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Protective Effect of Nicotinamide on Neuronal Cells under Oxygen and Glucose Deprivation and Hypoxia/Reoxygenation

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Key Words
Oxygen-glucose deprivation · Hypoxia/reoxygenation · Nicotinamide · Apoptosis · Caspase-3 · Reactive oxygen species · c-*fos*

Abstract
Nicotinamide (vitamin B_3) reduces the infarct volume following focal cerebral ischemia in rats; however, its mechanism of action has not been reported. After cerebral ischemia and/or reperfusion, reactive oxygen species (ROS) and reactive nitrogen species may be generated by inflammatory cells through several cellular pathways, which can lead to intracellular calcium influx and cell damage. Therefore, we investigated the mechanisms of action of nicotinamide in neuroprotection under conditions of hypoxia/reoxygenation. Results showed that nicotinamide significantly protected rat primary cortical cells from hypoxia by reducing lactate dehydrogenase release with 1 h of oxygen-glucose deprivation (OGD) stress. ROS production and calcium influx in neuronal cells during OGD were dose-dependently diminished by up to 10 m*M* nicotinamide ($p < 0.01$). This effect was further examined with OGD/reoxygenation (H/R). Cells were stained with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI) or antibodies against anti-microtubule-associated protein-2 and cleaved caspase-3. Apoptotic cells were studied using Western blotting of cytochrome c and cleaved caspase-3. Results showed that vitamin B_3 reduced cell injury, caspase-3 cleavage and nuclear condensation (DAPI staining) in neuronal cells under H/R. In addition, nicotinamide diminished c-*fos* and *zif*268 immediate-early gene expressions following OGD. Taken together, these results indicate that the neuroprotective effect of nicotinamide might occur through these mechanisms in this in vitro ischemia/reperfusion model.

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Introduction

Cerebral ischemia due to blockage of cerebral blood flow to the brain is one of the leading causes of morbidity and mortality in developed countries, including Taiwan [23]. After cerebral ischemia, reactive oxygen species (ROS) and reactive nitrogen species may be generated by inflammatory cells through several different cellular path-

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ways, including calcium activation of phospholipases, nitric oxide (NO) synthase and xanthine oxidase. If cellular defense systems are weak, these free radicals may oxidize DNA, proteins, lipids and carbohydrates [28, 34], which alter cellular functions in critical ways and can cause neuronal death [5]. Recent studies suggest that apoptosis is involved in ischemic neuronal damage based on morphological and biochemical evidence [6, 27]. Excessive generation of NO and ROS is believed to be a fundamental mechanism in many acute and chronic neurodegenerative disorders and cerebral ischemia and/or reperfusion injury [22].

Early DNA damage following cerebral ischemia/reperfusion may cause excessive activation of poly(ADP-ribose) polymerase (PARP). Pronounced activation of PARP can rapidly deplete the intracellular concentration of its substrate, nicotinamide adenine dinucleotide (NAD^+), slowing the rates of glycolysis and electron transport, and therefore ATP formation, resulting in cell dysfunction with eventual necrotic-type cell death. Caspases have been implicated as common executors of a variety of death signals. Inhibition of PARP has a neuroprotective effect [11, 37]. Cytochrome c is reported to be released from mitochondria to the cytosol [12], where it interacts with the protein Apaf-1 and activates caspase-9, which then activates other caspases, including caspase-3. Increasing the activity of caspases causes cleavage or excessive activation of PARP, which plays a role in apoptotic and necrotic neuronal cell death elicited by ischemia and/or reperfusion injury [8].

Nicotinamide, a soluble B group vitamin (vitamin B_3), is a precursor of NAD^+ and a PARP inhibitor, which can potentially boost energy reserves in tissue at risk during cerebral ischemia or hepatic ischemia/reperfusion [1, 2, 7]. Vitamin B_3, as a precursor for NAD^+, prevents apoptosis in the mouse brain [26, 32] and in cell culture [9], is an effective antioxidant against oxidative damage in rat brain mitochondria [16, 25], inhibits inducible NO synthase mRNA in glial cells and protects against trauma and NO exposure in rat hippocampus cells [15, 40], and has been reported to be an inhibitor of lipid peroxidation [4]. A recent study showed that nicotinamide reduced the infarct volume following focal cerebral ischemia [36]. However, the neuroprotective mechanism of nicotinamide during ischemia/reperfusion remains unclear. Therefore, we studied the protective mechanism of vitamin B_3 on rat cortical cell cultures. The effects of nicotinamide on ROS generation and caspase-3 activation under oxygen-glucose depletion (OGD) and OGD/reoxygenation (H/R) were investigated.

Materials and Methods

Chemicals

Chemicals were obtained from the following companies: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, neurobasal medium and supplement B27 from GIBCO (Grand Island, N.Y., USA); horse serum from Hyclone (Logan, Utah, USA); nicotinamide, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), boric acid, 3,3′-diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate tablet, 4,6-diamidino-2-phenylindole (DAPI), DNase I, ethylenediaminetetraacetic acid (EDTA), papain, penicillin and streptomycin from Sigma (St. Louis, Mo., USA); lactate dehydrogenase (LDH) kits and monoclonal anti-microtubule-associated protein-2 (MAP-2) from Boehringer Mannheim (Mannheim, Germany); polyclonal anti-cleaved caspase-3 from Cell Signaling (Beverly, Mass., USA); anti-rat caspase-3 monoclonal antibody from MBL (Nagoya, Japan); anti-mouse cytochrome c polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, Calif., USA); fura-2 acetoxymethyl ester (fura-2 AM) from Calbiochem (San Diego, Calif., USA); a Vectastain ABC kit from Vector (Burlingame, Calif., USA); 6-carboxy-2′,7′-dichlorodihydrofluorescein acetoxymethyl ester (DCF-AM) from Molecular Probes (Eugene, Oreg., USA), and a chemiluminescence detection kit from Amersham International (Little Chalfont, UK). Primers for rat c-*fos* and *zif*268 were obtained from Clontech Laboratory (Palo Alto, Calif., USA).

