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A NEUROPROTECTIVE ROLE OF EXTRACELLULAR SIGNAL-REGULATED KINASE IN *N*-ACETYL-*O*-METHYLDOPAMINE-TREATED HIPPOCAMPAL NEURONS AFTER EXPOSURE TO *IN VITRO* AND *IN VIVO* ISCHEMIA

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Abstract—In response to cerebral ischemia, neurons activate survival/repair pathways in addition to death cascades. Activation of cyclic AMP-response-element-binding protein (CREB) is linked to neuroprotection in experimental animal models of stroke. However, a role of the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MAPK/ERK or MEK), an upstream kinase for CREB, and its relation to CREB phosphorylation in neuroprotection in cerebral ischemia has not been delineated. Previously, we reported that *N*-acetyl-*O*-methyldopamine (NAMDA) significantly protected CA1 neurons after transient forebrain ischemia [J Neurosci 19 (1999b) 87.8]. The current study is to investigate whether NAMDA-induced neuroprotection occurs via the activation of ERK and its downstream effector, CREB. NAMDA induced ERK1/2 and CREB phosphorylation with increased survival of HC2S2 hippocampal neurons subjected to oxygen-glucose deprivation. These effects were reversed by U0126, a MEK kinase inhibitor. Similarly, animals treated with NAMDA following ischemia showed increased ERK and CREB phosphorylation in the CA1 subregion of the hippocampus during early reperfusion period with increased number of surviving neurons examined 7 days following ischemia. The NAMDA-induced neuroprotection was abolished by U0126 administered shortly after reperfusion. The results showed that the ERK-CREB signaling pathway might be involved in NAMDA-induced neuroprotection following transient global ischemia and imply that the activation of the pathway in neurons may be an effective therapeutic strategy to treat stroke or other neurological syndromes.

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Abbreviations: ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; CREB, cyclic AMP-response-element-binding protein; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's Modified Eagle medium; DMSO, dimethyl sulphoxide; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK/ERK or MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; NAMDA, *N*-acetyl-*O*-methyldopamine; OD, optical density; OGD, oxygen-glucose deprivation; PB, phosphate buffer; PBS, phosphate-buffered saline; pCREB, phosphorylated CREB; pERK, phosphorylated ERK; PLSD, protected least significant difference; RSK, ribosomal S6 kinase; SDS, sodium dodecyl sulphate; TBS, tris-buffered saline; 4-VO, four-vessel occlusion.

Key words: ERK and CREB phosphorylation, neuroprotection, *N*-acetyl-*O*-methyldopamine, selective neuronal injury, oxygen-glucose deprivation, transient forebrain ischemia.

Upon pathological stress, cells activate multiple signaling pathways that determine their fate. Although the inhibition of targeted death cascades could provide neuroprotection, promoting the endogenous survival/repair mechanism may bear equal, if not greater, importance in achieving neuroprotection.

Activation of cyclic AMP-response-element binding protein (CREB) by phosphorylation at the Ser133 residue is critical in regulating the expression of many downstream genes containing CRE elements (Frank and Greenberg, 1994; Davis et al., 2000). CREB is phosphorylated by multiple kinases including protein kinase A, calcium-calmodulin kinases, protein kinase B (PKB) (Du and Montminy, 1998) and mitogen-activated protein kinase/extracellular signal regulated kinase kinase (MAPK/ERK, MEK) via ribosomal S6 kinase (RSK). Functionally, CREB phosphorylation is involved in nerve cell excitation, CNS development, long-term memory formation, and circadian rhythm as well as neuroprotection (Walton et al., 1996, 1999; Riccio et al., 1999; Somers et al., 1999; Tanaka et al., 1999, 2000; Walton and Dragunow, 2000; Mabuchi et al., 2001). *In vitro* studies suggested that CREB regulates genes that mediate neurotrophin-induced neuronal survival (Bonni et al., 1999; Riccio et al., 1999). In an animal model of transient forebrain ischemia, where CA1 hippocampal neurons degenerate selectively (Kirino, 1982; Pulsinelli, 1985), the degree of CREB phosphorylation (pCREB) was positively correlated with surviving neurons and also shown to be a protective response (Walton et al., 1996; Hu et al., 1999; Mabuchi et al., 2001). Moreover, a neuroprotective role of pCREB has been recently defined by disrupting of CREB function in the mouse CNS (Mantamadiotis et al., 2002).

