanciu *et al.* independently demonstrated that MEK inhibitors block oxidative glutamate toxicity in cortical neurons (33, 34). Enhancement by brain-derived neurotrophic factor (BDNF) in NO-induced neuronal apoptosis has been shown to be mediated by ERK activation (35). Recently, it has been shown that cisplatin-induced apoptosis in HeLa cells requires ERK activation (36).

We observed that U0126 up to 10 μM does not affect p38 MAPK, JNK, and $p70^{S6}$ kinase in primary cultured cortical neurons. In addition, p38 MAPK inhibitor SB203580 was not protective against SNP. Therefore, we can exclude the possibility that U0126 protected by nonspecific inhibition of these proapoptotic molecules. We found that U0126 did not reduce the phosphorylation levels of MEK1/2 in cortical neurons exposed to oxygen deprivation. Rather, U0126 was associated with an increase of MEK1/2 phosphorylation levels. These results suggest that U0126 does not affect upstream components of MEK1/2. Furthermore, the protection afforded by U0126 is not due to scavenging of free radicals because NO elicits phosphorylation of ERK2 by direct activation of Ras, an upstream effector of the MEK/ERK pathway (11). Another MEK inhibitor, PD98059, also attenuated SNP-induced neuronal death. Hence, we believe that the neuroprotective efficacy of U0126 results from its inhibition of MEK1/2.

U0126 inhibited phosphorylation of ERK2 in a dose-dependent manner; however, U0126 had no effect on phosphorylation of ERK1 during 2 h after reoxygenation after 9 h of oxygen deprivation. The inhibition of ERK2 phosphorylation was correlated with inhibition of neuronal death. These findings suggest that ERK1 and ERK2 play differential roles in cell survival and cell death, respectively. Sutherland *et al.* have shown that activation of ERK2 but not ERK1 is associated with B cell antigen receptor-induced apoptosis in B lymphoma cells (37). We cannot presently explain this differential effect of U0126 on the phosphorylation states of ERK1 and ERK2. It is possible that phosphorylated ERK1 may be more stable than phosphorylated ERK2 in cortical neurons during these reoxygenation periods.

In conclusion, we demonstrated brain protection against ischemia by i.v. injection of MEK inhibitor U0126. These results constitute further evidence for the crucial role of MEK/ERK in brain injury resulting from ischemia and reperfusion in experimental models. In addition, as demonstrated in our animal models, phosphorylation of ERK2 was dramatically increased in the brain by reperfusion after ischemia. These data, together with accumulating evidence that the MEK/ERK pathway contributes to oxidative injury (31, 33, 34, 36), suggest that U0126 may protect brain from reperfusion injury.

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