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Title	Nitric Oxide Produced During Ischemia Is Toxic but Crucial to Preconditioning-Induced Ischemic Tolerance of Neurons in Culture
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Nitric oxide produced during ischemia is toxic but crucial to preconditioning-induced ischemic tolerance of neurons in culture.

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Abstract

The present study investigated the roles of nitric oxide (NO) in preconditioning (PC)-induced neuronal ischemic tolerance in cortical cultures. Ischemia in vitro was simulated by subjecting cultures to both oxygen and glucose deprivation (OGD). A sublethal OGD (PC) significantly increased the survival rate of neurons when cultures were exposed to a lethal OGD 24 hr later. Both the inhibition of nitric oxide synthase (NOS) and scavenging of NO during PC significantly attenuated the PC-induced neuronal tolerance. In addition, exposure to an NO donor emulated the PC. In contrast, the inhibition of NOS and the scavenging of NO during lethal OGD tended to increase the survival rate of neurons. This study suggested that NO produced during ischemia was fundamentally toxic, but critical to the development of PC-induced neuronal tolerance.

Key words: ischemia, preconditioning, nitric oxide, neurotoxicity, cortical culture

Introduction

Preconditioning (PC) to ischemic tolerance is a phenomenon in which a brief subtoxic insult induces robust protection against the deleterious effects of a subsequent, prolonged, lethal ischemia (1). In the brain, Kitagawa et al. (2) first reported that gerbils subjected to a sublethal transient global ischemia exhibited reduced hippocampal neuronal death after a more severe ischemic insult 24~28 hr later. Since then, numerous studies have been performed on this phenomenon, but the mechanistic basis of PC-induced ischemic tolerance has not been fully delineated. For the induction of ischemic tolerance, a triggering role of neuronal NMDA receptor activation has been suggested (3,4). We have recently found that the PC-induced immediate enhancement in the phosphorylation of CREB in the penumbra region during subsequent lethal ischemia is crucially involved (5), although the triggering mechanisms for the enhancement remain unknown. Cinani et al. (6) recently demonstrated that nitric oxide (NO) signaling is functionally coupled to the phosphorylation of CREB, and is involved in the survival of neurons.

NO is generated in various mammalian tissues, and acts as an intercellular messenger associated with various physiological and pathological events (7). Recently, we have

demonstrated that the activity of nitric oxide synthase (NOS) varies associated with the differentiation of a neuronal cell line (NG108-15 cells), suggesting that NO functions as an important signaling molecule in differentiated NG108-15 cells (8). We have also found that NO modulates the propagation of astrocytic Ca^{2+} waves induced by the local photolysis of caged Ca^{2+} ionophore (9,10). In addition, Yun et al. (11) have recently demonstrated that the activation of p21^{RAS} induced by NO derived from nNOS is responsible for the PC-induced tolerance of neurons, suggesting that NO is a key mediator in the process leading to tolerance against lethal ischemia.

Here we provide experimental evidence suggesting that NO produced during ischemia was fundamentally toxic to neurons, but critical to the development of PC-induced ischemic tolerance of neurons in culture.

Experimental Procedure

The animal experiments conformed to the “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1996), as well as the “guide for the care and use of laboratory animals”, Hokkaido University School of Medicine (Hokkaido, Japan).

Cell culture.

Culture methods were described elsewhere in detail (12). In brief, neurons were prepared from 16 to 18 day old embryonic rat cortices and grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY) which was supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 10 % Ham’s F12, and 0.24 % penicillin/streptomycin (culture medium). Cells were plated at a uniform density of 3.0×10^5 cells/cm² onto poly-L-lysine (100 μ g/ml)-coated plastic dishes and maintained in a 5 % CO₂ incubator at 37 °C. The cultures were fed a filtered (0.22 μ m; Millipore, Bedford, TX) conditioned medium (CM) twice a week. To obtain the CM, cells from the 16-18 day old embryonic rat cortices were plated onto poly-L-lysine-coated 6-well dishes and cultured for more than 2 weeks. The cultures were then fed a cooled culture medium and incubated for an additional day. The

culture medium was then filtered and used as a CM. The experiments described here were performed on neurons maintained for 13-15 days in culture.

