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Neuroprotective MK801 Is Associated With Nitric Oxide Synthase During Hypoxia/Reoxygenation in Rat Cortical Cell Cultures

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Abstract The neuroprotective effect of MK801 against hypoxia and/or reoxygenation-induced neuronal cell injury and its relationship to neuronal nitric oxide synthetase (nNOS) expression were examined in cultured rat cortical cells. Treatment of cortical neuronal cells with hypoxia (95% N_2/5% CO_2) for 2 h followed by reoxygenation for 24 h induced a release of lactate dehydrogenase (LDH) into the medium, and reduced the protein level of MAP-2 as well. MK801 attenuated the release of LDH and the reduction of the MAP-2 protein by hypoxia, suggesting a neuroprotective role of MK801. MK801 also diminished the number of nuclear condensation by hypoxia/reoxygenation. The NOS inhibitors 7-nitroindazole (7-NI) and N (G)-nitro-L-arginine methyl ester (L-NAME), as well as the Ca^{2+} channel blocker nimodipine, reduced hypoxia-induced LDH, suggesting that nitric oxide (NO) and calcium homeostasis contribute to hypoxia and/or the reoxygenation-induced cell injury. The levels of nNOS immunoactivities and mRNA by RT-PCR were enhanced by hypoxia with time and, down regulated following 24 h reoxygenation after hypoxia, and were attenuated by MK801. In addition, the reduction of nNOS mRNA levels by hypoxia/reoxygenation was also diminished by MK801. Further delineation of the mechanisms of NO production and nNOS regulation are needed and may lead to additional strategies to protect neuronal cells against hypoxic/reoxygenation insults. J. Cell. Biochem. 84: 367–376, 2002.

Key words: nNOS; apoptosis; MAP-2; necrosis; NMDA

Focal ischemia or hypoxia activates glutamate release, which subsequently interacts with the *N*-methyl-D-aspartate (NMDA) receptor and leads to an increase in intracellular concentration of Ca^{2+}. These events lead to neuronal death [Koh and Choi, 1987; Choi and Rothman, 1990]. Under normal conditions, powerful neuronal and glial uptake systems rapidly remove synaptically released glutamate from the extracellular space before toxicity occurs [Drejer et al., 1982, 1985]. However, under hypoxic and ischemic conditions, the over-release of glutamate will cause neurotoxicity by a toxic influx of extracellular calcium. The glutamate neurotoxicity may be blocked by antagonists, in particular those which are effective against the receptor-ionosphere complexes [Rothman and Olney, 1986; Nicholls and Attwell, 1990; Choi, 1995]. Hypoxia is a

Abbreviations used: DAB, 3,3′-diaminobenzidine tetrahydrochloride; DAPI, 4′,6′-diamidino-2-phenylindole; MK801, dizocilpine maleate; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminotetraacetic acid; GFAP, glial fibrillary acidic protein; LDH, lactate dehydrogenase; MAP-2, microtubule associated protein-2; nNOS, neuronal nitric oxide synthase; L-NAME, N (G)-nitro-L-arginine methyl ester; NMDA, *N*-methyl-D-aspartate; 7-NI, 7-nitroindazole; TBS, Tris-buffered saline.

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pathophysiological condition that occurs during injury, ischemia, and stroke. In this study, we examined whether hypoxia and/or reoxygenation under glucose deprivation would induce necrotic and apoptotic neuronal death and whether MK801 could attenuate these processes. We also tested neuronal nitric oxide synthase (nNOS) expression during hypoxia and/or reoxygenation underlying the basis of the MK801 neuroprotective mechanisms in cultured cortical neuronal cells.

There is increasing evidence that nitric oxide (NO) may play complex roles in the pathophysiology of cerebral ischemia. Activation of the NMDA receptor generates NO [Garthwaite et al., 1988], and overexpression of nNOS leads to the excessive production of NO, which is the link between the excitatory amino acids and subsequent cell damage [Dawson et al., 1991; Jones et al., 1998]. Hypoxia also induces NO release from nNOS in cerebral capillary flow [Hudetz et al., 1998] and increases nNOS [Higuchi et al., 1996]. The selective inhibitor of nNOS, 7-nitroindazole (7-NI), reduces delayed neuronal damage after transient focal cerebral ischemia in rats [Escott et al., 1998; Nanri et al., 1998].

The mechanisms of hypoxia/reoxygenation are poorly understood, and both necrosis and apoptotic cell death pathways may occur. Necrotic lesions have been associated with hypoxic ischemic and excitotoxic neuropathy [Dessi et al., 1993; Gwag et al., 1995]. Cortical neurons, which possess high concentrations of glutamate receptors, are directly coupled to calcium influx. It is likely that an alteration in the regulation of calcium homeostasis participates in the neurodegenerative process. Oxygen–glucose deprivation causes marked acute cell body swelling followed by neurodegeneration, consistent with necrosis-type death. In addition to necrosis, apoptosis is also associated with delayed neuronal cell death in ischemia [Nitatori et al., 1995; Pulera et al., 1998] or chemical hypoxia [Mills et al., 1996], and internucleosomal DNA cleavage has also been demonstrated in both global and focal ischemia [Heron et al., 1993; MacManus et al., 1994]. Hypoxia followed by reoxygenation triggers sequential changes in the synthesis of specific proteins, leading to delayed and mainly apoptotic neuronal death [Bossenmeyer et al., 1998; Tagami et al., 1998].