Cell Culture

Cultured cortical cells were prepared from the cerebral cortices of 1-day-old Sprague-Dawley rats under pentobarbital anesthesia as previously described [20], and this study was approved by the Committee of Animal Study Protocol Review, Taichung Veterans General Hospital. After the brains were dissected, the blood vessels and meninges were removed under a microscope. Then, the cortices were placed in ice-cold DMEM and minced. Tissue chunks were incubated in a papain solution (100 U/ml papain, 0.5 m*M* EDTA, 0.2 mg/ml cysteine, 1.5 m*M* $CaCl_2$ 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, pellets were plated onto poly-*D*-lysine-coated petri dishes 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 B27, 25 μ*M* glutamate and 0.5 m*M* glutamine. On the 4th day in vitro, the medium was changed and replaced with neurobasal-B27 medium without glutamate. Cultured cells were grown for approximately 10 days, and contained 70% neurons and 30% astrocytes, as determined by immunostaining with monoclonal antibodies against MAP-2 and glial fibrillary acidic protein.

OGD and H/R

On the day of the experiment (10th day in vitro), the culture media were replaced with glucose-free DMEM, then gassed with 5% CO_2-85% N_2-10% H_2 for various time periods in the absence or presence of various concentrations of nicotinamide (1, 5, 10, 20, 50, 100 or 200 m*M*) [20]. H/R consisted of OGD for 60 min, then reoxygenation for 2, 4 or 8 h. Hypoxia without glucose deprivation was also included as a control group. Each experiment consisted of duplicate samples in the presence or absence of nicotinamide. Four independent experiments were carried out to determine the effect of nicotinamide.

Measurement of LDH

Cytotoxicities, as indicated by cell membrane integrities, were assessed by measuring the activity of LDH in the culture media by colorimetric detection of formazan, using an LDH diagnostic kit (Boehringer Mannheim). Cultures on 96-well plates (5×10^4 cells) were treated with OGD for 1 h or H/R for 1/8 h (H1/R8) from the preliminary test in the presence or absence of various concentrations of nicotinamide (1, 5, 10, 20, 50 or 100 m*M*), then the supernatants were collected and centrifuged at 200 *g* for 5 min. Fifty microliters of supernatants were transferred to another 96-well microtiter plate, and LDH was determined, according to the manufacturer's instructions. The activity of LDH was spectrophotometrically measured at 492 nm (Spectra Max340; Molecular Devices, Sunnyvale, Calif., USA).

Measurement of Mitochondrial Impairment

The MTT assay is based on cleavage of the yellow tetrazolium salt, MTT, to purple formazan crystals by mitochondrial succinate dehydrogenase in living cells. Formazan crystals were solubilized and quantified spectrophotometrically (Spectra Max340). Cell cultures (5×10^4 cells) were treated with OGD or H/R. Cell viability was determined at 540 nm. A standard curve for cell numbers was constructed, and data were expressed as percentages of the control values.

Measurement of ROS

5×10^4 cells were washed with phosphate-buffered saline (PBS) at pH 7.4 and then incubated with 10 μ*M* DCF-AM in DMEM in 5% CO_2 at 37 °C for 1 h. After DCF-AM was removed, cells were washed and incubated with glucose-free DMEM in the presence or absence of vitamin B_3 for the indicated time periods. The fluorescence was measured by a fluorescence plate reader (Fluoroskan Ascent; Labsystems, Helsinki, Finland). The excitation filter was set at 485 nm, and the emission filter was set at 538 nm. The percentage increase in fluorescence per well was calculated by the formula $[(Ft_{OGD} - Ft_0)/Ft_0] \times 100$, where Ft_{OGD} is the fluorescence at the indicated time periods of OGD and Ft_0 is the fluorescence at time 0 min.

Measurement of $[Ca^{2+}]_i$

Cells were seeded onto 24-mm poly-*D*-lysine-coated glass coverslips and grown for 10 days before the start of the experiments. Cells on coverslips were loaded with 2 μ*M* fura-2 AM in DMEM for 30 min at 37 °C. After loading, cells on coverslips were washed with HEPES buffer to remove any excess fluorescent dye. Then cells were incubated with glucose-free DMEM in the absence or presence of vitamin B_3 for the indicated time periods of OGD and H/R. The fluorescence of cells from each coverslip was measured and recorded using an inverted IX-70 Olympus microscope. $[Ca^{2+}]$ was monitored by alternating excitation wavelengths of between 340 and 380 nm and by an emission wavelength of 510 nm with a Delta Scan System (Photon Technology International, Princeton, N.J., USA) and calculated using the method of Grynkiewicz et al. [18].

Immunocytochemistry

Morphological observations were made by staining with a monoclonal antibody against MAP-2 and a polyclonal antibody against cleaved caspase-3. Briefly, on day 10 in vitro, cells on chamber slides at a seeding density of 2×10^5 cells per chamber were exposed to OGD in the presence or absence of 20 m*M* vitamin B_3. After 60 min of OGD, the media were replaced with conditioned media and continued to be incubated in 5% CO_2 for another 8 h (H1/R8). Then cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Cells were permeabilized, and the endogenous peroxidase activity was quenched by incubation in 3% H_2O_2-10% methanol at room temperature for 15 min. After extensive rinses with PBS, cells were then incubated with 5% skim milk in 0.05 *M* TBST (0.1 *M* Tris buffer, pH 7.4, 0.9% NaCl and 0.05% Tween 20) for 30 min to reduce nonspecific binding. Subsequently, cells were incubated with monoclonal anti-MAP-2 (1:1,000) or polyclonal anti-active caspase-3 (1:500) antibodies overnight at 4 °C, followed by incubation with the secondary antibody, biotinylated anti-mouse IgG or biotinylated anti-rabbit IgG (1:500), for 2 h at room temperature. Cells were washed with TBST and incubated with avidin-biotinylated horseradish peroxidase (Vectastain ABC kit) for 30 min. Reaction products were visualized with DAB staining, followed by hematoxylin counterstaining.

