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N-ethylmaleimide-Induced Death of Astrocytes: A Cytotoxic Role of Na^+ and Cl^-

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ABSTRACT

N-ethylmaleimide (NEM), which is experimentally used to alkylate the sulfhydryl groups, has been shown to induce cytotoxicity in various kinds of cells. Our preliminary experiments showed that NEM induced cytotoxicity in primary cultured astrocytes. In the present study, we investigated the mechanism for NEM-induced cytotoxicity in astrocytes. NEM markedly depolarized the mitochondrial transmembrane potential. The mitochondrial permeability transition inhibitor Cyclosporin A completely blocked the NEM-evoked depolarization of mitochondrial transmembrane potential, but did not reduce the cytotoxicity of NEM. NEM also depolarized the plasma membrane potential. Removal of extracellular Na^+ prevented NEM-evoked depolarization of plasma membrane potential and eliminated the cytotoxicity of NEM. Removal of extracellular Cl^- also protected the NEM-induced death of astrocytes, but with no blockade of NEM-evoked depolarization of plasma membrane potential. The present study suggested that both Na^+ and Cl^- play a critical role in NEM-induced astrocyte death regardless of the plasma membrane potential.

Key words: N-ethylmaleimide (NEM), LDH, astrocytes, Na^+, Cl^-, plasma membrane potential (PMP), mitochondrial transmembrane potential (MTP), death

INTRODUCTION

The sulfhydryl groups are ubiquitously found in membrane proteins and cytosolic and nuclear proteins. The sulfhydryl moieties in proteins play crucial roles in various critical cellular processes including enzymatic activities, ligand binding, signal transduction pathways and maintenance of membrane integrity. Thus, the modification of sulfhydryl groups by NEM can cause various kinds of cellular effects. N-ethylmaleimide (NEM) causes cytotoxicity in various kinds of cells by disrupting protein structure and function through cysteine modification (van Engeland et al., 1997; Song et al., 2000; Kim et al., 2001).

Alteration in cellular ionic balance can signal a cell to divide and differentiate. Recently, a loss of intracellular monovalent ions has also been reported to play a pivotal role in cell death (Dallaporta et al., 1998; Hughes et al., 1997; Wang et al., 1999; Yu et al., 1999). Cells were known to depolarize very early during apoptosis, prior to a loss in cell volume (Bortner et al., 2001). A dramatic loss of intracellular ions, particularly Na^+ and K^+, was as-

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sociated with the shrinkage of cells during apoptosis, this altering the intracellular environment and permitting nuclease activity and effecter caspase activation (Hughes et al., 1997).

Transmembrane movement of ions is important for the maintenance of cellular physiological functions in a wide variety of cell types. Alkylation of sulfhydryl groups of amino acids by NEM in ion channel proteins is known to affect their functional properties (Song et al., 2000). NEM was shown to altere the Na^+-Ca^{2+} exchanging activity in membrane preparations. NEM stimulated 10～20 fold the activity of K^+-Cl^- co-transport. K^+ ions have been shown to play an important role in the regulation of apoptosis. Apoptotic cells appear to have a much lower intracellular K^+ concentration ($[K^+]_i$) than normal cells (Hughes et al., 1997). NEM induces apoptosis in HepG2 human hepatoblastoma cells, and the mechanism of action of NEM includes intracellular K^+-Cl^- co-transport (Kim et al., 2001). In the present study, we investigated that Na^+ and Cl^- ions play a critical role in pathway of NEM-induced cytotoxicity in astrocytes death.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's media (DMEM/ F12), trypsin/EDTA, penicillin/streptomycin and Fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). N-ethylmaleimide (NEM), Cadmium chloride, N-methyl-D-glucamine, sodium gluconate and calcium gluconate were purchased from Sigma Chemical Co (St. Louis, MO). Cyclosporin A (CsA) were purchased from Calbiochem (La Jolla, CA). 5,5'',6,6''-tetrachloro-1,1'',3,3''-tetraethylbenzimidazolyl-carbocyanineiodide (JC-1) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) were purchased from Molecular Probes (Eugene, OR).