Immunocytochemistry.

Neurons were identified by immunostaining with an antibody against microtubule-associated protein 2 (MAP-2; Sigma, St Louis, MO). The nitric oxide synthases (NOS) in cultured cells were detected by immunostaining with anti-neuronal NOS (nNOS) (Euro-Diagnostica, Sweden), anti-endothelial NOS (eNOS) (Transduction Lab., Lexington KY), or anti-inducible NOS (iNOS) (Transduction Lab.). For the labeling of MAP-2, nNOS, eNOS, and iNOS, the cortical cells were fixed with 4 % paraformaldehyde for 5 minutes at 4 °C, followed by 95 % methanol in PBS for 10 minutes at -20 °C. The cells were then incubated with a primary antibody over a 24 hour period using a dilution of 1:1,000 for MAP-2, 1:1,000 for nNOS, 1:1,000 for eNOS, and 1:5,000 for iNOS. After being washed with phosphate-buffered saline (PBS), the cells were incubated with a secondary antibody containing 1.0 % goat serum for 30 minutes. For labeling, a 1:500 dilution of biotinylated goat antibody against mice IgG (Vector Laboratories, Burlingame, CA) was used. Bound antibodies were

detected by the avidin-biotin-peroxidase complex (ABC) method, using a commercial ABC kit (Vector Laboratories). Observation of peroxidase activity was made possible by incubation with 0.1 % 3,3'-diaminobenzidine tetrahydrochloride (DAB) in a 50 mM Tris-HCl buffer (pH 7.4) supplemented with 0.02 % H₂O₂. The cells were dehydrated in 70 – 100 % ethanol, cleared in xylene, and mounted on glass coverslips in Permount (Fisher Scientific, Fair Lawn, NJ) for light microscopic observation.

Oxygen-Glucose deprivation.

Cortical cultures were subjected to oxygen-glucose deprivation (OGD) injury using a protocol described previously (13). In brief, cultures were placed in an anaerobic chamber and washed two times with balanced salt solution (BSS: 116 mM NaCl, 0.8 mM MgSO₄, 5.4 mM KCl, 1.0 mM NaH₂PO₄, 26.2 mM NaHCO₃, 1.8 mM CaCl₂, 0.01 mM glycine, and 10 mg/l phenol red) lacking glucose. Near anoxic conditions were achieved using an Anaero-Pack System (Mitsubishi Gas Chemical, Tokyo, Japan). After pre-gassing with 95 % N₂-5 % CO₂ for at least 5 min to remove residual oxygen, glucose-free BSS was added to the cells, which were then placed in a purpose-built sealed chamber containing the deoxygenation reagent

(Kenki for Cells, Mitsubishi Gas Chemical). The catalytic reaction of the reagent resulted in the consumption of O₂ and production of CO₂. This Anaero-Pack System provided near anaerobic conditions with an O₂ concentration of < 1 % and a CO₂ concentration of about 5 % within 1 h of incubation at 37 °C. Cells were exposed to these conditions for a designated period to produce either mild (sublethal) or lethal OGD. To terminate OGD, cultures were carefully washed with glucose (20 mM) containing DMEM, and then incubated again at 37 °C in 95 % air-5 % CO₂ (reperfusion). Cultures with sham treatment not deprived of oxygen and glucose were placed in BSS containing 20 mM glucose.

Survival rate of neurons.

Neuronal death was analyzed following observation of the nuclear morphology using the fluorescent DNA-binding dyes, Hoechst 33342 (H33342) and propidium iodide (PI). Cells were incubated with these dyes for 15 minutes at 37°C. Individual nuclei were observed using fluorescent microscopy (Olympus, IX70, Tokyo, Japan) and subsequently analyzed. PI was used to identify nonviable cells. More specifically, an average of 450-500 neurons from random fields were analyzed in each experiment. The survival rate of neurons -- meaning the

percentage of viable neurons remaining -- was determined by placing images of nuclear staining on phase-contrast images, and calculating (viable neurons/total neurons before drug treatment) $\times 100$, since some neurons came off the dishes at the time of inspection. At least 4 independent experiments ($n \geq 4$) were conducted and analyzed.