MEK, an upstream kinase of CREB, is a member of the MAPK family that is involved in cell differentiation and proliferation, learning, long-term potentiation, and neuronal plasticity (English and Sweat 1996, 1997; Atkins et al., 1998; Impey et al., 1999; Davis et al., 2000). Unlike CREB, the role of the MEK pathway in pathologic insults such as cerebral ischemia is controversial. Whereas the inhibition of MEK pathway was shown to be either neuroprotective or had no effect in ischemia (Alessandrini et al., 1999; Sugino

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et al., 2000; Namura et al., 2001), MEK activation was linked to neuroprotection in ischemic tolerance and hypothermia, and in estrogen treatment against excitotoxic lesions (Hicks et al., 2000; Strohm et al., 2000; Kuroki et al., 2001).

We previously reported that treating animals with *N*-acetyl-*O*-methyldopamine (NAMDA) increased the number of surviving CA1 neurons after transient global ischemia (Cho et al., 1999b). The purpose of the current study is to establish a role for ERK and its downstream CREB signaling in neuroprotection following ischemia. Specifically, we focused to investigate whether NAMDA-induced neuroprotection occurs via an ERK–CREB pathway in *in vitro* (oxygen glucose deprivation, OGD) and *in vivo* ischemic conditions.

EXPERIMENTAL PROCEDURES

Materials

Cell culture media and antibiotics were obtained from Media Tech (Herndon, VA, USA). N2 supplement, basic fibroblast growth factor (bFGF), gentamicin, and fungizone were purchased from Gibco (Grand Island, NY, USA). Poly-L-ornithine and laminin were obtained from Sigma (St. Louis, MO, USA) and BD Bioscience (Bedford, MA, USA), respectively. NAMDA was synthesized from methyldopamine hydrochloride (Aldrich Chemical Co., Milwaukee, WI, USA) according to the method described previously by Cho et al. (1999b). The antibodies directed against phosphorylated Ser133 and phosphorylation state-independent CREB were obtained from Upstate Biotechnology (Lake Placid, NY, USA). The antibodies directed against dually phosphorylated Thr202 and 204 and phosphorylation state-independent ERK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). U0126 (a MEK1/2 inhibitor), SB203580 (a p38 MAPK inhibitor), LY294002 (a phosphatidylinositol 3(PI3)-kinase inhibitor) and other chemicals were obtained from Sigma.

Cell cultures

The immortalized neuronal progenitor cell line HC2S2 was derived from adult rat hippocampus (Hoshimaru et al., 1996) and was obtained from Dr. Fred Gage at the Salk Institute (San Diego, CA, USA). HC2S2 cells were grown in serum-free Dulbecco's Modified Eagle medium (DMEM)/Ham's /Ham's F-12 (50/50) with glutamine containing N2 supplement (insulin at 5 μg/ml, human transferrin at 50 μg/ml, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite), bFGF at 2 ng/ml, gentamicin and fungizone on a plastic tissue culture dish coated with poly-L-ornithine and laminin. Cells were split when they were 70–80% confluent.

OGD

This system is used to generate an ischemic-like condition *in vitro*. Cultured HC2S2 cells (1×10^5) were replaced with either glucose containing or glucose-free DMEM/Ham's F-12 (50/50) and treated with either one or combination of following drugs: NAMDA (5 mM), U0126 (20 μl), SB203580 (10 μl) or LY294002 (10 μl). The cultures were immediately placed in a chamber (Billups-Rothenberg, Del Mar, CA, USA). Except non-OGD controls, chambers containing cultures for OGD were flushed with 95% N_2/5% CO_2 gas mixture for 15 min at a flow rate of 4 l per minute (LPM) to create a hypoxic condition. Both normoxic and hypoxic chambers were incubated at an 37 °C incubator for 12 h. HC2S2 neurons were then resuspended in 200 μl of 0.05% Trypan Blue solution and surviving neurons were counted using a hemocytometer. To examine neuronal morphology, HC2S2 neurons (1×10^3) in chamber slides after 12 h OGD were fixed with 2% cold formaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.2), and stained with 0.1% Cresyl Violet.

