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NMDA Receptor Activation Induces Mitochondrial Dysfunction, Oxidative Stress and Apoptosis in Cultured Neonatal Rat Cardiomyocytes

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Summary

Glutamate is a well-characterized excitatory neurotransmitter in the central nervous system (CNS). Recently, glutamate receptors (GluRs) were also found in peripheral tissues, including the heart. However, the function of GluRs in peripheral organs remains poorly understood. In the present study, we found that N-methyl-D-aspartate (NMDA) could increase intracellular calcium ($[Ca^{2+}]i$) level in a dose-dependent manner in cultured neonatal rat cardiomyocytes. NMDA at 10^{-4} M increased the levels of reactive oxygen species (ROS), cytosolic cytochrome c (cyto c), and 17-kDa caspase-3, but depolarized mitochondrial membrane potential, leading to cardiomyocyte apoptosis. In addition, NMDA treatment induced an increase in bax mRNA but a decrease in bcl-2 mRNA expression in the cardiomyocytes. The above effects of NMDA were blocked by the NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), and by ROS scavengers glutathione (GSH) and N-acetylcystein (NAC). These results suggest that stimulation of NMDA receptor in the cardiomyocyte may lead to apoptosis *via* a Ca^{2+}, ROS, and caspase-3 mediated pathway. These findings suggest that NMDA receptor may play an important role in myocardial pathogenesis.

Key words
Calcium • Cytochrome c • Caspase-3 • Heart

Introduction

Glutamate monosodium has been used as a flavor enhancer for many years. However, the safety of glutamate monosodium has been questioned by scientists in the world because certain persons developed serious cardiac symptoms such as ventricular tachycardia (Gann 1977), supraventricular tachyarrhythmia (Goldberg 1982) and other adverse effects which are designated as "Chinese Restaurant Syndrome".

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Glutamate (Glu) is an important excitatory neurotransmitter in the central nervous system (CNS), which mediates a wide variety of neuronal functions depending on the specific type(s) of glutamate receptors (GluRs). GluRs include ionotropic receptors (iGluRs, ligand-gated channels) and metabotropic receptors (mGluRs, G protein-coupled receptors). The iGluRs are classified into three subtypes according to their selective agonists: N-methyl-D-aspartate receptor (NMDA-R), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPA-R) and kainite receptor (KA-R) (Meldrum 2000). In addition to the CNS, GluRs are also found in peripheral tissues, including the heart (Gill *et al.* 1998, 1999, Seeber *et al.* 2000, 2004), pancreatic island cells (Inagaki *et al.* 1995), keratinocytes (Morhenn *et al.* 1994, 2004). Although the role of each specific type of GluRs in the CNS has been extensively studied, the probable role of iGluRs in the heart remains unknown.

NMDA-R is known to act in concert with the receptor-gated cation channels in the postsynaptic membrane of nerve cells. When neurons are excited, this channel becomes permeable to Ca^{2+} and some monovalent cations. NMDA-R activation in the brain induces Ca^{2+} overload, ROS production, mitochondrial dysfunction, and apoptotic cell death. For example, NMDA induces increase of intracellular Ca^{2+} in rat in cortical neurons (Nakamichi *et al.* 2002). NMDA-R activation induces production of ROS in cerebrocortical neurons (Tenneti *et al.* 1998) and cerebellar granule cells (Boldyrev *et al.* 1999). Calcium overload and mitochondrial membrane potential (ΔΨm) depolarization occurs when cultured hippocampal cells are exposed to NMDA (Schinder *et al.* 1996). NMDA is also known to induce apoptosis in cerebrocortical neurons (Tenneti *et al.* 1998) and rabbit retinas (Kwong and Lam 2000). In addition, NMDA-R localization in the heart is mostly concentrated in the nerve terminals, ganglia, conducting fibers and atrial myocytes (Gill *et al.* 1998, 1999). Based on these findings, we hypothesized that activation of the NMDA-R in the heart may play a pathological role through alterations in calcium homeostasis, oxidative stress, mitochondrial function, subsequently leading to apoptosis.

Methods

Cell cultures

Primary cultures of neonatal rat cardiomyocytes were prepared according to a reported protocol (Iwaki *et al.* 1990) with minor modifications. Briefly, hearts were isolated from 1-3 days old Wistar rats. The myocardial cells were dispersed by digestion with collagenase II (0.4 mg/ml, GIBCOBRL) and pancreatin (0.6 mg/ml, Sigma). Cardiomyocytes were purified using a differential attachment technique.