DAPI Staining

H/R-induced nuclear condensation was visualized following DNA staining with the fluorescent dye DAPI, as previously described [20]. Briefly, cells on chamber slides at a seeding density of 2×10^5 cells per chamber were exposed to OGD in the presence or absence of 20 m*M* nicotinamide. After 60 min of OGD, the media were replaced with conditioned media and cells continued to be incubated in 5% CO_2 for another 8 h. Then cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Fixed cells were incubated with DAPI (at a final concentration of 1 μg/ml in methanol) for 15 min and then rinsed and visualized under UV light using fluorescence microscopy. Cells with condensed nuclei (DAPI stained) were considered to be apoptotic.

Western Blot Analysis

After OGD treatment, mitochondrial and cytosolic fractions of cells were isolated by a slight modification of a previously published method [21]. Briefly, treated cells were washed with ice-cold PBS and incubated with 0.3 ml of lysis buffer (in m*M*: HEPES-KOH 20 (pH 7.5), $MgCl_2$ 1.5, EDTA 2, EGTA 5, dithiothreitol 0.1, phenylmethylsulfonyl fluoride 0.1, and sucrose 250) on ice for 30 min before scraping. Cell lysates were homogenized with a Teflon homogenizer (30 strokes). Homogenates were centrifuged at 500 *g* for 10 min to remove unbroken cells and nuclei. Supernatants were centrifuged at 10,000 *g* for 30 min, and pellets (mitochondria) were resuspended in lysis buffer. Supernatants were centrifuged at 100,000 *g* (Beckman TLX ultracentrifuge, Beckman Instrument, Taipei, Taiwan) for 60 min to yield the cytosolic fraction. Samples of 20-μg aliquots were loaded on each lane of a 12.5% SDS-polyacrylamide gel for electrophoresis. After the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, Mass., USA), the membrane was incubated in blocking buffer (1 × PBS and 5% nonfat dry milk) for 1 h at room temperature and then probed with a primary antibody in blocking buffer overnight at 4 °C. The membrane was washed 4 times in PBS containing 0.3% Tween 20, probed with the secondary antibody in blocking buffer for 1 h at room temperature, and washed again in PBS containing 0.05% Tween 20. Signals were detected with an enhanced chemiluminescence detection kit (Amersham). The primary antibodies used were anti-rat caspase-3 monoclonal antibody (MBL) and anti-mouse cytochrome c polyclonal antibody (Santa Cruz).

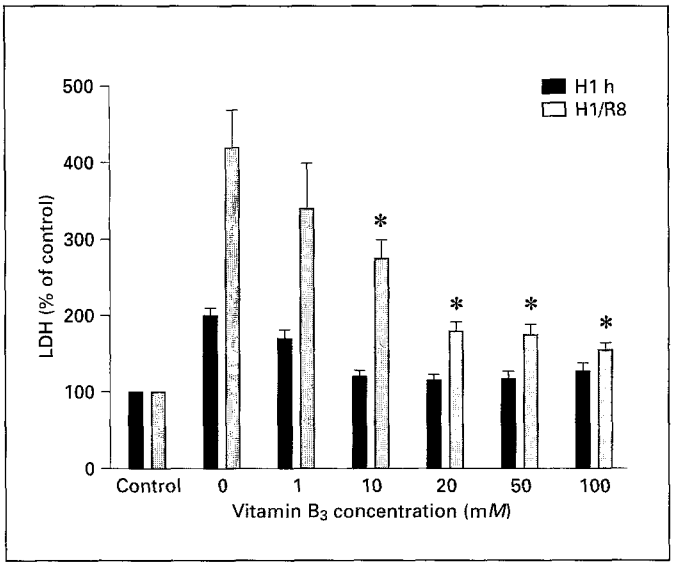

Fig. 1. Nicotinamide (vitamin B_3) dose-dependently reduced LDH release from rat cortical cells after OGD and OGD/reoxygenation (H1/R8). The control groups were not treated with OGD or H/R and had an OD 0.5 unit of LDH (100%). * $p < 0.01$ compared with the vitamin B_3 control under OGD or H1/R8 by ANOVA followed by Student's t test.

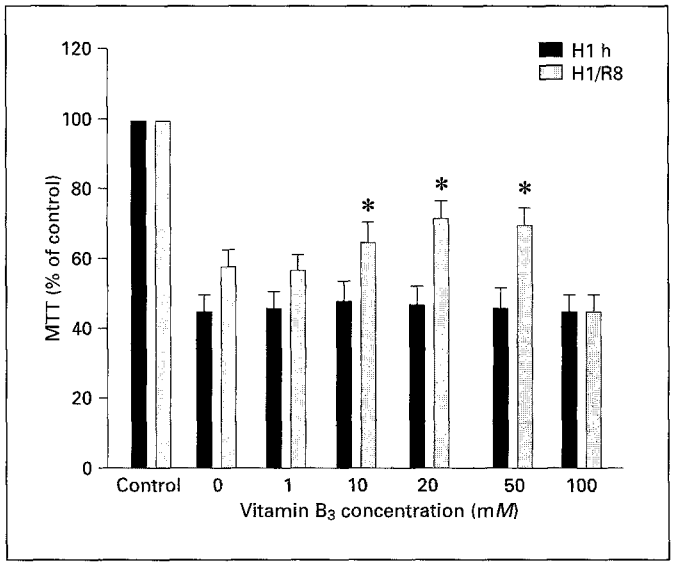

Fig. 2. Vitamin B_3 dose-dependently enhanced the cell viability of rat cortical cultures after hypoxia (OGD) and/or reoxygenation. The control groups received no treatment. Data represent the mean ± SEM from four separate experiments. * $p < 0.01$ compared with the control without vitamin B_3 under OGD or H/R.