Chemicals.

N^G -monomethyl-L-arginine (L-NMMA), Bisbenzimidazole (Hoechst 33342), and propidium iodide (PI) were obtained from Sigma. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (Carboxy-PTIO), and S-nitroso-N-acetyl-DL-penicillamine (SNAP) were obtained from Dojindo Lab., Inc. (Kumamoto, Japan). The other chemicals were from Wako Chem. (Tokyo, Japan).

Statistics.

Data are represented as the mean \pm S.D. Inter-group comparisons were made using the one-way analysis of variance (ANOVA) followed by a paired t-Test. Differences with a value of $P < 0.05$ were considered significant.

Results

We first investigated whether sublethal oxygen-glucose deprivation (OGD) induced neuronal ischemic tolerance in murine cortical cultures containing both neurons and glia (days *in vitro* 13-15). To do this, we have tried to determine both the sublethal and lethal exposure periods of OGD for cultured neurons (Fig. 1). Exposure to OGD for 1 hr did not result in significant neuronal death when examined 24 hr later (Fig. 1C and E). However, exposure for 2 hr produced massive neuronal death without glial degeneration 24 hr later (Fig. 1D and E). Thus, we adopted the 1 hr OGD as the preconditioning (PC), and 2hr OGD as the lethal insult. We then tried to determine the time interval between the PC and the lethal OGD for the effective development of ischemic tolerance in neurons. Cultures were preconditioned with 1 hr OGD and then exposed to lethal (2 hr) OGD 6, 24, and 48 hr later (Fig. 1F). Exposure of preconditioned cultures to lethal OGD 24 hr later resulted in a significant increase in the survival rate of neurons inspected 24 hr after the end of the lethal insult. However, no significant protective effect on neuronal death was observed in the preconditioned cultures exposed to the lethal insult 6 and 48 hr later. Thus, the time interval between the PC and the lethal OGD was set at 24 hr (reperfusion period).

We then investigated whether nitric oxide (NO) is involved in the development of PC-induced ischemic tolerance in neurons. We first performed an immunohistochemical analysis to delineate the expression patterns of nitric oxide synthase (NOS) isoforms (Fig. 2A). nNOS proteins were detected in the cultured neurons (A1), but neither iNOS (A2) nor eNOS (A3) proteins were clearly identified. We next analyzed whether the PC-induced ischemic tolerance of neurons was attenuated when the activity of NOS was inhibited by treatment with L-NMMA or the NO produced was scavenged by treatment with carboxy-PTIO (Fig. 2B). Treatment of cortical cultures with either L-NMMA or carboxy-PTIO during the exposure to sublethal OGD (PC) resulted in a significant decrease in the survival rate of neurons (PC NMMA + LI, PC PTIO + LI), suggesting that NO is crucially involved in the development of PC-induced ischemic tolerance in neurons. We then tried to determine whether NO was critical to the development of ischemic tolerance; that is, whether NO is produced during the period of sublethal OGD (PC) or during the period between the sublethal and lethal OGD (reperfusion period). When the production was inhibited (PC&rep NMMA + LI) or the NO produced was scavenged (PC&rep PTIO + LI) throughout the PC and the reperfusion period in the preconditioned cultures, a marked reduction in the survival rate of neurons was observed,

but there was no significant difference in the survival rate of neurons as compared to the cultures with either NOS inhibition or NO scavenging only during sublethal OGD. These results suggested that NO produced during the period of sublethal OGD (PC) played a critical role in the development of neuronal tolerance.

We further investigated whether the NO produced during PC is crucial to the development of PC-induced tolerance. To do this, cultures were exposed to SNAP, a donor of NO, instead of the sublethal OGD. Treatment with SNAP resulted in a significant increase in the survival rate of neurons, but that with decomposed SNAP did not (Fig. 2C).