Cell viability assessment by MTT assay

Cell viability was measured by estimating the mitochondrial respiration. Cardiomyocytes were treated with different concentration of NMDA in 96-well plates, MTT was added to each well under sterile conditions (final concentration 0.5 mg/ml), and the plates were incubated for 4 h at 37 °C. Formazan was quantified spectroscopically at 540 nm using a Bio-Rad automated EIA Analyzer.

Measurement of $[Ca^{2+}]i$ by confocal microscopy

Cardiomyocytes were cultured for 5 days and then loaded with 3 μM fluo-3 acetoxymethyl ester (fluo-3/AM) and 30 nM pluronic F-127 in dark for 1 h at 37 °C (Xu *et al.* 2003). Cells were then exposed to NMDA, MK-801 (added 20 min before the exposure to NMDA) or Ca^{2+}-free medium ($[Ca^{2+}]_o$ free). The $[Ca^{2+}]i$ was estimated by the relative fluorescence intensity.

Measurement of intracellular ROS generation by flow cytometry

For analysis of the generation of intracellular ROS, the oxidation-sensitive probe 2',7'-dichloro-fluorescein diacetate (DCFH-DA, Sigma) was used as previously described (Sureda *et al.* 1996). Cells were cultured for 5 days, then were detached by 0.125 % trypsin and suspended in Hank's solution. DCFH-DA (10 μmol/l) was added to the solution and the cells were incubated at 37 °C for 30 min. The changes of ROS production in response to different stimuli were estimated by the fluorescence intensity, with excitation wavelength at 488 nm and emission wavelength at 525 nm. A rightward shift of the fluorescence tracing curve indicates an increase in ROS and *vice versa*.

Measurement of mitochondrial membrane potential depolarization (ΔΨm) by flow cytometry

Rhodamine 123 (Rh123) is a frequently used fluorescent probe for measuring ΔΨm in mammalian cells (Juan *et al.* 1994). Briefly, cells were trypsinized in

the plate, resuspended in PBS and treated with 25 μmol/l Rh123 for 30 min at 37° C and then washed three times with PBS. The ΔΨm was assessed by flow cytometry with excitation at 480 nm and emission at 530 nm.

Morphological assessment of apoptosis

Cells were exposed to NMDA (10^{-4} M), MK-801 (30 μM), GSH (10 mM) and NAC (5 mM) for 24 h, washed three times with PBS and stained with acridine orange (200 μg/ml) for 3 min. Morphological features of apoptotic death (cell shrinkage, chromatin condensation and fragmentation) were monitored by fluorescent microscopy. The percentage of cardiomyocytes with apoptotic death was measured by counting these cells at 20x power in 10 randomly chosen fields (1 mm^2) in each slide. Three slides were checked in each group.

Flow cytometric analysis of apoptosis

Flow cytometric studies were performed on a FACScan with Lysis II software (Becton-Dickinson, USA). The cells were washed with D-Hanks, trypsinized, washed with PBS and fixed in 70 % ethanol for 30 min at 4 °C. The cells were again rinsed with PBS and resuspended in 1 ml PBS containing 50 μg/ml RNase A and 50 μg/ml propidium iodide (Sigma). The samples were kept in the dark at 4 °C for 30 min and then analyzed by flow cytometry with excitation at 488 nm and emission measured at 560-640 nm (FL2 mode). Cells undergoing apoptosis stained positive with propidium iodide and exhibited a reduced DNA content with a peak in the hypodiploid region (Fraker *et al.* 1995, Darzynkiewicz *et al.* 1997). The percentage of cells with apoptosis was taken as the fraction of cells with hypodiploid DNA content.

DNA fragmentation analysis

Cardiomyocytes were washed with PBS and pelleted by centrifugation. The cell pellets were then treated for 10 s with 50 μl lysis buffer (1 % Nonidet P-40 in 20 mmol/l EDTA and 50 mmol/l Tris-HCl, pH 7.5). After 5 min of centrifugation at 1600 *g*, the supernatant was collected and extracted with 50 μl lysis buffer. SDS (1 %) was added to the mixture, and the supernatant was treated for 2 h with 5 μg/ml RNase A (at 56 °C), followed by digestion with 2.5 μg/ml proteinase K for 2 h at 37 °C (Herrmann *et al.* 1994). After the addition of 1/2 vol of 10 M ammonium acetate, DNA was precipitated with 2.5 vol ethanol and dissolved in a gel loading buffer. The DNA fragments were then separated by electrophoresis with a 1.5 % agarose gel.