In this study, our results indicate that hypoxia and hypoxia/reoxygenation under glucose deprivation would induce necrotic and apoptotic neuronal death. Neuroprotective effect of MK801 during hypoxia and/or reoxygenation is associated with nNOS expression.

MATERIALS AND METHODS

Chemicals were obtained from the following companies: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, neurobasal, and supplement B27 from GIBCO (Grand Island, NY); horse serum from Hyclone (Logan, UT); boric acid, 3,3′-diaminobenzidine tetrahydrochloride (DAB), peroxidase substrate tablets, 4,6-diamidino-2-phenylindole (DAPI), DNase I, ethylenediaminotetraacetic acid (EDTA), antibodies to glial fibrillary acidic protein (GFAP) and microtubule associated protein-2 (MAP-2), papain, penicillin, and streptomycin from Sigma Chemical Co. (St. Louis, MO); antibodies to nNOS lactate and lactate dehydrogenase (LDH) kits from Boehringer Mannheim (Mannheim, Germany); Ultraspec RNA isolation kit from Biotecx Laboratory, Inc. (Houston, TX); and Vectastain ABC kit from Vector (Burlingame, CA).

Cell Culture

Cultured cortical cells were prepared from the cerebral cortices of 1-day-old Sprague–Dawley rats as previously described [Huang et al., 2000]. After the brains were dissected, the blood vessels and meninges were removed under a microscope. Then, the cortices were placed in ice-cold Dulbecco's modified Eagle's medium (DMEM) and minced. The tissue chunks were incubated with papain solution (100 U/ml papain, 0.5 mM EDTA, 0.2 mg/ml cysteine, 1.5 mM $CaCl_2$, and DNase I) at 37°C for 20 min to dissociate the cells, and the reaction was terminated by adding heat-inactivated horse serum. After the cell suspensions were centrifuged at 6,000*g*, the pellets were resuspended in DMEM supplemented with 10% horse serum. Cells were plated onto poly-D-lysine-coated 35-mm petri dishes at a seeding density of $2-4 \times 10^5$/dish and incubated at 37°C in a humidified incubator with 5% CO_2. Two hours after plating, the medium was replaced with neurobasal medium containing 0.5 mM glutamine, 25 μM glutamate, and B27 supplement. The medium was changed to neurobasal/

B27 medium without glutamate after 4 days. Cell cultures were grown for approximately another 10 days prior to the experiment.

Measurement of LDH

Fifty microliters of the supernatants were transferred to a 96-well microtiter, and the activity of LDH was determined using a LDH kit, according to the manufacturer's instructions by previously described methods [Huang et al., 1998, 1999]. The activity of LDH released from cells was measured at a wavelength of 492 nm and at a reference wavelength of 630 nm, using a spectrophotometric microtiter plate reader (MRX, Dynatech).

DAPI-Staining

The condensation of nuclear chromatin was visualized following DNA staining with the fluorescent dye DAPI (4′,6′-diamidino-2-phenylindole) as described previously [Lee et al., 1998]. After treatments, the cells were washed with phosphate-buffered saline (PBS: NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 8 mM; KH_2PO_4, 1.5 mM) at pH 7.4, and fixed with 4% paraformaldehyde in PBS for 10 min. Cells were then incubated with DAPI (final concentration, 1 μg/ml in methanol) for 15 min, rinsed in PBS, and examined under UV light using fluorescent microscopy. Apoptotic nuclei were counted and photographed.

Immunocytochemistry

After fixation with 4% paraformaldehyde in phosphate-buffered saline as described above, cells were permeabilized, and endogenous peroxidase activity was quenched by incubation in 3% H_2O_2/10% methanol at room temperature for 15 min followed by extensive PBS rinses. The cells were then incubated with 5% skim milk in 0.05 M Tris-buffered saline (TBS) for 30 min to reduce nonspecific binding. Subsequently, the cells were incubated with monoclonal anti-mouse MAP-2 (1:50) or anti-rabbit nNOS (1:250) antibodies overnight followed by incubation with the secondary antibody, horse anti-mouse IgG (1:25) and goat anti-rabbit IgG (1:200), respectively, for 2 h at room temperature. The cells were washed with TBST (0.1 M Tris-buffer, pH 7.4; 0.9% NaCl; and 0.1% Tween 20) and incubated with avidin-biotinylated horseradish peroxidase (Vectastain ABC kit) for 30 min. Reaction products were visualized with 3,3′-diamidinobenzidine tetrahydrochloride (DAB) staining followed by hematoxylin counter-staining.