We finally analyzed whether the NO produced during the period of lethal OGD played any role in the protection of neurons. Treatment of preconditioned cortical cultures with either L-NMMA or carboxy-PTIO during the exposure to lethal OGD resulted in an increase in the survival rate of neurons (PC + NMMA LI, PC + PTIO LI), although the increase was not significant (Fig. 3A). These results suggested that the NO produced during lethal OGD is toxic to neurons, although the NO produced during PC was crucial to the PC-induced neuronal tolerance. There is a possibility that the PC-induced increase in NOS expression was responsible for the deteriorative effect of NO on neurons during lethal OGD. Thus,

immunohistochemical analysis was performed on the cortical cultures 24 hr after the end of the sublethal OGD (PC) to clarify whether the expression patterns of NOS isoforms were altered by the PC insult (Fig. 3B). No detectable change in the expression patterns was observed, suggesting that NO produced by nNOS expressed in neurons was probably toxic to the neurons themselves. In fact, treatment of the non-preconditioned (control) cultures with either L-NMMA or carboxy-PTIO during the 2 hr period of lethal OGD also increased the survival rate of neurons, although the increase was not significant (Fig. 3C).

Discussion

The present study has demonstrated that NO produced during sublethal OGD (PC) was crucial in triggering the signal transduction pathway responsible for the development of the PC-induced neuronal tolerance (Fig. 2). NO was probably produced by the activation of nNOS expressed in neurons. However, both the inhibition of NOS and the scavenging of NO during lethal OGD tended to increase the survival rate of neurons (Fig. 3), suggesting that NO produced during ischemia is fundamentally toxic to neurons.

A previous study (14) has revealed that the induction of neuronal ischemic tolerance is dependent on new protein synthesis, and the development of tolerance is blocked by cycloheximide. The authors postulated that the NO/p21^{Ras}/Raf/Erk pathway is critically involved in the PC-induced ischemic tolerance of neurons. However, it is still unclear which Erk-activated transcription factor(s) or which protein(s) regulates the neuronal tolerance (1). We have recently found that the PC-induced immediate enhancement in the phosphorylation of CREB in the penumbra region during subsequent lethal ischemia is crucially involved in the protective effects in the rat model of cerebral infarction (5). The activation of Erk has been known to stimulate nuclear transcription factors such as CREB (15). These results may

support our previous finding that the enhanced phosphorylation of CREB is involved in the PC-induced ischemic tolerance of neurons. In the cortical cultures used here, sublethal OGD (PC) produced increased tolerance to lethal OGD (Fig. 1). However, the protective effect was transient; that is, the effects reached near maximum 24 hr after the end of the PC insult, and decreased thereafter (Fig. 1F). Since the PC-induced protective effects were short-lived in our cultures, the possibility exists the signaling pathway triggered by NO was protein synthesis-independent, and was probably mediated by post-translational protein modification. The mechanisms of the NO-induced ischemic tolerance of neurons are now being investigated.

In the present study, both the inhibition of NOS and scavenging of NO during lethal OGD resulted in an increase in the survival rate of neurons cultured with (Fig. 3A) or without prior sublethal OGD (PC) (Fig. 3C), suggesting that the NO produced during ischemia is fundamentally toxic to neurons. The expression patterns of NOS isoforms remained unchanged by the sublethal PC insult (Fig. 3B); in particular, no marked induction of iNOS was clearly observed in neurons or astrocytes. Thus, NO produced by nNOS expressed in neurons seemed critical to the development of ischemic tolerance as well as to the neurotoxicity. The role that NO plays in the mechanisms of ischemic brain injury has long been a source of much

debate (16). NO is known to be detrimental or beneficial to the injured brain; that is, NO functions like a double-edged sword against neurons (16). Inhibiting neuronal NOS either pharmacologically or genetically renders cultured neurons resistant to NMDA-induced death (17,18), and also reduces infarct volume in rodent models of transient focal ischemia (19). However, recent studies have revealed that NO inhibits the activation of caspase-3, an enzyme crucial for apoptosis, via S-nitrosylation of the active-site cysteine of the enzyme (20,21), and prevents neurons from undergoing apoptosis (22). The exact mechanism behind the PC-induced neuronal tolerance is currently unknown, but the ischemia-produced NO, fundamentally toxic to neurons, might trigger self-defense signaling in neurons against subsequent lethal ischemia.