RT-PCR for Early-Response Gene Expression

Cortical cell culture dishes for control and various concentrations of vitamin B_3 were treated with OGD for 2 h. Poly(A)$^+$ RNA was extracted with Straight A's mRNA Isolation System (Novagen) as described previously [19]. Briefly, extracted mRNA aliquots (0.1 µg) from each sample were used for RT to generate cDNA. Primers for rat c-*fos* and *zif*268 (Clontech) were as follows:
sense c-*fos* 5′-AATAAGATGGCTGCAGCCAA,
antisense c-*fos* 5′TTGGCAATCTCGGTCTGCAA;
sense *zif*268 5′GAAGCCCTTCCAGTGTGGAATCTG,
antisense *zif*268 5′GGAAGAGGCAGCTGAGGAGGGGGCCA.

To detect the c-*fos* PCR products, amplification consisted of 10 cycles of 45 s each of denaturation at 94 °C and annealing at 58 °C, with extension for 2 min at 72 °C, and 15 cycles of 45 s each of denaturation at 94 °C and annealing at 63 °C, with extension for 2 min at 72 °C. To detect the *zif*268 PCR products, amplification was carried out for 10 cycles of 45 s each of denaturation at 94 °C and annealing at 55 °C, with extension for 2 min at 72 °C, and 10 cycles of 45 s each of denaturation at 94 °C and annealing at 60 °C, with extension for 2 min at 72 °C in a Perkin Elmer Cetus thermocycler (Norwalk, Conn., USA). The reaction product was visualized by electrophoresis in a 2% agarose gel, and by staining with 0.5 µg/ml ethidium bromide. The gene amplification cycles were within the linear range for both the target gene and the control (β-actin).

Statistical Analysis

Data were expressed as the mean ± SEM from 4 separate experiments. For single variable comparisons, Student's t test was used. For multiple variable comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by Student's t test.

Results

Nicotinamide Dose-Dependently Increased Neuronal Survival during OGD or H/R

The neuroprotective effects of vitamin B_3 against OGD- and H/R-induced toxicity were evaluated by measuring the LDH released from the cytosol and the mitochondrial functions using the MTT assay. Cells in glucose-free DMEM without or with various concentrations of vitamin B_3 (1, 5, 10, 20, 50 and 100 m*M*) were exposed to OGD for 1 h followed by reoxygenation for 8 h. Results indicated that vitamin B_3 dose-dependently protected cells against OGD-induced toxicity by significantly reducing LDH release ($p < 0.01$) (fig. 1), but not by reducing mitochondria impairment ($p > 0.05$) (fig. 2). Vitamin B_3 (10 m*M*) significantly ($p < 0.01$) reduced LDH release, and this correlated well with the increased cell viability under H1/R8. Under the H1/R8 condition, cell viability in the vitamin B_3 group between concentrations of 10 and 50 m*M* was significantly better than that of the untreated group (fig. 2).

Nicotinamide Diminished OGD-Generated ROS

DCF-AM-loaded cells in glucose-free DMEM with or without vitamin B_3 were treated with OGD for 1 h or the

indicated time periods. Vitamin B_3 significantly diminished the DCF signal, which increased over time (fig. 3A). The increased DCF during OGD was dose-dependently diminished in the presence of vitamin B_3 (at concentrations of ≥ 10 m*M*; $p < 0.001$) (fig. 3B).

Nicotinamide Diminished the OGD-Induced Intracellular Calcium Rise in Cultured Cortical Cells

The fura-2 AM-loaded cells in glucose-free DMEM were subjected to OGD in the presence or absence of vitamin B_3 for the indicated time periods. The increased intracellular calcium concentration during OGD was significantly diminished in the presence of vitamin B_3 (fig. 4).

Nicotinamide Prevents H/R-Induced Procaspase-3 Cleavage

Morphologic observation of H/R-induced neurotoxicity was performed by staining with MAP-2 antibody (fig. 5A–C). Apoptotic cells were visualized by immunostaining with polyclonal antibody against cleaved caspase-3. Results demonstrated that vitamin B_3 prevented H1/R8-induced toxicity (fig. 5C) and procaspase-3 cleavage in neuronal cells (fig. 6A–C).

Nicotinamide Prevents H/R-Induced Apoptosis as Determined by Chromatin Condensation in Nuclei of Cultured Neuronal Cells

Both necrotic and apoptotic cell death occurred with hypoxia- and/or reoxygenation-induced neurotoxicity. Cellular apoptosis is morphologically characterized by