Western Blot Analysis

After treatment, the cell culture media were aspirated, and the cells on a 100-mm petri dish were hydrolyzed by a lysis buffer (50 mM Tris, 10% glycerol, 1% Triton X-100, 100 mM NaF, 0.25% SDS, 5 mM EDTA, 2 mM PMSF, 20 μg/ml leupeptin, and 20 μg/ml aprotinin). Twenty or one hundred micrograms of protein per lane was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) overnight. Resolved proteins were electroblotted onto immobilon polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA) for 3 h at 300 mA. The blots were incubated overnight in anti-MAP-2 (1:20,000) monoclonal antibody diluted in TBST containing 5% dry skim milk, followed by incubation with secondary antibody horse radish peroxidase conjugated affinity goat anti-mouse IgG (1:10,000) for 1 h. The bound immunoproteins were detected by enhancer chemiluminescent assay (ECL; Amersham, Berkshire, UK), and intensities were quantified with a densitometry analysis (Digital Protein DNA Imagineware, Huntington Station, NY).

Total RNA Extraction and RT-PCR

After treatment, the cells on a 35-mm petri dish were lysed by adding 1 ml of Ultraspect RNA extraction solution to isolate total RNA according to the manufacturer's instructions. Total RNA was quantified spectrophotometrically by absorbance at 260 nm. All RT-PCRs were performed using a GeneAmp PCR system 2400 (Perkin Elmer, Wellesley, MA) and a Titan one Tube RT-PCR system kit (Boehringer Mannheim, Mannheim, Germany). The sequence for the nNOS sense primer was 5′GAATACCAGCCTGATCCATGGAA3′ and the antisense primer was 5′TCCTCCAGGAGGGTGTCCACCGCATG3′; for actin the sense primer was 5′TTGTAACCAACTGGGACGATATGG3′ and the antisense primer was 5′GATCTTGATCTTCATGGTGCTAGG3′. RT-PCR was performed with these primers using 1 μg of total RNA as a template. The following RT-PCR protocol was employed: incubating at 50°C for 30 min; pre-melting at 94°C for 2 min; and 30 and 35 cycles, respectively for β-actin and nNOS; melting at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 2 min.

The reaction products were visualized by electrophoresis in a 2% agarose gel (consisting of 1% Nusieve and 1% agarose) and by staining with 0.5 μg/ml ethidium bromide. Size markers were included with the gel. Verification of the specific genes was established by its predicted size (602 bp for nNOS or 764 bp for β-actin) under UV light.

RESULTS

Effects of Hypoxia/Reoxygenation on the Cell Toxicity

Cultured cortical cells were treated with DMEM without glucose under 95% N_2/5% CO_2 for various time periods (0, 1, 2, or 4 h), reoxygenated and changed to the Neurobasal medium supplemented with B27 for 24 h. Cell toxicity was then evaluated by measuring the activity of LDH present in the culture medium. As shown in Figure 1, LDH activity increased 2-, 5-, and 30-fold after cell exposure to hypoxia for 1, 2, and 4 h, respectively. Furthermore, LDH release in the medium increased significantly during reoxygenation.

MK801 Prevents Hypoxia/Reoxygenation-Induced Neurotoxicity in Cultured Cortical Neuronal Cells

The neuroprotective effects of MK801 against hypoxia/reoxygenation-induced neurotoxicity were evaluated by measuring the activity of released LDH. The cells in glucose-free DMEM with or without MK801 (10 μM) were gassed with either 95% air/5% CO_2 or 95% N_2/5% CO_2 for 2 h, then were grown in neurobasal medium in a CO_2 incubator for 24 h. The supernatants were collected, and the activity of LDH released from cytosol was measured. The results indicated that MK801 protected the cells against hypoxia-induced toxicity by significantly reducing LDH release in the cultured medium (Fig. 2).

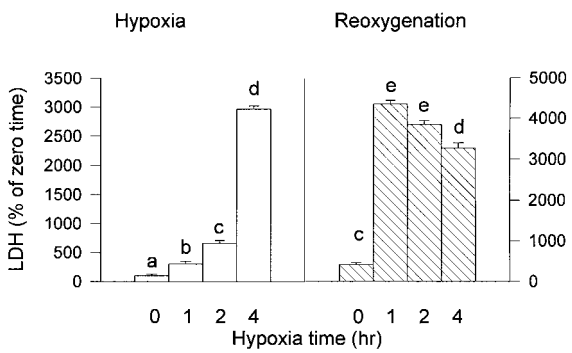

Fig. 1. Effect of hypoxia/reoxygenation on the activity of lactate dehydrogenase in cultured cortical cells. On the day of the experiment, the media were removed and the cells in DMEM without glucose were treated with either 95% air/5% CO_2 or gassed with 95% N_2/5% CO_2 for the indicated time (0, 1, 2, or 4 h), then reoxygenated for 24 h following hypoxia. The supernatants were collected and the activity of LDH released from cytosol was measured according to the methods as described. Data are the means ± SEM from two separate experiments (n = 8). Numbers with different letters show a statistically significant difference from each other ($P < 0.05$).