In neuronal cells, NO is synthesized by neuronal Ca^{2+} /calmodulin-dependent nNOS, and is released in response to the activation of N-methyl-D-aspartate (NMDA)-type glutamate receptors (23). Previous studies (3,4) have found that the activation of NMDA receptors is essential to the PC-induced ischemic tolerance of neurons. All these findings have suggested that a sublethal ischemic insult (PC) activates neuronal NMDA receptors, and the resultant Ca^{2+} influx then activates nNOS. The ischemia-induced marked increase in the concentration of

extracellular glutamate seems necessary for the excessive activation of NMDA receptors. The mechanisms responsible for this increase in glutamate are now being investigated in cortical cultures.

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Figure legends

Fig. 1

Preconditioning (PC)-induced ischemic tolerance of neurons. Immunostaining of cultures with an anti-MAP2 antibody shows the existence of MAP2-positive neurons (A). Photomicrographs B, C, and D show the control culture (B), and cultures exposed to oxygen-glucose deprivation (OGD) for 1 hr (C), and 2 hr (D). Photomicrographs C1 and D1 show the cultures before exposure to OGD, whereas C2 and D2 illustrate their state 24 hours after the exposure for 1 and 2 hr, respectively. Cell nuclei were stained with bisbenzimidazole (Hoechst 33342) and propidium iodide (PI) (B3, C3, and D3). Red nuclei in D3 indicate dead PI-positive neurons. Exposure to OGD for 2 hr produced significant death of cultured neurons, but exposure for 1 hr did not (E). Figure F shows the effective time interval between the sublethal 1 hr OGD (PC) and the lethal 2hr OGD for the development of PC-induced neuronal ischemic tolerance. The scale bar indicates 200 μ m. Data are expressed as the mean+SD (n>4). * p<0.05. Abbreviations: rep, reperfusion (time interval between PC and LI); LI, lethal ischemia (2 hr OGD).

Fig. 2

Nitric oxide (NO) is crucially involved in the development of preconditioning (PC)-induced neuronal ischemic tolerance. Immunocytochemical analysis using anti-nNOS (A1), anti-iNOS (A2), and anti-eNOS (A3) antibodies indicates that there were nNOS-positive neurons, but not iNOS- or eNOS-positive cells in the cortical cultures. PC-induced neuronal tolerance was significantly attenuated by treatment with either 1 mM L-NMMA, an NOS inhibitor, or 10 μ M carboxy-PTIO, an NO scavenger, during sublethal OGD (PC) (B). Exposure of cultures to 100 μ M SNAP, a donor of NO, produced neuronal tolerance to subsequent lethal OGD, but decomposed SNAP, pre-incubated for 24 hr to exhaust all the NO, did not (C). SNAP was dissolved in DMSO, and the final concentration of DMSO (1 %) was added to the sham control. The scale bar indicates 200 μ m. Data are expressed as the mean \pm SD (n>4). * p<0.05 compared to sham. + p<0.05 compared to PC (B) or SNAP (C).

Abbreviations: NMMA, L-NMMA; PTIO, carboxy-PTIO; decSNAP, decomposed SNAP.

Other abbreviations are the same as those in Fig. 1. See text for details.

Fig. 3

Nitric oxide produced during ischemia is fundamentally toxic to neurons. Treatment of preconditioned cultures with either 1 mM L-NMMA or 100 μ M carboxy-PTIO during lethal OGD resulted in an increase in the survival rate of neurons (A). Immunocytochemical analysis using anti-nNOS (B1), anti-iNOS (B2), and anti-eNOS (B3) antibodies performed 24 hr after the end of the sublethal OGD (PC) indicates that the expression patterns of NOS isoforms were essentially the same as those in the cultures before the PC insult (Fig. 2A1-A3). Treatment of non-preconditioned cortical cultures with either L-NMMA or carboxy-PTIO during lethal OGD also resulted in an increase in the survival rate of neurons, although the increase was not significant (C). The scale bar indicates 200 μ m. Data are expressed as the mean+SD (n>4).