Western blot hybridization

Cells (1×10^6) were washed with phosphate-buffered saline (PBS; 0.1 M, pH 7.4), collected, and centrifuged to pellet. To lyse the cell pellet, 0.5 ml of RIPA buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS)) with freshly added phenylmethyl sulphonyl fluoride (PMSF 0.4 mM), aprotinin (30 μl/ml), and sodium orthovanadate (1 nM) were added to the pellet, incubated for 30 min on ice, centrifuged at $1000\times g$ for 15 min at 4 °C. Protein concentration from the supernatant was determined (Bio-Rad Laboratories, Hercules, CA, USA) and 30 μg of proteins were loaded for SDS-polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed and proteins were transferred from the gel to low protein binding Durapore (PVDF) (Amersham Phamarcia Biotech Inc., Piscataway, NJ, USA) using an electroblotting apparatus. Membranes were blocked overnight in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% dry milk, incubated with primary antibodies that recognize pCREB (1:1000), pERK (1:2000), and c-Fos (1:1000; Oncogene Research Products, Cambridge, MA, USA), followed by horseradish peroxidase-conjugated secondary antibodies for 1 h. Membranes were washed three times (30 min each) with TBS containing 0.1% Tween-20 between each step. Protein bands were visualized by an ECL Plus Western blotting detection system (Amersham Pharmacia Biotech Inc.). The membrane is reblotted to visualize non-phosphorylated form of ERK (1:1000) and CREB (1:1000). For quantification, densities of phosphorylated ERK and CREB were normalized by corresponding non-phosphorylated forms of ERK and CREB. The values were then expressed in relation to that of non-OGD cultures.

Transient forebrain ischemia

Animal surgery was in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and The Institutional Animal Care and Use Committee of Weill Medical College of Cornell University. All efforts were made to minimize the number of animals used and their suffering. Adult male Wistar rats (200–250 g; Hill Top, Scottsdale, AZ, USA) were anesthetized with a mixture of halothane (1%), oxygen, and nitrogen and surgically prepared for four-vessel occlusion (4-VO) according to the method described by Pulsinelli et al. (1982). Briefly, both common carotid arteries were occluded using reversible clasps and vertebral arteries were occluded by electrocauterization. Collateral blood flow to the brain was controlled by an adjustable neck suture. A burr hole on the skull (1 mm posterior to bregma, 1.5 mm lateral) was drilled for post-ischemic administration of U0126 or vehicle after ischemia. Animals were deprived of food overnight, but water was freely available. On the following day, 10 min 4-VO ischemia was induced by tightening the clasps around the common carotid arteries and the suture. To minimized variability, the following criteria were applied: loss of righting reflex and bilateral pupil dilation during the entire ischemic period and 20±5 min of post-ischemic coma after 10 min ischemia. Animals were treated with either triple injection of saline or NAMDA (10 mg/kg) at 0, 1/2, and 2 h after ischemia. In addition, while animals were in post-ischemic coma, 5 μl of 100 μM U0126 (total 0.5 nmoles) or vehicle (0.4% dimethyl sulphoxide, DMSO) were injected over 5 min at the lateral ventricle (3.5 mm from dura). The body temperature of all animals was kept at 37.5±0.5 °C by a thermocouple-regulated heating lamp during ischemia and reperfusion until the animals regained consciousness and re-established thermohomeostasis.

Tissue preparation

Animals were anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with saline containing 0.5% sodium nitrite and 10 U/ml heparin sulfate followed by 4% cold formaldehyde in 0.1 M PB (pH 7.2). The brains were further post-fixed for 2 h and stored in a 30% sucrose solution overnight. Using a sliding microtome, the dorsal hippocampus between bregma −3.0 mm and −4.0 mm was sectioned at a thickness of 20 μm for Cresyl Violet staining and 40 μm for immunocytochemistry.

Immunocytochemistry

Immunocytochemistry was performed using an avidin/biotin peroxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) method (Cho et al., 1999a). Briefly, sections were incubated in 0.1 M PBS containing 3% H_2O_2 and 10% methanol, followed by 2% normal horse or goat serum, and subsequently incubated overnight with one of the following specific antibodies in 1% normal serum: phosphorylated CREB (1:500), phosphorylated ERK (1:300), and c-Fos (1:10,000). On the following day, the sections were incubated with appropriate biotinylated secondary immunoglobulin G (anti-rabbit goat antibody or anti-mouse horse antibody; 1:200) for 1 h, and avidin/biotin/peroxidase for 1 h in a humidified chamber. PBS was used to wash sections between all steps. The antigen–antibody complexes were visualized by incubation for 5 min in 0.05% 3,3′-diaminobenzidine (DAB) or with 0.1% Ni (Ni-DAB to enhance color reaction) and 0.003% H_2O_2. To test the specificity of antibodies, primary antibodies were preabsorbed with respective cognate peptides (2–3 mg/ml of antibody working solution) in PBS for 2 h and then followed immunocytochemical procedures described above. For the quantification of pCREB immunoreactivity, the mean intensity of immunoreactivity in the entire CA1 pyramidal layer (mean intensity per pixel) was obtained after subtracting mean intensity of strata radiatum as background.