MK801 Prevents Hypoxia/Reoxygenation-Induced Apoptosis as Determined by the Chromatin Condensation in Nuclei of Cultured Neuronal Cells

Chromatin condensation was visualized by the uptake of fluorochrome DAPI, which binds with DNA, to test whether the neuronal death following hypoxia or reoxygenation is apoptotic or necrotic. As shown in Figure 3, nuclear condensation and shrinkage of cell nuclei were evident even after the cultured neurons were exposed to glucose-free DMEM/reoxygenation. The number of apoptotic cells (percent of chromatin condensation in total number of nuclei showing DAPI-uptake) following 2 h of normal oxygen in glucose-free DMEM/24-h reoxygenation was 35% ± 6%, and that following 2 h of

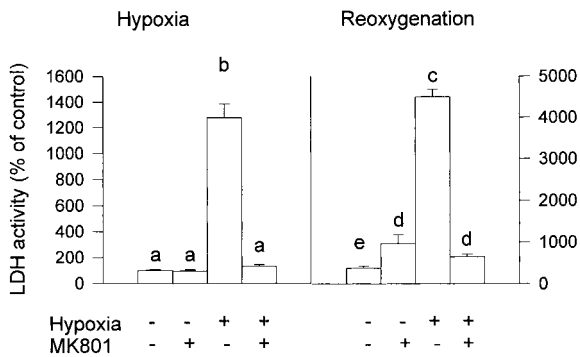

Fig. 2. MK801 prevents hypoxia/reoxygenation-induced LDH release from cultured cortical cells. Cultured cortical cells were grown as described in Materials and Methods. On the day of the experiment, the cells in glucose-free DMEM with or without MK801 (10 μM) were gassed with either 95% air/5% CO_2 or 95% N_2/5% CO_2 for 2 h, then were grown in neurobasal medium in a CO_2 incubator for 24 h. The supernatants were collected and the activity of LDH released from cytosol was measured according to the methods described. Values are presented as the means ± SEM of two separate experiments with five dishes per experiment for LDH. Numbers with different letters show a statistically significant difference from each other as determined by ANOVA followed by F-test ($F(7, 81) = 28$: $P < 0.01$).