Astrocyte cultures

Astrocytes were cultured from the prefrontal cortices of 2- to 5-d-old Sprague-Dawley rat pups as previously described (Choi et al., 2002). In brief, cells were dissociated by mild trypsinization (10 min at 37°C, with Dulbecco's modified Eagle's medium (DMEM) containing 0.1% trypsin) and passed through sterile nylon sieves (130μm pore size) into DMEM containing 10% heat-inactivated FBS. Cells were then plated onto poly-L-lysine (20μg/ml) coated 75-cm^2 culture bottles and maintained for one week in DMEM/F12 supplemented with 10% FBS. Astrocytes were then trypsinized, washed and plated in the growth medium onto poly-L-lysine (20μg/ml)-coated 48 well plates. Cells were used for the experiments 5 to 7 d later.

Measurement of lactate dehydrogenase

Astrocytes injury and death was assessed by morphological examination of cells using phase-contrast microscopy and quantified by measuring the amount of lactate dehydrogenase (LDH) released into the bathing medium (Choi et al., 2002). Activity of LDH was measured using the diagnostic kit (Sigma Chemical Co., St. Louis, MO, USA). In brief, NADH and pyruvate (0.1 w/v %) were added and the samples incubated at 37°C for 30 min. The samples then were incubated with the coloring reagent for 20 min. The reaction was stopped by adding 0.4 N NaOH, and the activity (unit/ml) of LDH in each sample was calculated from the standard curve.

Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential (MTP) was measured according to the previous report (Choi et al., 2002). In brief, astrocytes cultured on 48-well culture plates were loaded for 20 min at 37°C with JC-1 (1.0μg/ml) in culture medium. Depolarization of MTP was assessed with measuring the fluorescence intensities at emission wavelengths of 530 and 590 nm using a fluorescence microplate reader (SPECTRAmax GEMINXS, Molecular Devices, California, USA). During the measurements, cells were protected from light and remained in a 5% CO2/95% air chamber at 37°C. Fluorescence intensity was acquired at indicated times for $<$2s to minimize photobleaching and was corrected for autofluorescence. The intensity of autofluorescence (i.e., fluorescence of cells not loaded with JC-1) was unchanged during the whole experimental period. In control experiments, no photobleaching was observed during the whole period of fluorescence monitoring.

Treatment of Na^+ -free or Cl^--free buffer

Astrocytes were cultured for 5～7 d on 48- well culture plates, rinsed twice with Na^+-containing buffer solution (120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, 25 mM HEPES, pH 7.4) or Na^+-free buffer solution and then experiments were performed in the same buffer solution. Na^+-free buffer solution was made by replacement of Na^+ with iso-osmotic N-methyl-D-glucamine (NMDG) (Wolf et al., 2001). Similarly, cells were experimented in Cl^--containing buffer solution (125 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 6 mM glucose, 1 mM $CaCl_2$, 25 mM HEPES, pH 7.4) or Cl^--free buffer solution. In Cl^--free condition, external Cl^- replaced with gluconate (Kim et al., 2001).

Measurement of plasma membrane potential (PMP)

The plasma membrane potential was measured according to the previous report of Gopalakrishnan his colleagues. (1999) with minor modifications. Astrocytes cultured on 48-well culture plates were rinsed twice with Krebs-Ringer buffer (125 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 6 mM glucose, 1 mM $CaCl_2$, 25 mM HEPES and pH 7.4) and loaded for 30 min at 37°C with 5μM bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) in the same solution. The test compounds was added and then depolarization of plasma membrane was assessed with measuring the fluorescence intensities at excitation and emission wavelengths of 493 and 517 nm using a fluorescence microplate reader. During the measurements, cells were protected from light and remained in a 5% CO_2/95% air chamber 37°C. Fluorescence intensity was acquired at indicated times for ＜2s to minimize photobleaching and was corrected for autofluorescence. The intensity of autofluorescence (i.e., fluorescence of cells not loaded with ($DiBAC_4(3)$) was unchanged during the whole experimental period. In control experiments, no photo-bleaching was observed during the whole period of fluorescence monitoring.