Neuron density measurement

Neuron density in the CA1 pyramidal layer was assessed according to the method described previously by Cho et al. (1999b). Briefly, digital images of the CA1 hippocampus were acquired on a Zeiss Axiophoto microscope fitted with an Axiocam video camera, using KS400 image analysis software (Zeiss, Thornwood, NY, USA). Images were overlaid with a grid composed of 100 μm×100 μm boxes to facilitate systematic counting along the *x*- and *y*-axes. Two sections at the level of dorsal hippocampus (bregma −3.8 mm) at least 100 μm apart were evaluated bilaterally by an investigator who was blinded to the experimental conditions to obtain mean density for each animal.

RESULTS

ERK-induced CREB phosphorylation in NAMDA-treated OGD neurons

ERK phosphorylation in rat hippocampal-derived HC2S2 neurons subjected to OGD was first assessed in the presence and the absence of the neuroprotectant NAMDA (Fig. 1). There were high basal levels of phosphorylated ERK (pERK) and pCREB in non-OGD control cultures (Figs. 1 and 2), probably due to the presence of bFGF in the culture media. However, compared to non-OGD control cultures, cultures exposed to 12 h OGD showed reduced expression of pERK (Fig. 1). Treating cultures with NAMDA during OGD reversed the reduction. The NAMDA-induced ERK phosphorylation was significantly repressed by co-

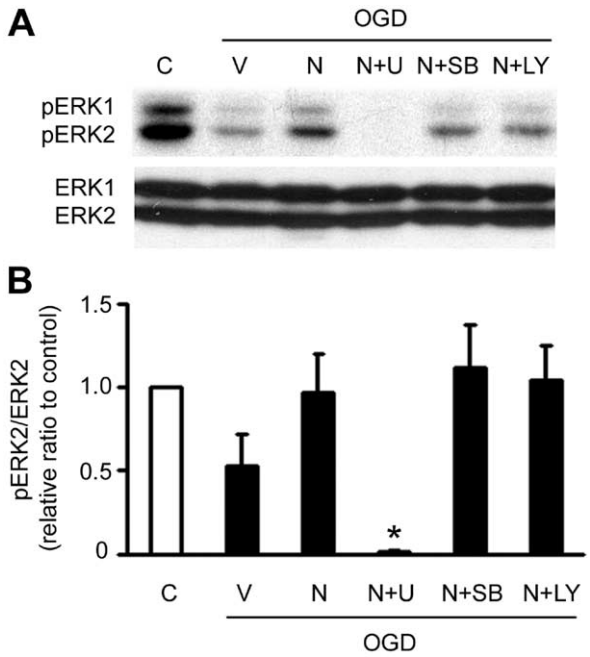

Fig. 1. ERK phosphorylation in HC2S2 neurons. *A,* Shown are Western blots of ERK1/2 in neuronal cultures exposed to 12 h OGD. Cultures were treated with NAMDA or NAMDA with an indicated inhibitor prior to subject to 12 h OGD. Note that the changes of pERK2 were much more pronounced. ERK2 was used to normalize the densities of pERK2. *B,* For quantification, the optical density (OD) values were expressed as ratios of non-OGD control cultures and averaged from five sets of independent experiments (mean±S.D.). V, vehicle (0.01% DMSO); N, 5 mM NAMDA; U, 20 μM U0126; S, 10 μM SB203580; L, 10 μM LY294002. * $P<0.05$ vs. N (NAMDA), one-way analysis of variance (ANOVA), Fisher's protected least significant difference (PLSD) multiple comparison test.

treatment with U0126, a specific MEK inhibitor. Treatment with SB203580 (a p38 MAPK inhibitor), or LY294002 (a *PI*3 kinase inhibitor) produced little repression of NAMDA-induced ERK phosphorylation, indicating no cross-reactivity of these kinases with the MEK signaling pathway. CREB phosphorylation was also assessed under the identical culture conditions (Fig. 2). There was a 40% reduction of pCREB expression in OGD exposed as compared to non-OGD control cultures (Fig. 2B). Similar to ERK phosphorylation, treating cultures with NAMDA during OGD reversed the reduction. In addition, the NAMDA-induced CREB phosphorylation was significantly repressed by co-treatment with U0126, but neither by SB203580 nor LY294002. The expression level of c-Fos, a target transcription factor of CREB, was parallel with that of pCREB response to these agents (Fig. 2A).