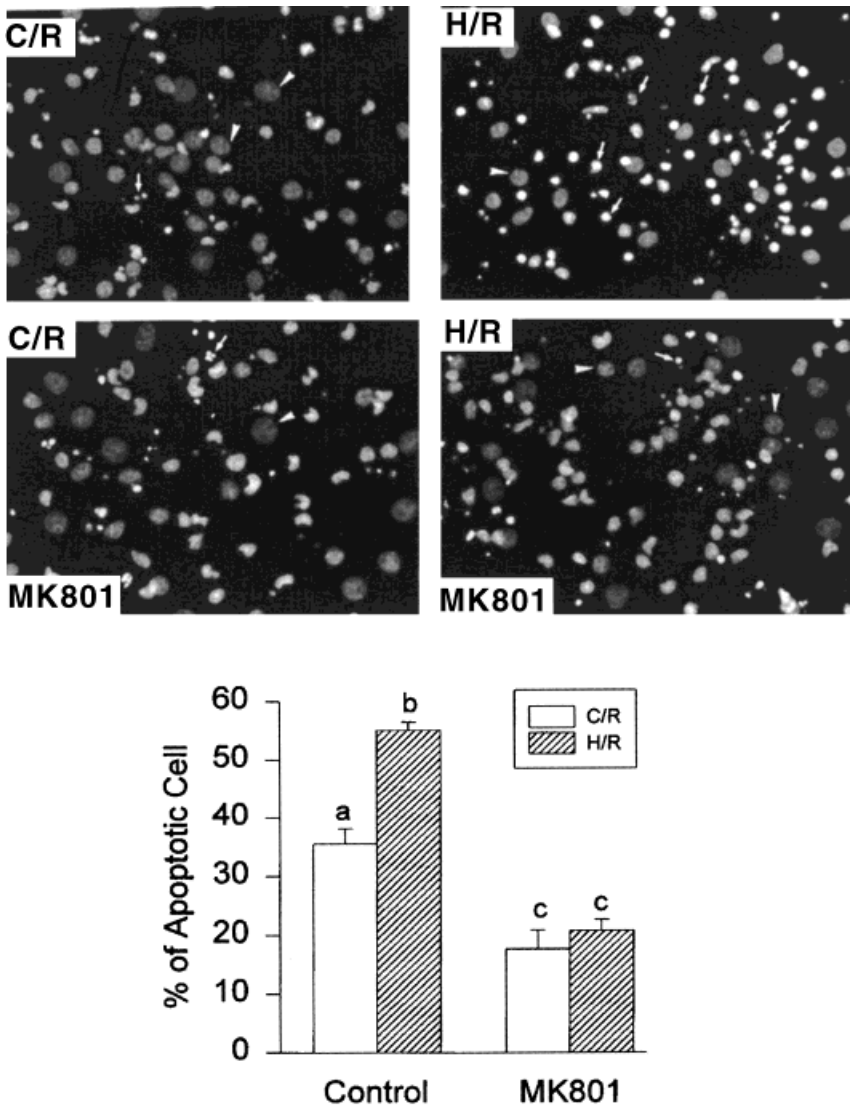

Fig. 3. Neuroprotective effect of MK801 against hypoxia/reoxygenation-induced nuclei condensation. Cultured cortical cells in glucose-free DMEM were treated with 95% air/5% CO_2or 95% N_2/5% CO_2 for 2 h, then changed to growth media for 24 h in CO_2 incubator (C/R, H/R). The nuclei condensation was determined by DAPI DNA. **A**: Typical normal nuclei are indicated by arrowheads, and condensed nuclei are indicated by arrows. **B**: Data are the mean ± SEM from three separate experiments. Numbers with different letters show a statistically significant difference from each other as determined by ANOVA followed by F-test ($F(5, 107) = 42$: $P < 0.01$).

hypoxia in glucose free DMEM/24-h reoxygenation was further increased to 54.9% ± 1.1%. On the other hand, cells pretreated with MK801 (10 μM) had fewer apoptotic bodies (Fig. 3B). The appearance of nuclear condensation in control/reoxygenated cells suggested that glucose deprivation alone also induced apoptotic cell death. Thus, significant nuclear condensation appeared significantly in the reoxygenated cells. The neuroprotective effect of MK801 was further confirmed by the expression of MAP-2, and the results are shown in Figure 4; cortical MAP-2 protein was reduced 27% ± 5.6% after 2 h hypoxia and markedly reduced 75% ± 2% after hypoxia/reoxygenation, whereas the increase of MAP-2 protein level by MK801 was observed.

In order to evaluate the role of NO and calcium in cell death during hypoxia, we also examined the effects of the nNOS specific inhibitor 7-NI, the nonselective NOS inhibitor L-NAME, and the L-type calcium channel blocker nimodipine on the hypoxia/reoxygenation-induced release of LDH. The release of hypoxia-induced LDH was attenuated

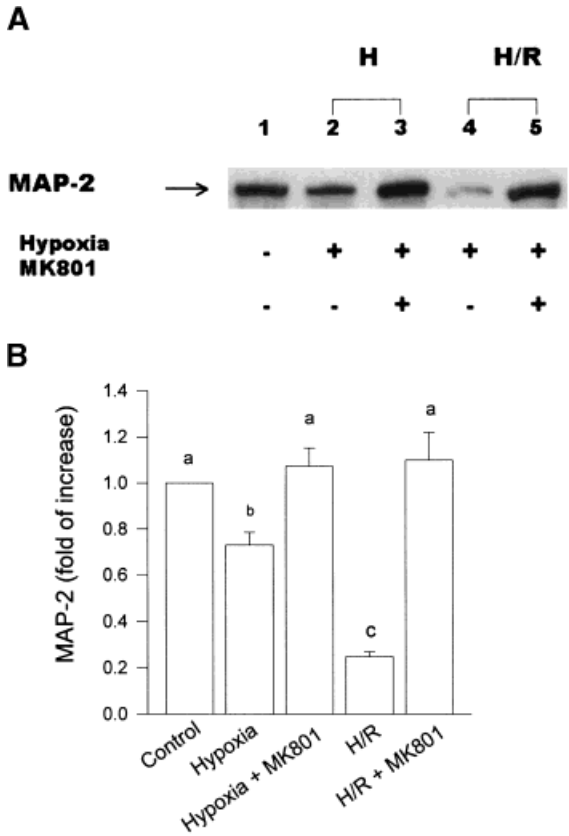

Fig. 4. Neuroprotective effect of MK801 on MAP-2 protein following hypoxia/reoxygenation. **A**: Cultured cortical cells in glucose-deprived DMEM were treated with hypoxia for 2 h in the presence or absence of MK801, and then were grown in the neurobasal medium for 24 h. Total cell lysate were prepared and subjected to Western blot using an anti-MAP-2 antibody. Each lane was loaded with 100 μg of protein. **B**: Data in the bottom panel are immunoreactivities of MAP-2 expressed as a percentage of the control from three separate experiments with two blots for each experiment. Values with different letters indicate significant difference from each other ($P < 0.05$).

significantly by adding 7-NI (10 μM), L-NAME (10 μM), and nimodipine (10 μM), to 53, 33, and 21% of the level without blockers, respectively (Fig. 5). Therefore, the results suggest that NO and calcium are involved in this hypoxia and/or reoxygenation-induced neuronal injury.