RT-PCR analysis

The mRNA levels of bcl-2 and bax were estimated by a semiquantitative RT-PCR assay. Total cellular mRNA was obtained using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One microgram of total RNA was reversely transcribed using random primers and Superscript II RT. The single-strand cDNA was amplified by PCR with Taq DNA polymerase. The sequences of sense and antisense primers, respective PCR conditions, and cycle counts were as follows: bax, 5'-GCAGAG GATGATTGCTGATG-3' and 5'-CTCAGCCCATCT TCTTCCAG-3', 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, 35 cycles (Sato *et al.* 1994), bcl-2, 5'-TCCATTATAAGCTGTCACAG-3' and 5'-GAA GAGTTCCTCCACCAC-3', 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, 35 cycles (Oltvai *et al.* 1993). GAPDH cDNA was amplified as a control.

Western blot analysis

Western blot analysis was used to detect the cytosolic level of cytochrome *c* and caspase-3. Cells were scraped off in PBS, pelleted by centrifugation at 1,000 *g* for 10 min at 4 °C and resuspended in a lysis buffer (50 mM Tris-HCl, pH 7.4), leupeptin (10 mg/ml) pepstatin A (2 mg/ml), aprotinin (10 mg/ml), PMSF (10 mg/ml), 0.5M EDTA) for 60 min at 4 °C. Lysates were clarified by centrifugation at 15,000 *g* for 20 min at 4 °C. The supernatant contained cytosolic protein extract. Protein concentration was determined by Bradford method. Forty micrograms of the cytosolic proteins were separated by SDS-12 % polyacrylamide gel electro-phoresis, transferred onto a nitrocellulose membrane, followed by incubation with anti-cyto-*c* (Santa Cruz) or anti-caspase-3 (Santa Cruz) primary antibody at a dilution of 1:500 and 1:1000 overnight respectively. After washing with TBST, the membrane was incubated for 1 h with a horseradish peroxidase-coupled secondary antibody (1:5000 dilution). Blots were then developed with the ECL system.

Statistical Analysis

Data are presented as mean ± S.D. Comparisons of parameters among different groups were made with Kruskal and Wallis H test. $P<0.05$ value was considered to be significant.

Results

NMDA-induced calcium transient or overload in cardiomyocytes

NMDA stimulated a transient reversible increase of $[Ca^{2+}]_i$ at 10^{-5} M and induced a sustained irreversible $[Ca^{2+}]_i$ increase (calcium overload) at 10^{-4} M in cultured cardiomyocytes of neonatal rats. Pretreatment with MK-801 (30 μM), a selective NMDA-R antagonist, partially inhibited the increase of $[Ca^{2+}]_i$. Removal of extracellular Ca^{2+} completely abolished the $[Ca^{2+}]_i$ increase (Fig. 1).

NMDA-induced ROS generation and mitochondrial membrane potential depolarization in cardiomyocytes

Figures 2A and 2B show the effect of NMDA on ROS generation in cardiomyocytes using DCFH-DA fluorescence probe. In DCFH-DA loaded cells, strong DCF fluorescence was detected after challenge with 10^{-4}M NMDA, suggesting an increase in the ROS content compared to the control. MK-801 (30 μM) or removal of extracellular free Ca^{2+} prevented the increase in DCF fluorescence intensity induced by NMDA, suggesting that the NMDA-R mediated generation of ROS in cardiomyocytes is Ca^{2+}-dependent. Figure 2C is an example of the original recordings of $\Delta\Psi m$ at baseline, exposed to NMDA, and to a combination of NMDA and MK-801. A leftward shift of the tracing indicates a decrease of $\Delta\Psi m$. The $\Delta\Psi m$, as indicated by the normalization of fluorescence intensity, decreased to about 43 % of the basal value after the exposure to NMDA (Fig. 2D). Pretreatment with MK-801 (30 μM) induced a 90 % recovery of the $\Delta\Psi m$ as indicated by the normalization of fluorescence intensity.

NMDA-induced cytochrome c release and caspase-3 activation

Cytosolic level of cytochrome *c* was estimated by Western blotting. After exposure to 10^{-4} M NMDA for 24 or 48 h, cytochrome *c* appeared in the cytosol of cardiomyocytes (Fig. 3A), suggesting a release of cytochrome *c* from the mitochondria to the cytosol. MK-801, GSH and NAC significantly inhibited this release of cytochrome *c* into cytosol.

Release of cytochrome *c* may lead to the activation of caspase-3, which can be detected by its cleavage from the inactive pro-CPP32 to the active 17-kDa product. We detected increased 17-kDa caspase-3 after 24 h or 48 h exposure to 10^{-4} M NMDA (Fig. 3B).