ERK-dependent neuroprotection in NAMDA-treated OGD neurons

To investigate whether NAMDA-induced ERK and CREB activation is involved in neuroprotection, the morphology and the number of surviving neurons were assessed in HC2S2 neurons following OGD treatment (Fig. 3). Compared to non-OGD control cultures (Fig. 3A, H), exposure to 12 h of OGD resulted in 70% neu-

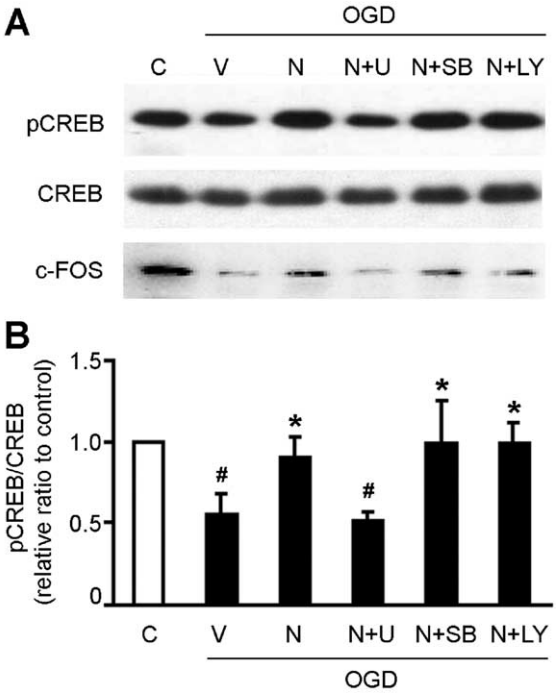

Fig. 2. CREB phosphorylation in HC2S2 neurons. *A,* Shown are Western blots of pCREB and CREB, and c-Fos in neuronal cultures exposed to 12 h OGD. Cultures were treated with NAMDA or NAMDA with an indicated inhibitor prior to subject to 12 h OGD. CREB was used to normalize the densities of pCREB. *B,* For quantification, the OD values were expressed as ratios of non-OGD control cultures and averaged from five sets of independent experiments (mean±S.D.). V, vehicle (0.01% DMSO); N, 5 mM NAMDA; U, 20 μM U0126; S, 10 μM SB203580; L, 10 μM LY294002. * $P<0.05$ vs. V (vehicle), # $P<0.05$ vs. N (NAMDA), one-way ANOVA, Fisher's PLSD multiple comparison test.

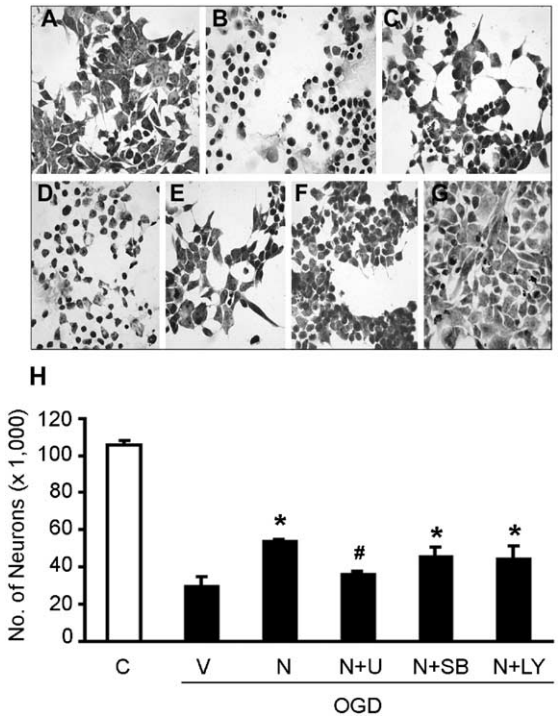

Fig. 3. ERK-dependent neuroprotection against OGD. HC2S2 neurons subjected to 12 h OGD were stained with Cresyl Violet to examine their morphology. *A,* Non-OGD control; *B,* OGD; *C,* OGD+5 mM NAMDA (N); *D,* OGD+N+20 μM U0126 (U); *E,* OGD+N+10 μM SB203580 (SB); *F,* OGD+N+10 μM LY294002 (LY); *G,* 20 μM U0126 without OGD; *H,* quantification of the number of surviving HC2S2 neurons. HC2S2 neurons were treated with NAMDA or NAMDA with an indicated inhibitor prior to subject to 12 h OGD. The number of surviving neurons was counted at the end of OGD. Values represent mean±S.D. * $P<0.05$ vs. V (vehicle), # $P<0.01$ vs. N, one-way ANOVA, Fisher's PLSD multiple comparison test ($n=5$–7). Images were adjusted for optimal contrast and sharpening using Adobe photoshop, version 6.0.

ronal death (Fig. 3B). NAMDA significantly increased the number of surviving neurons (Fig. 3C; $P<0.05$). The NAMDA-induced neuroprotection disappeared with U0126 co-treatment (Fig. 3D; $P<0.01$), but not with SB203580-or LY294002-treated cultures. The morphology of U0126-treated OGD cultures was not different from that of OGD culture shown in Fig. 3B. In addition, neurons retained normal morphology when the same concentration of U0126 was added to non-OGD-treated cultures (Fig. 3G), indicating the absence of non-specific cytotoxicity of U0126 at the concentration used.