Statistical Analysis

Data are expressed as the standard error of mean (S.E.M.) and analyzed for statistical significance by using paired t-test. A p value ＜0.05 was considered significant.

RESULTS

Astroglial cell death by NEM

Cell death was morphologically observed and quantitatively determined by measuring the release of lactate dehydrogenase (LDH) from injured or dead cells. As shown in Fig. 1, the thiol-alkylating agent NEM induced a significant death of astrocytes. A significant death of astrocytes was significantly evoked by as much as 10μM NEM, but not by 5μM.

Relationship between NEM-induced MTP depolarization and cytotoxicity

At low micromolar concenttations (10～20μM) NEM was previously reported to induce mito-

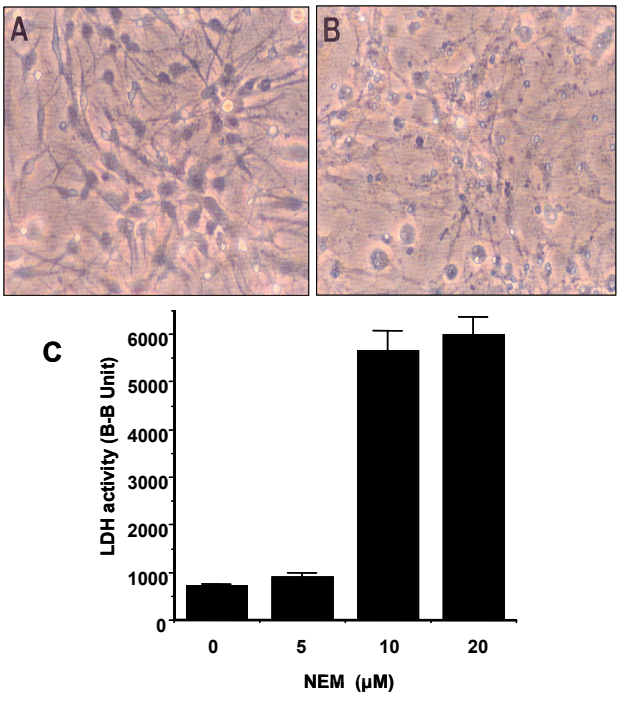

Fig. 1. NEM induced astrocyte death. Astrocytes were cultured for 5～7d on 48well culture plates before use. A. Control. B. 10μM NEM. Micrographs were taken 3h after NEM treatment and representative of five independent experiments. C. Concentration-response relationship of NEM-induced astroglial death. Astrocytes were treated with 0, 5, 10, 20μM NEM in glucose-containing DMEM. LDH levels were determined 3 h after NEM treatment. Data represent mean±S.E.M. of three independent experiments.

chondrial permeability transition (MPT) (Costantini et al., 1998). Therefore, we tested whether the NEM-induced depolarization of mitochondrial transmembrane potential (MTP) caused the astrocyte death. Alteration of the MTP was measured by using JC-1, a MTP-sensitive fluorescence dye. A