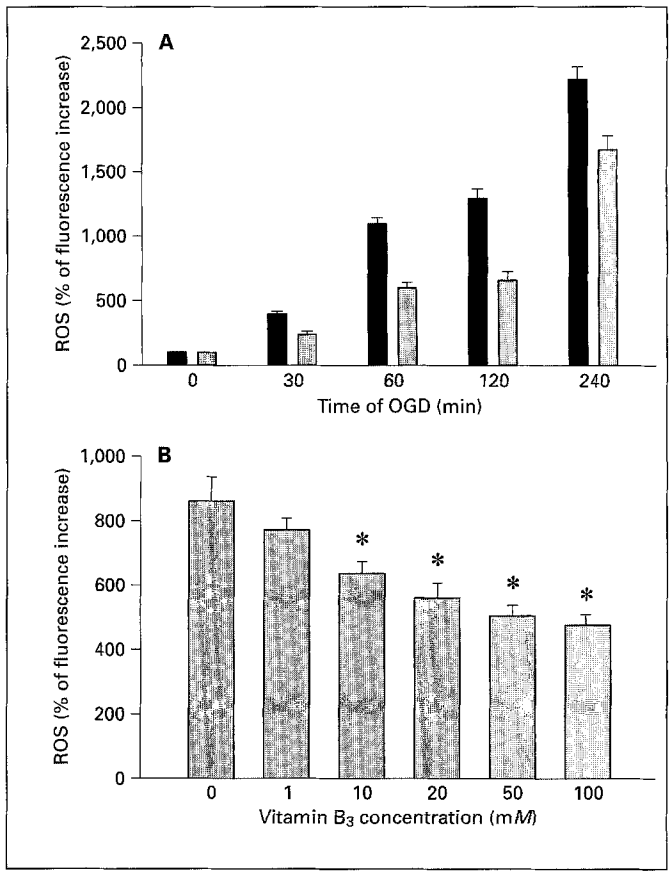

Fig. 3. Vitamin B_3 diminished OGD-generated ROS. The DCF-AM-loaded cells in glucose-free DMEM with or without nicotinamide were treated with 5% CO_2-85% N_2-10% H_2 for 1 h or the indicated time period. The increased DCF during OGD (black columns) were time- (**A**) and dose-dependently (**B**) diminished in the presence of vitamin B_3 (gray columns). * $p < 0.001$.

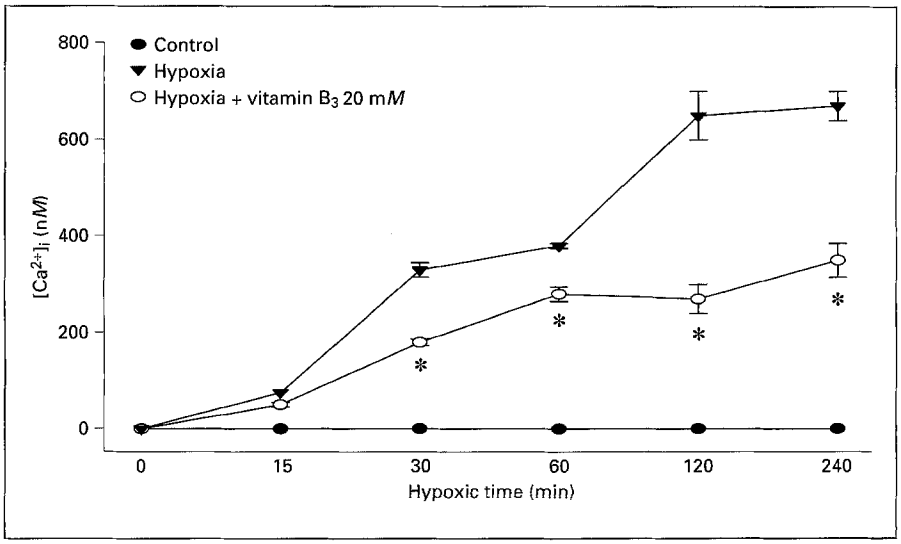

Fig. 4. Vitamin B_3 diminished the OGD-induced intracellular calcium rise in cultured cortical cells. The fura-2 AM-loaded cells were treated with or without vitamin B_3 under OGD as indicated. The increased $[Ca^{2+}]_i$ during OGD was significantly diminished in the presence of vitamin B_3 (* $p <$ 0.01).

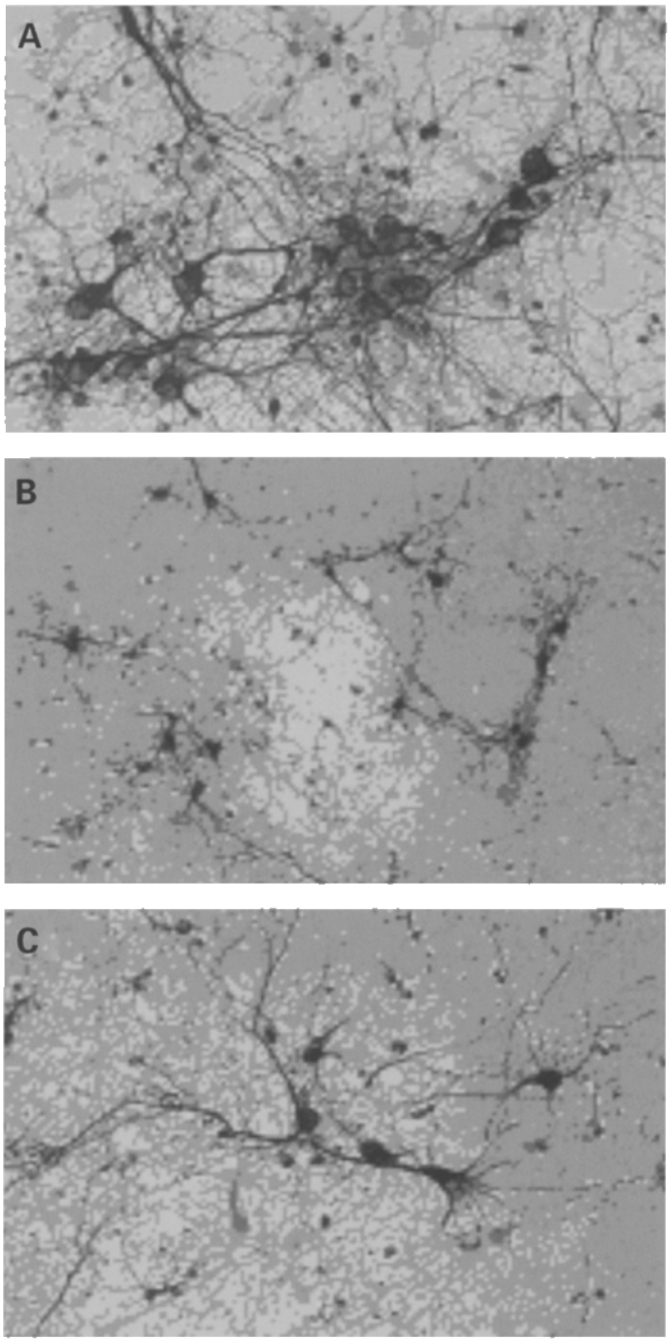

Fig. 5. Vitamin B_3 prevented H/R-induced neuronal death. H/R-induced neurotoxicity was observed by immunostaining with monoclonal antibody against MAP-2. The morphology of cortical cells of the normal control group (**A**), H1/R8 group (**B**) and the group with H/R in the presence of 20 m*M* vitamin B_3 (**C**) was significantly different.