NAMDA-induced ERK/CREB phosphorylation in the early post-ischemic hippocampus

Immunolocalization of pERK during early reperfusion hours was examined in rat hippocampus after 10 min of 4-VO ischemia. In the control and 3 h post ischemic hippocampus, pERK immunoreactivity (Fig. 4Aa, b) was observed in stratum oriens and stratum radiatum, but absent in the pyramidal layer of the CA1 sub-region. The immunoreactivity of pERK in the stratum oriens and stratum radiatum was increased in the animal treated with NAMDA at 0, 1/2 and 2 h of reperfusion (Fig. 4Ac). In order to determine whether increased pERK activity in the stratum oriens and stratum radiatum is associated with CREB activation in NAMDA-treated CA1 sub-region, CREB phosphorylation was assessed in ischemic hippocampus (Fig. 4Ad–f). pCREB positive CA1 pyramidal neurons were rarely found in the control hippocampus. At 3 h post-ischemia, pCREB immunoreactivity in the CA1 pyramidal layer was slightly increased along with a few pCREB positive neurons (Fig. 4Ae). Treatment of NAMDA at 0, 1/2 and 2 h of reperfusion significantly increased pCREB immunoreactivity in the CA1 pyramidal layer (Fig. 4A f, g; $P<0.05$). Preabsorption with respective cognate peptides to pERK and pCREB primary antiserum completely abolished the staining of pERK and pCREB in the adjacent sections (data not shown).

We further examined the expression of c-Fos, a protein product encoding CRE-dependent *c-fos* gene, to confirm that CREB activation leads to the transcription of target genes. Ischemia caused a slight induction of c-Fos in the CA1 pyramidal layer 6 h after ischemia (Fig. 4Bb) and the expression was further potentiated in the NAMDA-treated CA1 neurons (Fig. 4Bc).

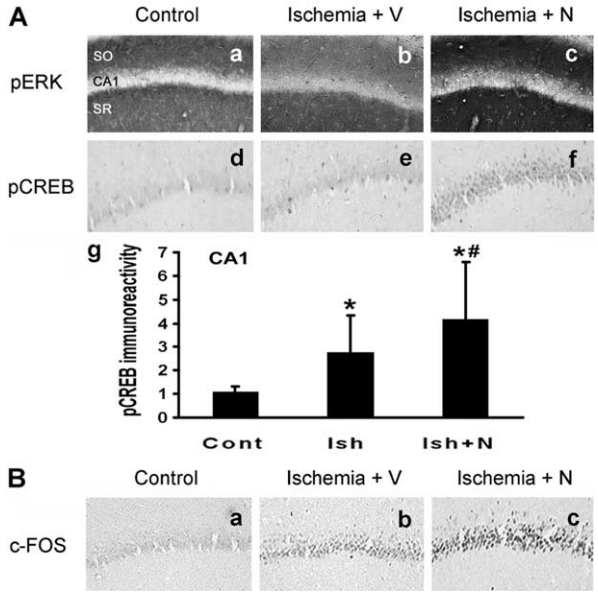

Fig. 4. Immunolocalization of pERK, pCREB and c-Fos in post-ischemic CA1 hippocampus. Comparable sections at the level of dorsal hippocampus of CA1 are used to localize pERK (*Aa–c*), pCREB (*Ad–f*), and c-Fos (*Ba–c*) at 3 h post-ischemia for pERK and pCREB, and 6 h post-ischemia for c-Fos immunohistochemistry. Each section is representative from three sets of paired saline- and NAMDA-treated animals after ischemia. *B, g,* Quantification of pCREB immunoreactivity in the CA1 pyramidal layers. * $P<0.05$ vs. control (Cont), # $P<0.05$ vs. ischemia (Ish), one-way ANOVA, Fisher's PLSD multiple comparison test ($n=3–4$). Triple i.p. injection of saline (V) or NAMDA (N; 10 mg/kg each time) were given at 0, 1/2 and 2 h of reperfusion. CA1, CA1 pyramidal layer; SO, stratum oriens; SR, stratum radiatum. Images were adjusted for optimal contrast and sharpening using Adobe photoshop, version 6.0.