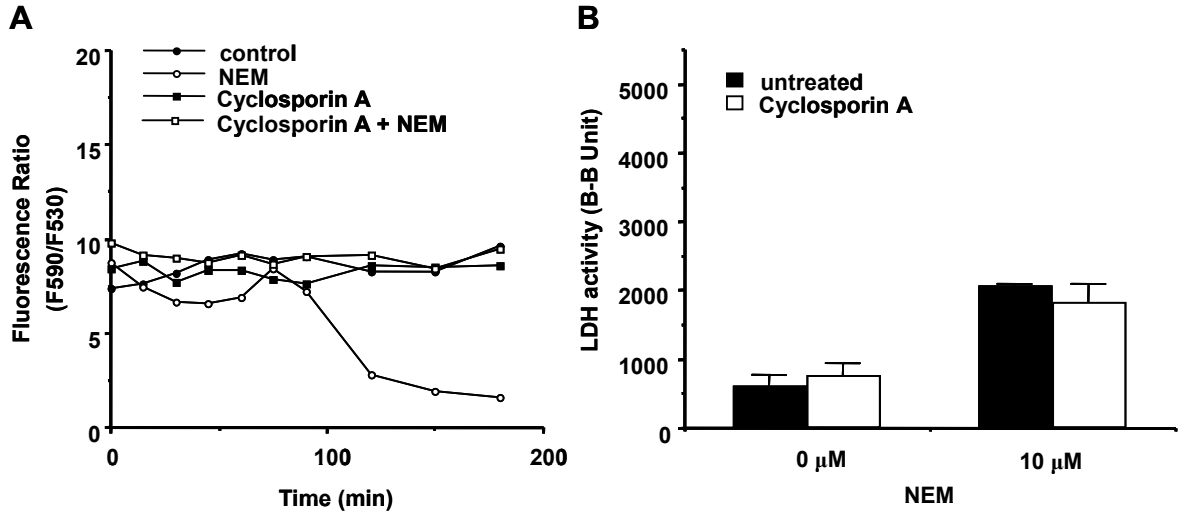

Fig. 2. Effect of Cyclosporin A (CsA) on the NEM-induced astrocytic death. (A). Astrocytes were preloaded with JC-1 (1.0 μg/ml) for 20 min at 37°C and then treated with 10μM NEM with or without 2 h-pretreatment of 2 μM CsA. Fluorescence intensity was acquired at indicated times after NEM treatment. Data were expressed as the mean±S.E.M. of the ratio of aggregate fluorescence (590 nm) to monomer fluorescence (530 nm). Traces are representative of four independent experiments. (B). Astrocytes were pre-incubated for 2 h with 2μM CsA and then treated with 10μM NEM. LDH levels were determined 3 h after NEM treatment. Data are mean± S.E.M. of four independent experiments.