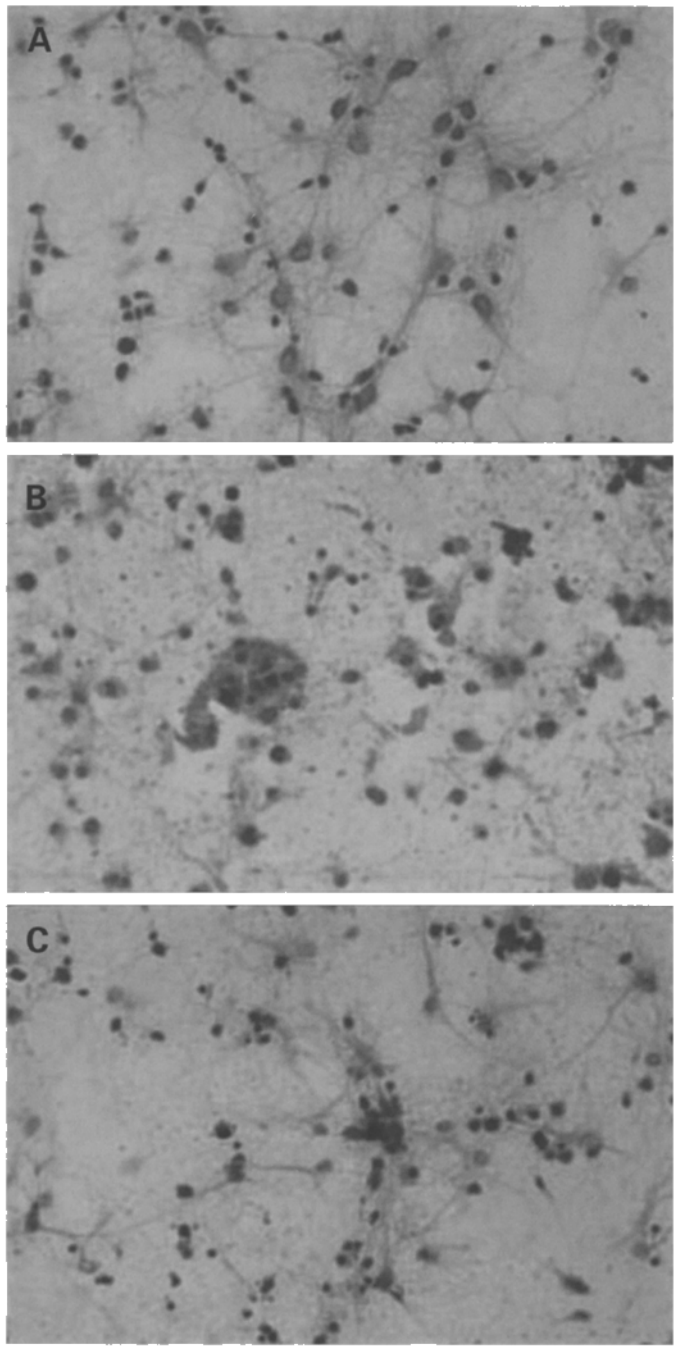

Fig. 6. Vitamin B_3 prevented H/R-induced procaspase-3 cleavage. Apoptotic cells were visualized by polyclonal antibody against cleaved caspase-3. The pictures show low caspase-3 expression in cortical cells of the normal control group (**A**), high caspase-3 expression in the H1/R8 group (**B**) and moderate caspase-3 expression in the H1/R8 group in the presence of 20 m*M* vitamin B_3 (**C**).