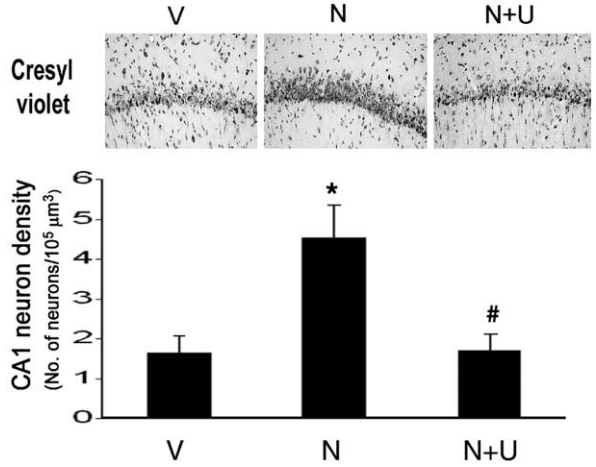

Fig. 5. ERK-dependent neuroprotection against transient global ischemia. Cresyl Violet staining in CA1 layer of vehicle-treated (V), NAMDA-treated (N) and NAMDA+U0126-treated (N + U) ischemic animals 7 days after ischemia. Mean neuron density in the CA1 hippocampus measured 7 days post-ischemia. Triple i.p. injection of saline or NAMDA (10 mg/kg each time) were given at 0, 1/2 and 2 h of reperfusion. U0126 (0.5 nmoles) was given intraventricularly 15 min after reperfusion. Neuron densities (number of neurons/$10^5\mu m^3$) are expressed as mean±S.D. $N=5–7$ for each group. * $P<0.05$ vs. V, # $P<0.05$ vs. N, ANOVA, post-hoc Fisher's PLSD. Images were adjusted for optimal contrast and sharpening using Adobe photoshop, version 6.0.

ERK-dependent neuroprotection in NAMDA-treated ischemic CA1 neurons

To investigate whether ERK activation is account for neuronal survival *in vivo*, we assessed the number of surviving neurons in animals treated with saline, NAMDA, and NAMDA+U0126. Neuronal survival was significantly greater in NAMDA- as compared to saline-treated ischemic animals as indicated by neuronal density measured 7 days after ischemia (Fig. 5). The NAMDA-induced neuroprotection was abolished by U0126 co-treatment.

DISCUSSION

The current study was directed to investigate whether ERK phosphorylation and its downstream target CREB cascade serve as an endogenous neuroprotective signaling pathway in NAMDA-induced neuroprotection following ischemia. We demonstrated that ERK-dependent CREB phosphorylation and CREB-mediated c-Fos expression occurs in NAMDA-treated ischemic neurons and NAMDA-induced neuroprotection following ischemia is ERK-dependent.

cAMP and its analogue via cAMP-dependent pathway are known to promote survival and neurite outgrowth in sympathetic and sensory neurons (Rydel and Greene, 1988) and to suppress programmed cell death in rat sympathetic and cerebellar granular neurons (Edwards et al., 1991; Chang et al., 1996; Chang and Korolev, 1997). Furthermore, elevated Ser133 phosphorylation of CREB protects neurons against apoptosis induced by okadaic acid (Woodgate et al., 1999). Neuroprotective role of CREB has been clearly delineated *in vitro* and *in vivo* studies (Bonni et al., 1999; Riccio et al., 1999; Mabucchi et al., 2001; Mantamadiotis et al., 2002). Consistent with these reports, we also showed that increasing CREB phosphorylation by a neuroprotective measure (e.g. NAMDA) was associated with increased survival of HC2S2 neurons subjected to OGD. In an experimental animal model of stroke, CREB phosphorylation was shown to be associated with surviving neurons both in global and focal ischemia (Walton et al., 1996; Hu et al., 1999; Tanaka et al., 1999, 2000; Walton and Dragnow, 2000; Tanaka, 2001). Similar to these results, we also observed more CREB phosphorylation in the resistant CA3 neurons and dentate granular cells compared to vulnerable CA1 in the transient global ischemic rat model (data not shown). In addition, our findings of increased neuronal survival with the increased number of pCREB positive neurons in the CA1 of NAMDA-treated ischemic animals support the view that CREB phosphorylation is associated with neuroprotection after ischemia *in vivo*.