NMDA-induced alterations in the mRNA expressions of bax and bcl-2

The mRNA level of bax significantly increased and that of bcl-2 decreased in NMDA treated cardiomyocytes compared with MK-801, GSH and NAC treated cells (Fig. 3C, D). The levels of mRNA encoding for GAPDH did not show significant difference between groups.

NMDA-induced decrease of cardiomyocyte viability

Cardiomyocyte viability was evaluated by MTT assay. NMDA exposure for 24 h at relatively lower concentrations (10^{-6} M and 10^{-5} M) had no significant influence on cell viability. However, NMDA at 10^{-4} M or higher, induced cell death and decreased cell viability in a dose-dependent manner (Fig. 4A). This effect could be inhibited by MK-801 (30 μM), GSH (10 mM) and NAC (5 mM), the scavengers of H_2O_2 (Fig. 4B). In addition, the absence of Ca^{2+} in the medium abolished the toxicity induced by NMDA (Fig. 4B). These results suggest that H_2O_2 is involved in NMDA-induced cell toxicity and Ca^{2+} takes a trigger role in the signaling cascade.

NMDA-induced cardiomyocyte apoptosis

As shown by acridine orange staining and fluorescence microscopy, cardiomyocytes treated with 10^{-4} M NMDA displayed morphological features of cell apoptosis (Fig. 4C). The percentage of cells with highly condensed chromatin or fragmented nuclei were higher in NMDA-treated cells (17.66±3.05 %) than in the control cells (3.66±1.53 %), MK-801-treated cells (5.00± 0.98 %), GSH- and NAC-treated cells (8.67±2.08 %) (Fig. 4D).

To confirm that NMDA induces apoptosis in cardiomyocytes, we examined DNA fragmentation by agarose gel electrophoresis. Extracted genomic DNA showed a distinct DNA ladder characteristic of apoptosis when cardiomyocytes were exposed to 10^{-4} M NMDA for 24 h and 48 h (Fig. 5A), confirming that NMDA can induce cardiomyocyte apoptosis.

Flow cytometric analysis also revealed 1.11 ±0.44 % of control cells had hypodiploid DNA content typical of apoptosis. Treatment with 10^{-4} M NMDA for 24 h increased the percentage of apoptotic cells to 22.8±2.65 %. A decrease of apoptotic cells was observed in cells treated with MK-801 (2.57±0.37 %) or GSH/NAC (12.16±1.81 %) (Figs 5B and 5C).