* p<0.05 compared to sham. Abbreviations are the same as those in Fig. 1 and Fig. 2.

Abbreviations:

carboxy-PTIO,

2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl3-oxide; CM, conditioned medium;

CREB, cAMP-responsive element binding protein; decSNAP, decomposed

S-nitroso-N-acetyl-DL-penicillamine; eNOS, endothelial nitric oxide synthase; Erk,

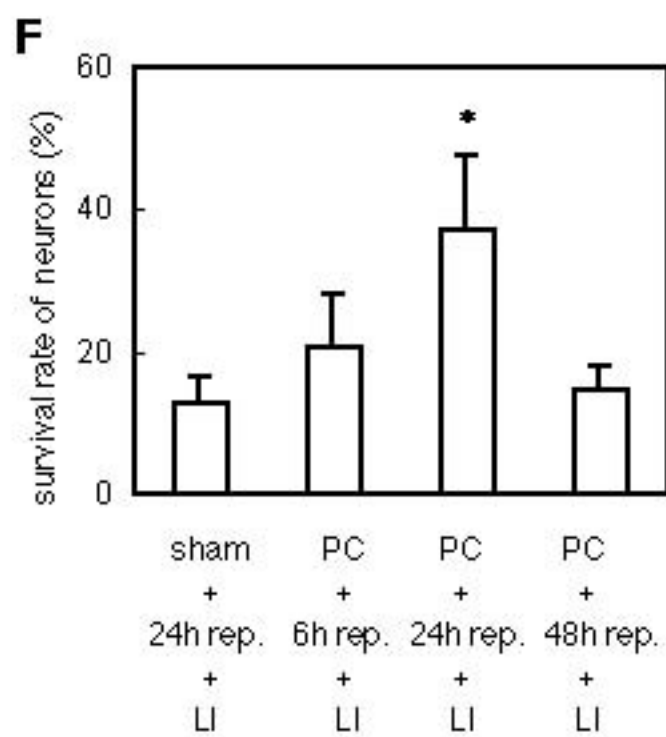
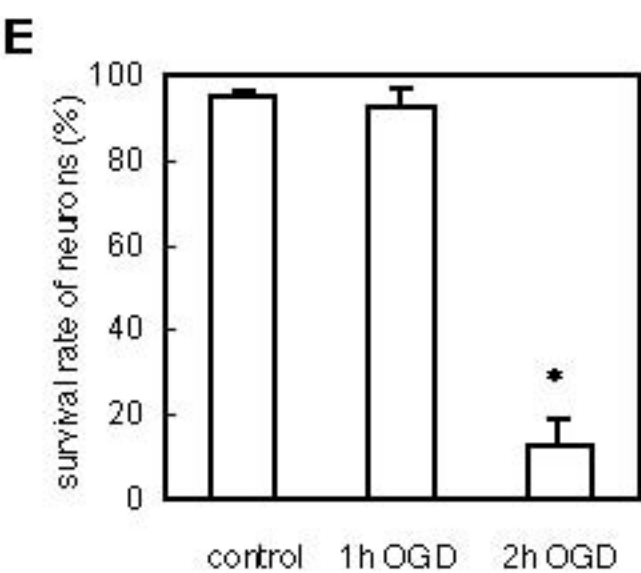
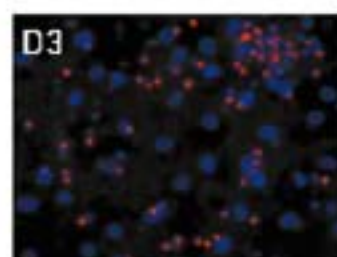
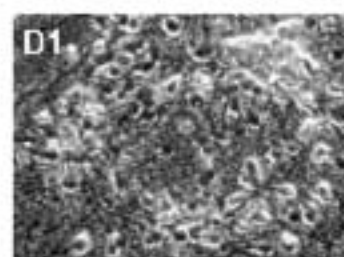
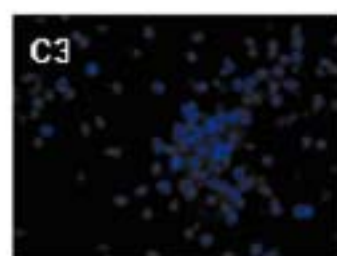
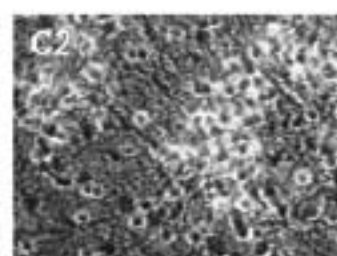
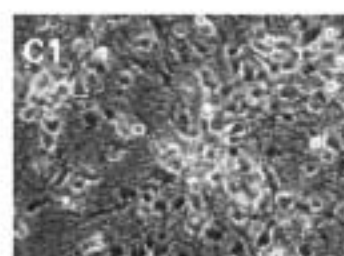
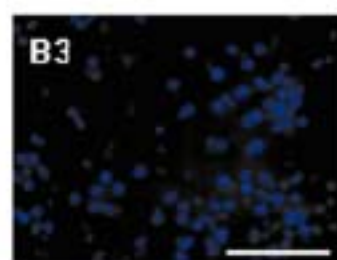
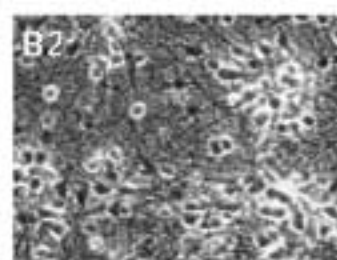
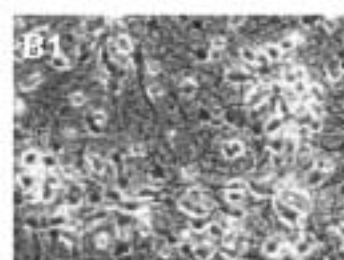
extracellular-signal-regulated kinase; iNOS, inducible nitric oxide synthase; L-NMMA,