MEK phosphorylates CREB via activation of sequential ERK and RSK-2. Unlike the delineated neuroprotective role of CREB, a role of ERK is debatable. Alessandrini et al. (1999) and Namura et al. (2001) reported that the inhibition of MEK1 protein kinase (thus inhibiting ERK activation) reduced damage in focal ischemic animal models. However, there are many reports that support the notion

that ERK activation is a neuroprotective signal (Ozawa et al., 1999; Shamloo et al., 1999; Hicks et al., 2000; Strohm et al., 2000). In ischemic animals, neuroprotective measures such as preconditioning and hypothermia were linked to ERK activation (Hicks et al., 2000; Strohm et al., 2000). ERK activation also protected neurons against quinolinate and glutamate toxicity (Singer et al., 1999; Kuroki et al., 2001; see review by Lee and McEwen, 2001). Furthermore, urocortin, a member of the corticotorpin releasing hormone family of neuropeptides, has been shown to activate ERK 1/2 and to protect cultured hippocampal neurons from oxidative and excitotoxic cell death (Grammatopoulos et al., 2000; Pedersen et al., 2002). The discrepancy between neuroprotective and adverse effects of ERK activation may lie in either difference in experimental animal models or dual potential of ERK activation. Ishikawa and Kitamura (1999) reported that inhibition of transient up-regulation of ERK protects neurons from H_2O_2-induced cell death. In contrast, the inhibition of prolongation of basal and constitutive activity of ERK resulted in cell death. Since ERK activation occurs within minutes of reperfusion after ischemia, inhibition of insult-induced transient ERK activation may afford neuroprotection as reported by Alessandrini (1999) and Namura et al. (2001). On the other hand, applying neuroprotective measures (i.e. pre-ischemic conditioning, hypothermia, and estrogen) may increase or prolong ERK activation, which subsequently turns on survival cascades. Stanciu and DeFranco (2002) recently reported that prolonged nuclear retention of activated ERK is a critical factor in eliciting proapoptotic effects in neurons subjected to oxidative stress. However, the authors were unable to link between nuclear retention of ERK and CREB phosphorylation in the study, suggesting the presence of downstream events other than CREB phosphorylation after ERK activation. Therefore, it is tempting to speculate that endogenous surviving cascades such as a ERK–CREB pathway can be enhanced by neuroprotective measure (or preconditioning) and that ERK activation caused by insult alone or insult with neuroprotective measures may differentially activate downstream-signaling pathways. Unlike the above studies that focused only on an ERK signaling pathway with neuronal death/survival, the present study was directed to define the role of ERK signaling in relation to CREB activation. Therefore, NAMDA-induced neuroprotection via ERK activation that leads to CREB phosphorylation during a critical period maybe a key step toward transcription of CRE-dependent genes necessary for survival.

Mechanistic studies using an *in vitro* ischemic condition revealed that the inhibition of other P38 MAPK did not affect the NAMDA-induced ERK–CREB signaling pathway and neuroprotection, indicating no cross-inhibition of ERK activation by the P38 inhibitor. In addition, the inhibition of the protein kinase B pathway had little effect on ERK-dependent CREB phosphorylation and on the number of surviving neurons in NAMDA-treated cultures. On the other hand, the inhibition of ERK activation by U0126 attenuated NAMDA-induced CREB activation and the survival of hippocampal neurons following OGD. The finding indicates that ERK–CREB signaling pathway might be involved in the survival of NAMDA-treated ischemic neurons.

Ischemia altered expression of a number of genes including immediate early genes, heat shock protein, basic FGF, nerve growth factor, brain-derived neurotrophic factors, and many other trophic factors and receptors (Finklestein et al., 1988, 1990; Lindvall et al., 1992; Nowak 1993; Takeda et al., 1993; Hsu et al., 1993, 1994), some of which are implicated in functional recovery after stroke. Phosphorylation of a critical CREB residue, Ser/133, results in transactivation of some target genes including *c-fos, bcl2, bdnf, Mcll*, as well as many other unidentified genes. Prolongation of CRE binding activity via ERK, thus, may be one of the critical steps necessary to prevent ischemia-induced neuronal injury. Supporting this idea is the unique action of NAMDA that increases expression of activated ERK and CREB and subsequently protected neurons from insults both *in vitro* and *in vivo*. In addition, increased CREB phosphorylation was followed by increased the number of c-Fos positive neurons in NAMDA-treated ischemic CA1 neurons at early reperfusion hours (Fig. 4B), indicating that CREB phosphorylation leads to the transcription of *c-fos*, a downstream CREB-mediated target gene. The finding supports a functional role of increased CREB activation *in vivo*. The previously reported a possible neuroprotective role of early c-Fos induction after cerebral ischemia (Cho et al., 2001) is consistent with our present finding that CRE-dependent gene transcription may be required for neuronal survival.

In summary, the current study demonstrates the role of ERK in hippocampal neuronal survival and provides a basis to develop therapeutic strategies aimed at augmenting the ERK–CREB cascade to enhance neuronal survival against pathological insults such as ischemia.

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