To evaluate further whether nNOS was involved in the acute phase of hypoxia-induced neuronal injury, cells were grown under 95% N_2/5% CO_2 for various time periods (0, 0.5, 1, or 2 h), then reoxygenated and transferred to the growth medium for 24 h. As shown in Figure 6, the protein level of nNOS by Western blot analysis increased after hypoxia for 1 h, then declined, showing that they were decreased significantly during the subsequent reoxygenation period. In addition, the cells after hypoxia

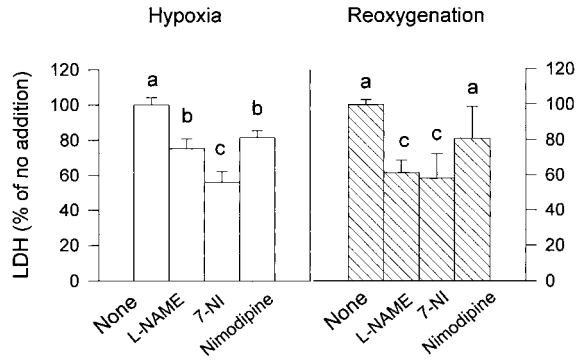

Fig. 5. NOS inhibitors and a Ca^{2+} channel blocker prevent hypoxia/reoxygenation-induced LDH release from cultured cortical cells. Cultured cortical cells were grown as described in Materials and Methods. On the day of the experiment, the cells in glucose-free DMEM with or without the NOS inhibitors 7-NI (10 μM) or L-NAME (10 μM), or the Ca^{2+} channel blocker nimordipine (10 μM), were gassed with either 95% air/5% CO_2 or 95% N_2/5% CO_2 for 2 h, then were grown in neurobasal media in a CO_2 incubator for 24 h. The supernatants were collected, and the activity of LDH released from cytosol into the media was measured according to the methods described. Values with different letters indicate significant difference from each other ($P < 0.05$).

and/or reoxygenation were immunostained with monoclonal antibodies against nNOS, and then photographed, and total numbers of nNOS-staining were counted. The results in Figure 7 show the level of immunoactivity of

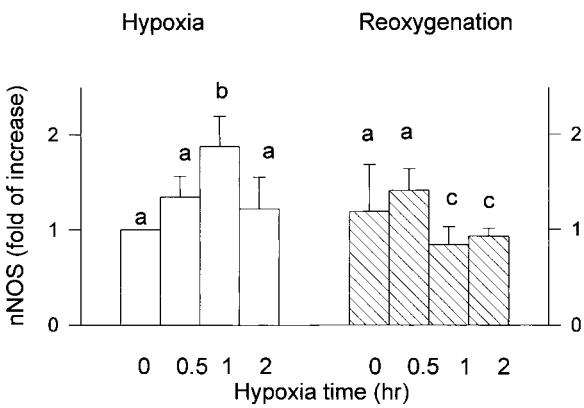

Fig. 6. Effects of hypoxia and/or reoxygenation on the protein level of nNOS in cultured cortical cells. Cultured cortical cells in glucose-deprived DMEM were treated with hypoxia for various time periods (0, 0.5, 1, 2 h), then were grown in the neurobasal medium for 24 h. Total cell lysate were prepared and subjected to Western blot using an anti-nNOS antibody. Each lane was loaded with 100 μg of protein. The bound immunoproteins were detected by enhancer chemiluminescent assay, the intensity of the bands was quantified by densitometry analysis and normalized to control, and the normalized signal obtained from the control was arbitrarily defined as 1. Values with different letters indicate significant difference from each other ($P < 0.05$).