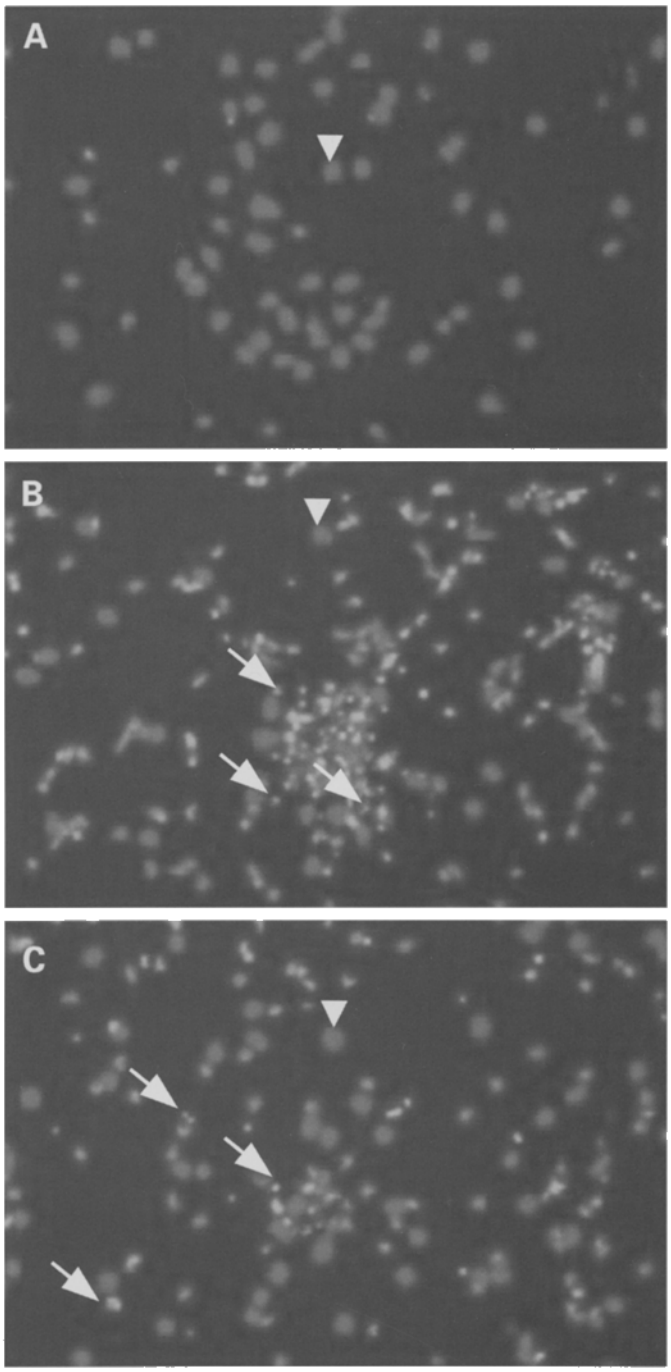

Fig. 7. Vitamin B_3 prevented H/R-induced neuronal apoptosis. H/R-induced apoptosis was observed by DAPI uptake. Cortical cells of the normal control group (**A**), and the cells exposed to H1/R8 (**B**) or in the presence of 20 m*M* vitamin B_3 (**C**) are shown. Typical normal nuclei are indicated by arrowheads and condensed nuclei by arrows.

cell shrinkage and chromatin condensation through a cascade of molecular and biochemical events, including activation of endonuclease, which cleaves DNA into oligonucleosomes. Chromatin condensation was visualized by the uptake of fluorochrome DAPI, which binds with DNA in apoptotic cells. Results showed that vitamin B_3 prevented H/R-induced nuclear condensation and shrinkage of cell nuclei (fig. 7A–C).

Nicotinamide Affected OGD-Induced Caspase-3 and Cytochrome c

Cell extracts for Western analysis were prepared from cortical cultures with or without vitamin B_3 treatment under ODG for 1 h. The primary antibodies used were anti-rat caspase-3 monoclonal antibody, anti-mouse cytochrome c polyclonal antibody and anti-mouse β-actin monoclonal antibody. Results showed no significant changes in cytochrome c or caspase-3 concentrations in mitochondria and cytosol between the control and nicotinamide-treated groups after 1 h of OGD (fig. 8).

Nicotinamide Decreased OGD-Induced Immediate-Early Gene Expression

Increased neuronal expression of c-*fos* and *zif*268 after ischemia has been demonstrated. The mRNA encoded by the c-*fos* gene and its protein product, Fos, provide an index of cell activation. Together with Fos, Zif268 and other related proteins, these transcription-regulating factors can couple diverse stimuli to widespread expression of other genes. The present study demonstrated alterations in expression of ischemia-induced immediate-early genes (IEGs). Vitamin B_3 dose-dependently reduced OGD-induced c-*fos* gene expression. However, inhibition of the *zif*268 gene was seen only at a concentration of 50 m*M* of vitamin B_3 after OGD (fig. 9).

Discussion

The complex responses in the brain following cerebral ischemia and/or reperfusion are related to both necrosis and apoptosis, and activation of caspase-3 involves the apoptotic pathway [6, 27, 29, 35]. Our results show that the mechanism by which nicotinamide reduced neuronal death (MTT and LDH release) under OGD and H1/R8 conditions was inhibition of ROS, Ca^{2+}, caspase-3 and IEGs.

Under physiological conditions, excessive ROS are neutralized by endogenous antioxidants (e.g. ascorbate, α-tocopherol, β-carotene and glutathione) and antioxidant

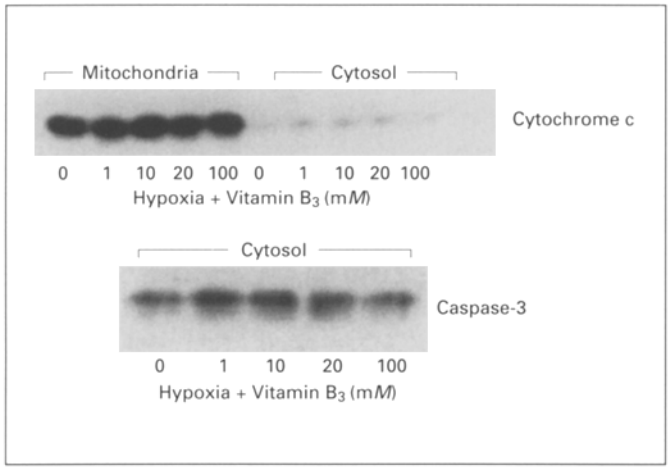

Fig. 8. Western blot of cytochrome c and caspase-3. Cell extracts for Western analysis were prepared from vitamin B_3-treated and nontreated cortical cultures under 1 h of OGD. The primary antibodies used were anti-rat caspase-3 monoclonal antibody and anti-mouse cytochrome c polyclonal antibody. The changes in cytochrome c and caspase-3 concentrations in the mitrochondria and cytosol in vitamin B_3-treated and nontreated groups after 1 h of OGD were not significantly different.