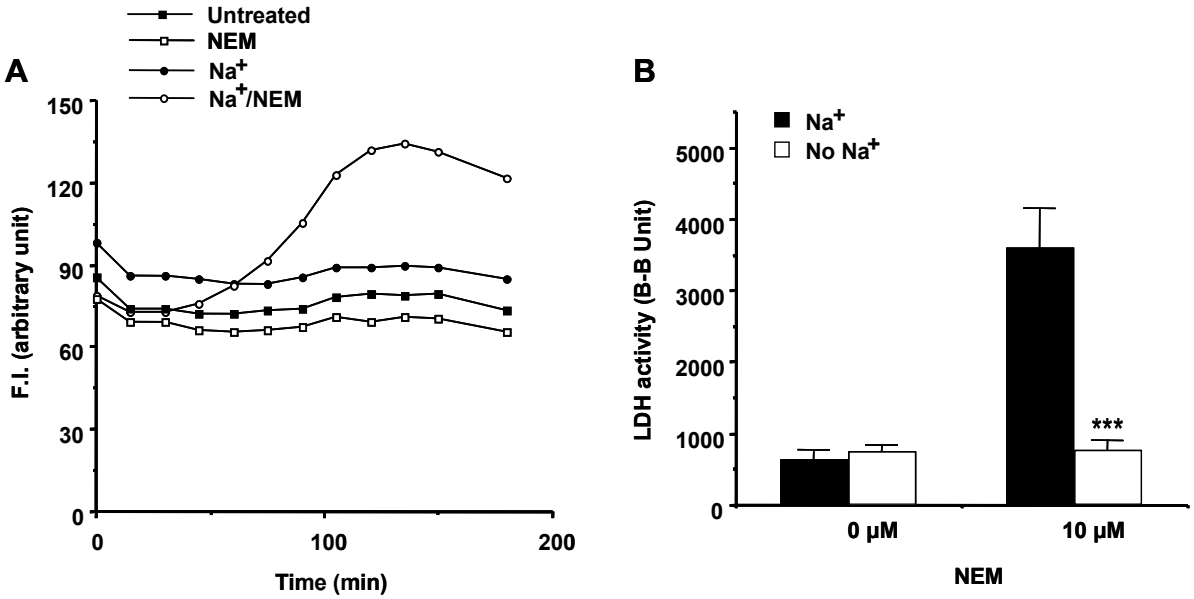

Fig. 3. Removal of extracellular Na^+ inhibited both PMP depolarization and NEM-induced astrocytes death. (A). Astrocytes were preloaded with 5μM $DiBAC_4(3)$ for 30 min at 37°C in the Na^+-containing or -free condition, washed twice with the same buffer solution and then treated with 10μM NEM. Fluorescence intensity was acquired every 15 min for 3 h. Data were expressed as fluorescence intensities (F.I.) and traces are representative of four independent experiment. (B). For extracellular Na^+-free buffer solution (No Na^+), external Na^+ was replaced with N-methyl-D-glucamine. Astrocytes were rinsed twice with Na^+-containing or free buffer solution and experimented in the same solution. LDH levels were determined 3 h after NEM treatment. N= 5. ***$p < 0.001$ (paired *t*-test)

significant depolarization of MTP was observed 90 min after exposure to NEM (Fig. 2A). Interestingly, however, NEM-induced LDH release was observed before the significant MTP depolarization occurred (data not shown). We further found that the inhibition of MTP depolarization by the MPT inhibitor CsA completely blocked the NEM-induced MTP depolarization but did not prevent the LDH release in NEM treated astrocytes (Fig. 2A and B). The data indicate that NEM-induced cytotoxicity is not mediated by inducing MTP depolarization.

Relationship between NEM-induced PMP depolarization and cytotoxicity

Plasma membrane potential (PMP) plays a critical role during apoptosis (Bortner et al., 2001). NEM alkylation exerts functional changes in various types of ion channels (Song et al., 2000). The PMP specific dye $DiBAC_4(3)$ was used to monitor changes of PMP. $DiBAC_4(3)$ is an anionic oxonal dye that responds with an increase in fluorescent intensity at 530 nm upon membrane depolarization (Bortner et al., 2001). To test the role of PMP change in NEM-induced astrocytes death, we compared the NEM cytotoxicity obtained under Na^+-containing or -free conditions. Under a Na^+-free condition, NEM induced neither PMP depolarization nor the augmented death (Fig. 3). Normal Cl^- homeostasis also plays an important role in the regulation of cellular pH, volume and membrane potential (O'Neill et al., 1999; Casey et al., 1998). Thus, alteration of intracellular Cl^- was also reported to be seriously involved in apoptosis (Lang et al., 2000). Interestingly, in the present study removal of extracellular Cl^- did not block the PMP depolarization but significantly inhibited the cell death caused by NEM (Fig. 4). In contrast to Na^+ and Cl^-, Ca^{2+} channel inhibitor Cd^{2+} did not prevent the augmented death of NEM-treated astrocytes, implying that Ca^{2+} is not involved in NEM cytotoxicity (Fig. 5.).

DISCUSSION

The sulfhydryl moieties are ubiquitously found in membrane proteins and play crucial roles in various membrane protein functions. Thus, the modification of sulfhydryl groups by NEM would cause various kinds of cellular effects. One of the cellular events caused by NEM is the opening of MPTP (Petronilli et al., 1994; Costantini et al., 1998). Previous reports