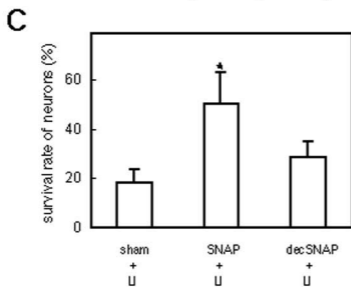
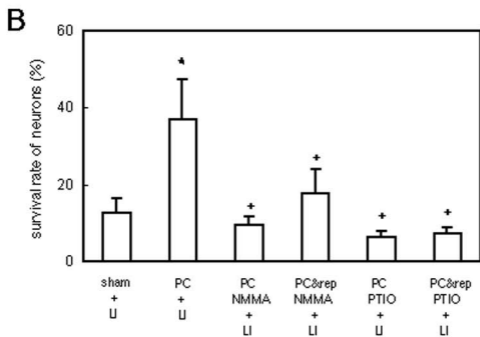
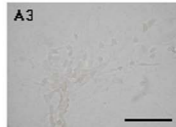
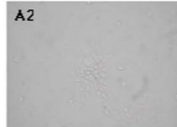
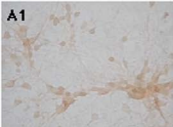
N^G-monomethyl-L-arginine; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide

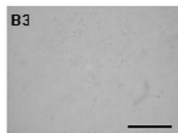
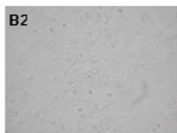
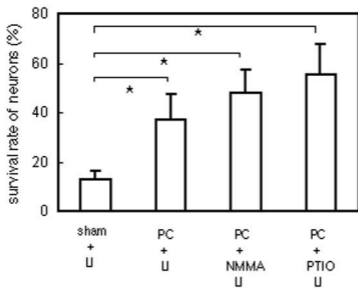
synthase; nNOS, neuronal nitric oxide synthase; MAP-2, microtubule-associated protein 2;

OGD, oxygen and glucose deprivation; PC, preconditioning; PI, propidium iodide; SNAP,

S-nitroso-N-acetyl-DL-penicillamine.





A**C**