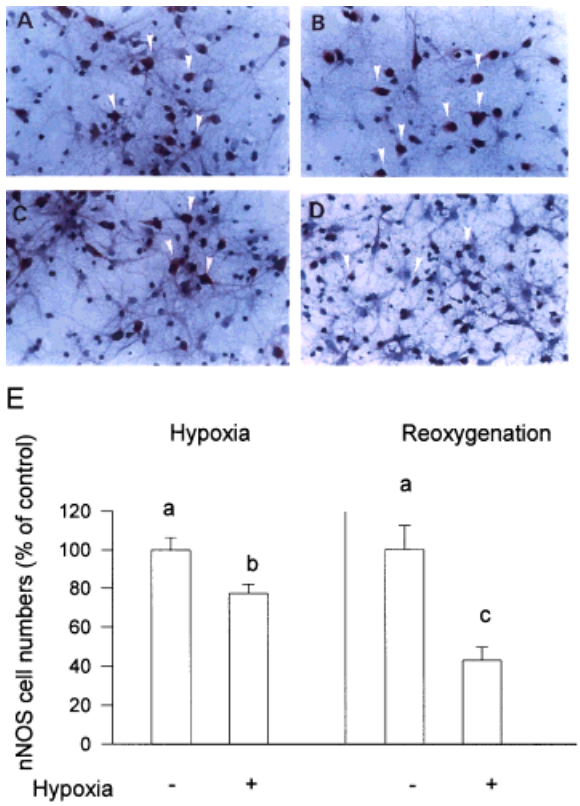

Fig. 7. Effects of hypoxia and/or reoxygenation on the nNOS expression in cultured cortical cells. Cultured cortical cells in glucose-deprived DMEM were treated with hypoxia for 1 h, then immunostained with monoclonal antibodies against nNOS and photographed. **A**: control culture; **B**: hypoxia; **C**: control/reoxygenation; **D**: hypoxia/reoxygenation. nNOS positive cells are indicated by arrowheads. **E**: Data expressed as a percentage of nNOS-positive cell number in the total cell populations. Values with different letters indicate significant difference from each other ($P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nNOS was enhanced slightly after hypoxia for 1 h (Fig. 7B), but the number of cells expressing nNOS was reduced 25% (Fig. 7E). Up-regulation of nNOS cells may result in the generation of NO, which in turn may contribute to hypoxic-ischemic neuronal injury. This may explain why NOS inhibitors reduced the LDH release by hypoxia, and supports the notion that NO production underlies hypoxia-induced neuronal injury. Furthermore, the number and immunoactivity for nNOS-positive cells were not affected significantly after control/reoxygenation (Fig. 7C,E). On the contrary, immunoactivity for nNOS (Fig. 7D) and the number of nNOS-positive cells were reduced 60% after hypoxia/reoxygenation (Fig. 7E), in agreement with the reduction of MAP-2 protein (Fig. 4), and increases in apoptotic cells (Fig. 3).

To evaluate further whether nNOS was involved in the neuroprotective MK801 against hypoxia/reoxygenation induced neuronal injury, cultured cortical cells in glucose-free DMEM were treated with hypoxia for 2 h, and subsequently following reoxygenation for 24 h, levels of nNOS and β-actin mRNA were analyzed. Although the nNOS expressions were not affected after hypoxia for 2 h, they were decreased significantly during subsequent reoxygenation period (Fig. 8). Furthermore, the reduction of nNOS mRNA by hypoxia/reoxygenation was upregulated 58% by MK801 (Fig. 8).

DISCUSSION

The present study demonstrated that MK801 prevents neuronal cell death following hypoxia and/or reoxygenation based on LDH activity, nuclear condensation, and immunochemical analysis. The LDH level in the medium was increased significantly after the cells were treated with hypoxia for 1 h (Fig. 1), but chromatin condensation did not occur during this acute hypoxic insult. Subsequently, chromatin condensation appeared during reoxygenation following oxygen and glucose-deprivation (Fig. 2). Furthermore, protection of the neuronal cells against hypoxia/reoxygenation-induced necrosis and apoptosis, and maintenance of MAP-2 proteins were observed by MK801 (Figs. 3 and 4). The NO inhibitors, 7-NI and L-NAME, and the Ca^{2+} channel blocker nimodipine reduced LDH release following hypoxia/reoxygenation (Fig. 5), which suggests that NO production and Ca^{2+} are involved in hypoxia-induced neuronal injury. Immunostaining results have shown that hypoxia-induced nNOS expression during hypoxia was attenuated by MK801. On the contrary, nNOS mRNA levels were reduced markedly after hypoxia/reoxygenation, and of that were prevented by MK801. Thus, the neuroprotective mechanism of MK801 involved regulating nNOS expression.

Both necrotic and apoptotic cell death occurred in the hypoxia and/or reoxygenation-induced neurotoxicity. Cellular apoptosis is characterized morphologically by cell shrinkage and chromatin condensation through a cascade of molecular and biochemical events, including