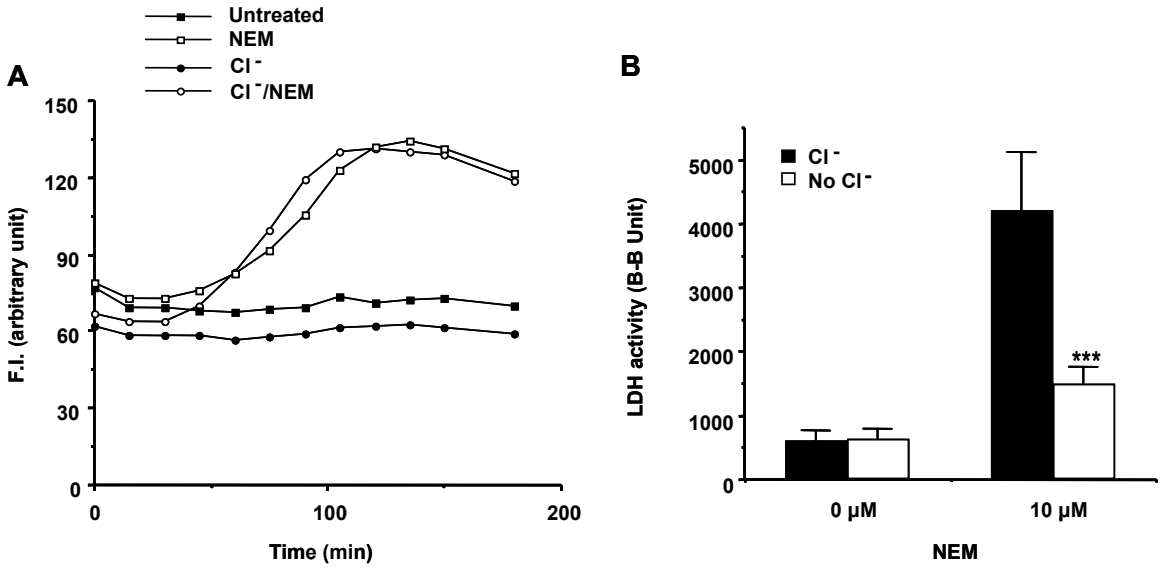

Fig. 4. Removal of extracellular Cl^- inhibited the death of astrocytes with no blockade of PMP depolarization in NEM-treated astrocytes. (A). For extracellular Cl^--free buffer solution (No Cl^-), external Cl^- was replaced with gluconate. Astrocytes were rinsed twice with Cl^- containing or free buffer solution and experimented in the same solution. LDH levels were determined 3 h after NEM treatment. (B). Astrocytes were preloaded with 5μM $DiBAC_4(3)$ for 30 min at 37°C in the Cl^--containing or free condition, washed twice with the same buffer solution and then treated with 10μM NEM. Fluorescence intensity was acquired every 15 min for 3 h. N=4. ***$p < 0.001$ (paired *t*-test)