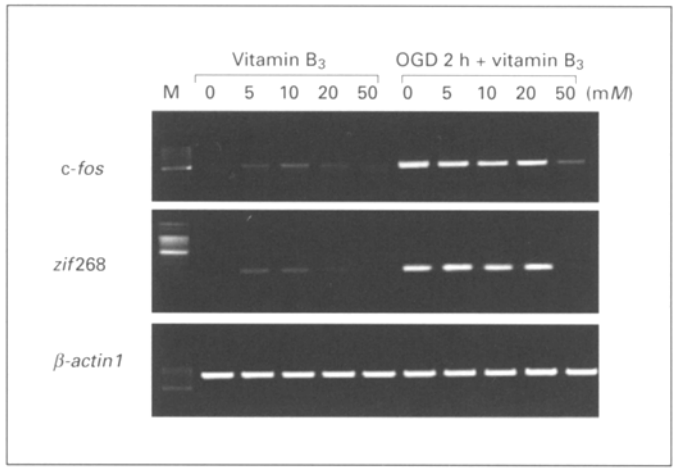

Fig. 9. Vitamin B_3 diminished OGD-induced IEGs, c-*fos* and *zif*268. Cortical cells were treated with 2 h of OGD and subjected to RT-PCR. Vitamin B_3 reduced OGD-induced c-*fos* mRNA expression dose-dependently. The inhibition of the *zif*268 gene was achieved at the concentration of 50 m*M* vitamin B_3.

enzymes (e.g. superoxide dismutase, catalase and glutathione peroxidase). Severe oxidative stress may overwhelm the antioxidant mechanisms and lead to oxidative DNA damage. DNA fragmentation occurs within minutes of induction of oxidative stress. DNA fragmentation probably results from attack by free radicals on DNA and from activation of endonucleases. Excitotoxicity and excess generation of NO are believed to be fundamental mechanisms in many acute and chronic neurodegenerative disorders as well as in cerebral ischemia and reperfusion injury. Disturbances of Ca^{2+} homeostasis and protein nitration/nitrosylation are key features in such conditions [30].

Nicotinamide (vitamin B_3) is an effective antioxidant against oxidative damage in rat brain cells [16, 25, 40]. Our results show that vitamin B_3 significantly reduced ROS generation and intracellular Ca^{2+} influx during hypoxia (OGD); these are similar to our findings that silymarin protects cells from injury [42]. However, these effects were not significant under the H1/R8 condition, because during reoxygenation, we replaced the medium with cell culture medium containing serum supplement B27, which has an antioxidative effect.

Oxidative stress can trigger DNA strand breakage, which then activates the nuclear enzyme PARP [37]. Rapid activation of this enzyme depletes the intracellular concentration of its substrate, NAD^+, thus slowing the rate of glycolysis, electron transport and subsequent ATP formation. This process can result in cell dysfunction and cell death [3]. Our results show that nicotinamide did protect against cell death from hypoxia and H/R injury. Nicotinamide is a precursor of NAD^+ and a PARP inhibitor, and it can potentially boost energy reserves to tissue at risk during cerebral ischemia [1, 2, 32, 41] and hepatic ischemia/reperfusion [7]. PARP has been implicated in ischemic cell death [11, 37] and was suggested to be a 'death substrate' for caspases, such as caspase-3. The cleavage of PARP by caspase-3 is an essential link in the apoptotic pathway in animal cells [17]. Cytochrome c was reported to be released from mitochondria to the cytosol under ischemia [12, 14], and activates other caspases, including caspase-3. One unstable substrate for caspase-3 is PARP, which contributes to repair or genomic maintenance. Therefore, inactivation of PARP can increase DNA cleavage and contribute to programmed cell death [10]. Triggering activities of caspases which cause cleavage of PARP or excessive activation of PARP plays a role in apoptotic and necrotic neuronal cell death elicited by ischemia and/or reperfusion injury [38]. The present results show that the prevention of H1/R8-induced procaspase-3 cleavage by vitamin B_3 might contribute to the reduced PARP activity that led to the neuroprotective effect.

We found that nicotinamide significantly protected cultured cortical cells against OGD- and H/R-induced LDH release and dose-dependently protected against mitochondrial impairment. Influx of intracellular calcium and production of ROS in cultured cortical cells during 1 h of OGD were also dose-dependently diminished in the presence of nicotinamide. Morphologic observation of H/R-induced neurotoxicity showed that vitamin B_3 reduced procaspase-3 cleavage (fig. 6A–C) as well as the nuclear condensation and shrinkage of cell nuclei (fig. 7). Our results indicate that vitamin B_3 fulfills roles as a precursor of NAD^+, a PARP inhibitor and a mild antioxidant, while also protecting against ROS and NO toxicity [15]. This may in turn protect against hypoxia- or H/R-induced neuronal injury through blockage of the activation of caspase-3 and the cleavage of PARP [25]. These findings may reveal the mechanisms contributing to the maintenance of genomic DNA integrity and the reversal of discrete pathways of programmed cell death, related to reductions in infarct volume following focal cerebral ischemia [31, 36].

Furthermore, we found that hypoxia-induced c-*fos* expression was dose-dependently diminished by vitamin B_3, suggesting that vitamin B_3 modulates the rapid response system of IEGs. The mRNA encoded by the c-*fos* gene and its protein product, Fos, provide an index of cell activation. Together with Fos, Zif268 and other related proteins, these transcription-regulating factors can couple diverse stimuli to widespread expression of other genes. For example, dimerization of Fos forms functional transcription factor complexes (e.g. AP-1) that bind to regulatory DNA sequences located in upstream regions of target genes and regulate gene transcription. The *zif*268 sequence encodes a 'zinc finger' protein that acts at another class of transcription-regulatory sites. Increased expression of c-*fos* and *zif*268 after ischemia has been demonstrated. In animals with middle cerebral arterial occlusion, IEG mRNAs (c-*fos*, c-*jun*, *nur77* and *zif*268) were induced in the ipsilateral forebrain, and, less frequently, in the contralateral forebrain, 1, 2 and 3 h after hypoxia [19]. Fos protein was identified in dying striatal neurons after hypoxic-ischemic brain injury [33]. Thus, the reduced hypoxia-induced IEG expression caused by nicotinamide may partially contribute to neuroprotection.

In conclusion, vitamin B_3 as an antioxidant inhibited both the excess generation of ROS and the influx of intracellular Ca^{2+}. Vitamin B_3 as a precursor of NAD^+ and a PARP inhibitor regulated caspase-3, which may modulate the DNA repair enzyme PARP. In addition, vitamin B_3 dose-dependently modulated the c-*fos* and *zif*268 genes. Taken together, these mechanisms of action of vitamin B_3 may contribute to neuroprotection under conditions of H/R injury.

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