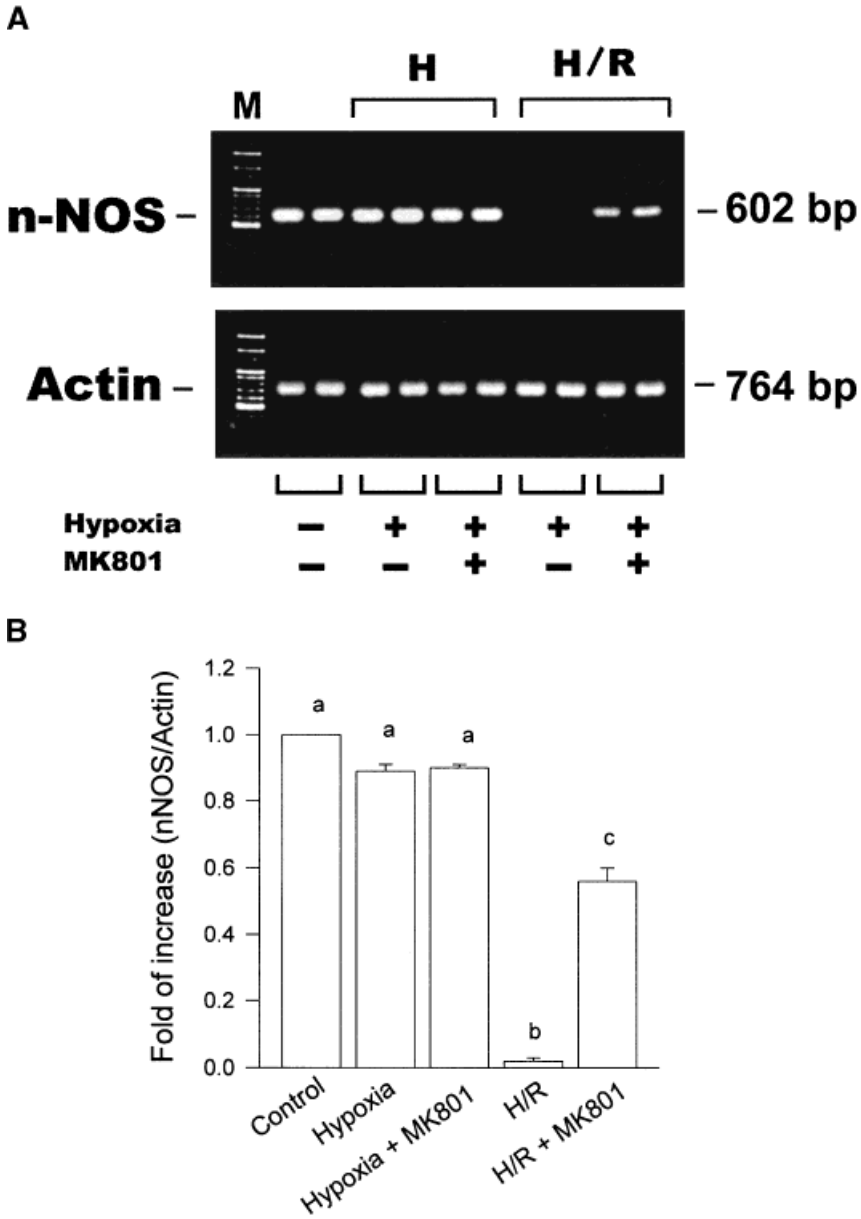

Fig. 8. Neuroprotective effects of MK801 on nNOS expression during hypoxia/reoxygenation in cultured cortical cells. Cultured cortical cells in glucose-deprived DMEM were treated with hypoxia for 1 h in the presence or absence of MK801, then were grown in the neurobasal medium for 24 h, and total RNA were prepared. **A**: nNOS (602 bp) and actin mRNAs (764 bp) were determined by RT-PCR. **B**: The RT-PCRs were semiquantified by densitometric analysis and normalized to the mRNA level of Actin (bottom lane), and the normalized signal obtained from the control was arbitrarily defined as 1. Data were obtained from three separate experiments. Numbers with different letters show a statistically significant difference from each other as determined by ANOVA followed by F-test.

the activation of endonuclease, that cleave DNA into oligonucleosomes [Cohen, 1993; Cohen et al., 1994]. We found that exposure of cortical cultures to hypoxia induced acute necrosis, while the reoxygenation was associated with delayed neuronal death characterized by nuclear condensation, one of the apoptotic features. Necrotic lesions have been associated with ischemic and excitotoxic neuropathy, usually detected in close temporal and spatial proximity to the hypoxic stimulus [Siesjo, 1992], and apoptosis is associated with neurodegeneration in ischemia [Heron et al., 1993], chemical hypoxia [Mills et al., 1996], and hypoxia and reoxygenation [Tagami et al., 1998]. By analogy, high concentrations of NMDA or

peroxynitrite induced relative rapid necrotic cell death in neurons [Bonfoco et al., 1995]. In contrast, low concentrations or a short duration of NMDA or peroxynitrite induced delayed apoptotic neuronal death [Nicotera and Lipton, 1999].

The neuroprotective effects elicited by MK801 may be due to its effect on NO production, at least in part. We have found that LDH release was attenuated more by the nNOS inhibitor 7-NI than by the nonspecific inhibitor L-NAME, indicating that NO production from a neuronal source may contribute to hypoxia-induced injury (Fig. 5), in agreement with increases in nNOS-positive cell numbers and their protein levels by immunocytochemical and Western blot analysis during hypoxia (Figs. 6 and 7). These results are consistent with in vivo studies showing that hypoxia (5% O_2, 95% N_2 for 30 min) induced neuronal damage with an enhancement of nNOS immunoreactivity in the rat hippocampus [Higuchi et al., 1996; Matsuoka et al., 1997] and in central and peripheral neurons [Prabhakar et al., 1996]. It has become well known that NO production from nNOS is caused by activation of the NMDA receptor [Dawson et al., 1992]. During middle cerebral artery ligation, cortical NO was released rapidly and then declined, and this NO release induced by acute cerebral ischemia could be attenuated by pretreating with NMDA antagonists [Lin et al., 1996]. Furthermore, our data showed that hypoxia/reoxygenation during oxygen–glucose-deprivation caused a decrease in the levels of nNOS mRNA expression (Fig. 8), in agreement with other's report [De Alba et al., 1999]. In addition, MK801 significantly attenuates NMDA-induced NO in human brain tissue [Liu et al., 1997] or Ca^{2+} directly acts on NOS that results in neuroprotection during hypoxia and/or the reoxygenation process.

In summary, we demonstrated that hypoxia and/or reoxygenation induced neuronal death by necrotic and apoptotic processes. MK801 protected against acute neuronal death, probably by attenuating nNOS and hypoxic necrosis. Furthermore, MK801 protected against delayed neuronal death by attenuating reoxygenated apoptosis concomitant with up regulation of MAP-2 and nNOS mRNAs. Further delineation of the mechanisms of NO production and NOS regulation are desirable to lead to additional strategies that may be used to protect neuronal cells against hypoxic/reoxygenated insults.

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