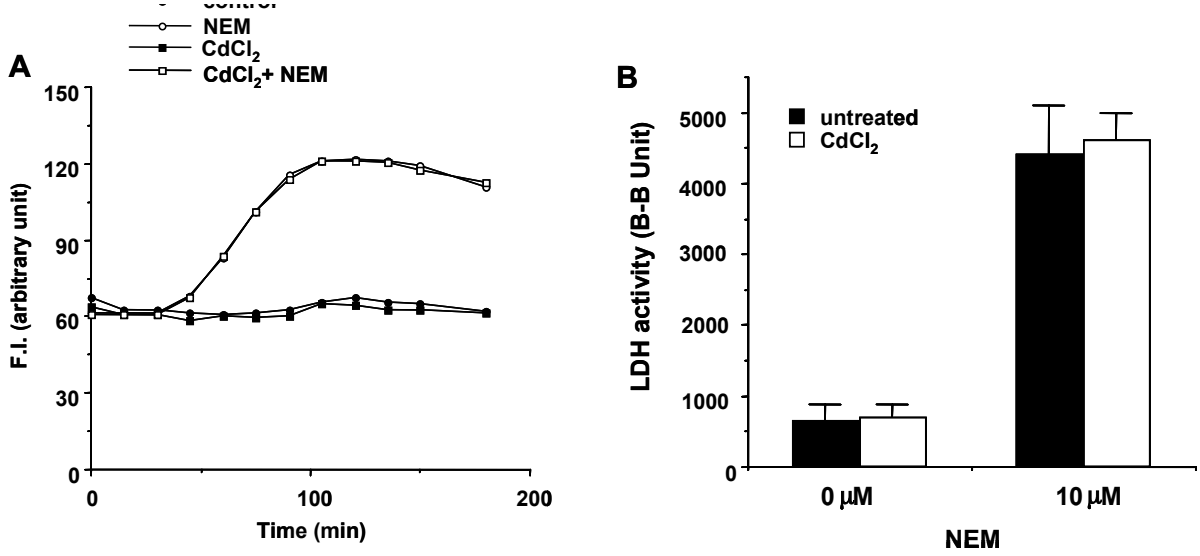

Fig. 5. A Ca^{2+} channel blocker cadmium chloride did not effect both PMP depolarization and NEM-induced astrocytes death. (A). Astrocytes were pre-incubated for 10 min with 0.1μM $CdCl_2$ and treated with 10μM NEM. LDH levels were determined 3 h after NEM treatment. (B) Astrocytes were preloaded with 4μM $DiBAC_4(3)$ for 30 min at 37°C in the KRH buffer solution, washed twice with the same buffer solution and then treated 10μM NEM. Fluorescence intensity was acquired every 15 min for 3 h. Data were expressed as fluorescence intensities (F.I.) and traces are representative of four independent experiments. N=4.

indicated that the opening of the mitochondrial permeability transition pore (MPTP) might be a causative event in necrotic or apoptotic cell death. In the present study, however, NEM-induced MPTP opening was not a direct reason for NEM-induced astrocytes death.

The PMP is the sum of charges resulting from the concentration gradients of various ions such as H^+, Na^+, K^+, Ca^{2+} and Cl^-. In most eukaryotic cells a Na^+/K^+-ATPase or an H^+/K^+-ATPase generates electrochemical gradients, maintains the PMP, and drives secondary transporters involved in moving ions and metabolites across the cell membrane (Van Der Heyden and Docampo, 2002). Modifications of amino acid sulfhydryl groups by NEM are known to affect functional properties in various types of ion channels (Nakajima et al., 1990; Aghdasi et al., 1997) and these alterations in a living organism result in pathological cell swelling or shrinkage, leading to the clinical states of edema, uncontrolled apoptosis and even tissue necrosis (Lauf et al., 2000). In most excitable cells, cellular depolarization occurs as a result of a movement of sodium ions, which can occur through Na^+ channels, suppression of the Na^+/K^+-ATPase activity and activation of Na^+-dependent amino acid co-transport systems. The present study demonstrates that NEM-induced PMP depolarization and cell death both are associated with Na^+ ion. Removal of extracellular Na^+, obtained by using Na^+-free condition, completely prevented both PMP depolarization and cell death by NEM. Dissimilar to Na^+, however, removal of extracellular Cl^-, obtained by using Cl^--free condition, did not reduce the PMP depolarization but significantly inhibited the cell death caused by NEM. The data indicate that regardless of the level of PMP both Na^+ and Cl^- are closely associated with the cytotoxicity induced by NEM. Recently, NEM was reported to increase the level of $[Ca^+]i$ (Wang, 2003). Like Na^+ and Cl^-, the level of intracellular Ca^+ ($[Ca^+]i$) has been known to be a critical factor for the cell viability (Orrenius et al., 2003; Tymianski, 1996; Orrenius and Nicotera, 1994). Since in our study the Ca^{2+} channel inhibitor Cd^{2+} did not prevent the augmented death of NEM-treated astrocytes, however, Ca^+ did not appear to be involved in the cytotoxicity of NEM.

Taken together, NEM significantly induced the death of primary cultured astrocytes. The influx of

Na^+ or Cl^-, but not Ca^+, are well associated with the.cytotoxic effect of NEM. Our study further indicates that the data obtained by using NEM should be carefully interpreted for its significant effect on cytotoxicity.

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