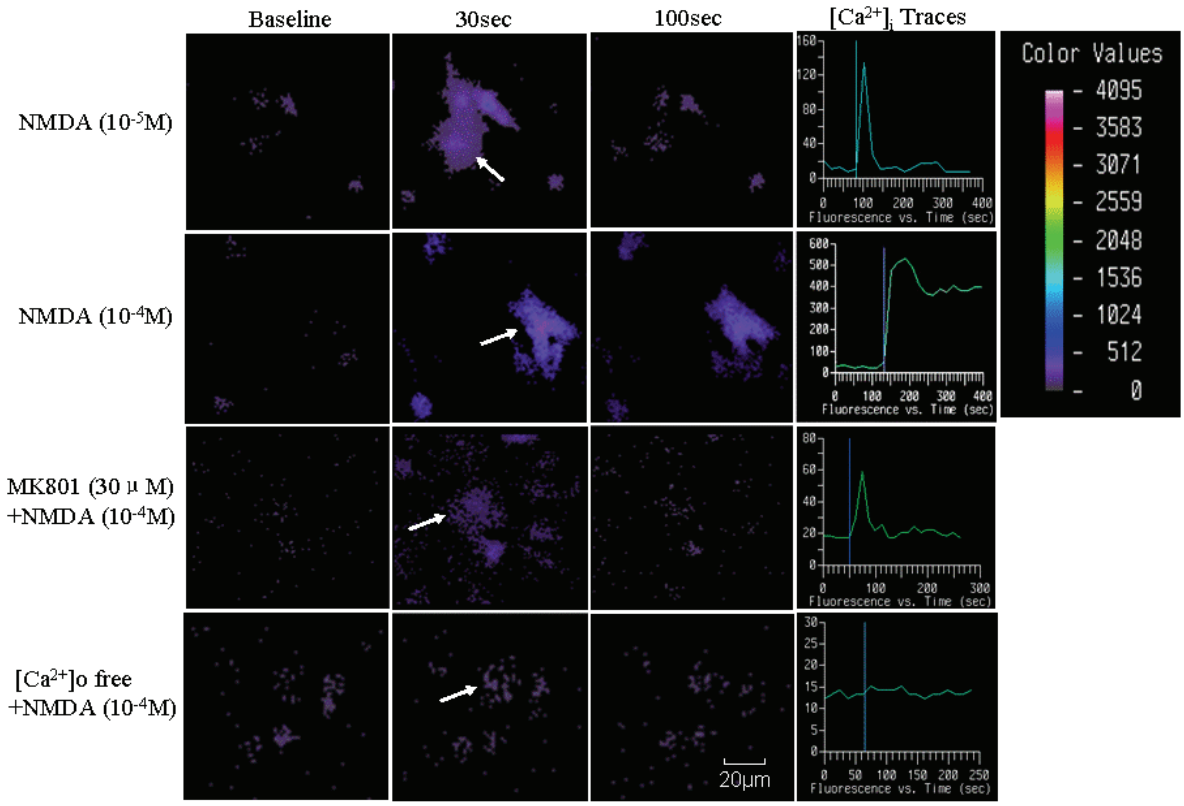

Fig. 1. NMDA-induced calcium mobilization in cultured neonatal rat cardiomyocytes. The three left columns are representative confocal fluo-3 fluorescent images at baseline, 30 s (roughly the peak response), and 100 s after NMDA exposure, respectively. The right column shows the respective $[Ca^{2+}]_i$ dynamic traces of the single cells marked by the white arrows. The blue straight lines mark the injection of NMDA into the sample chamber.

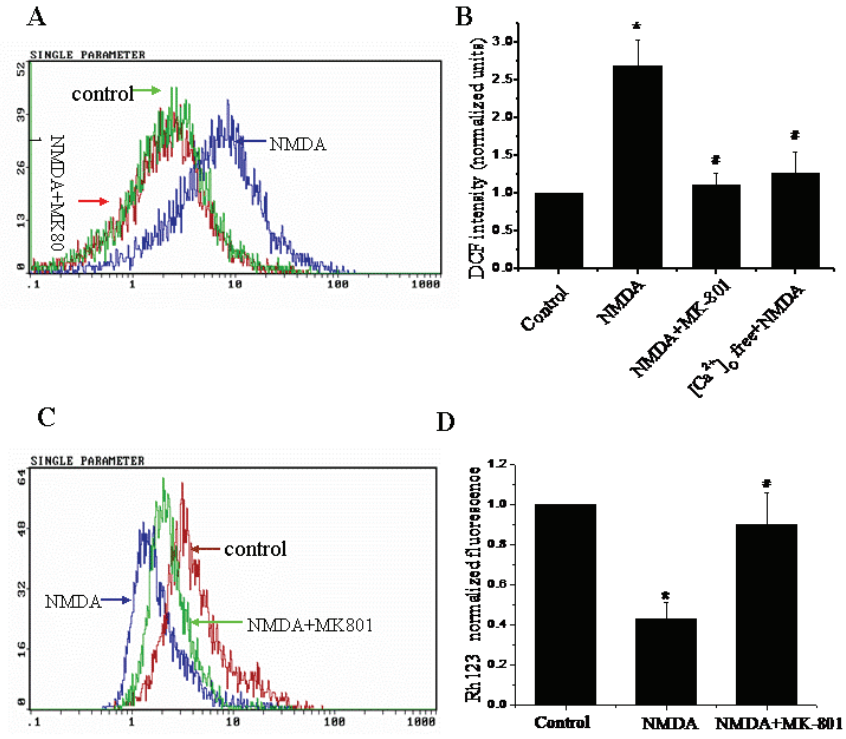

Figure 2. A: the original representative tracings showing the DCF fluorescence intensities. A rightward shift indicates an increase in ROS generation. Green, control. Blue, NMDA (10^{-4} M). Red, MK-801(30 μM) + NMDA (10^{-4} M). **B:** statistical data (normalized units) showing the differences in the DCF fluorescence intensities among treatments (n=6 for each treatment). **P*<0.05 *vs* control, # *P*<0.05 *vs* NMDA. **C**: the original tracings showing the Rh123 fluorescence intensities. A leftward shift of the traces indicates a decrease in Rh123 fluorescence intensity and therefore a depolarization of ΔΨm. The tracings show that cardiomyocytes displayed a prominent depolarization of ΔΨm after challenge with 10^{-4} M NMDA. Pretreatment with 30μM MK-801 partially restored the ΔΨm. **D**: statistical histograms (normalized fluorescence intensity) (n=6 for each treatment). **P*<0.05 *vs* control. # *P*<0.05 *vs* NMDA.

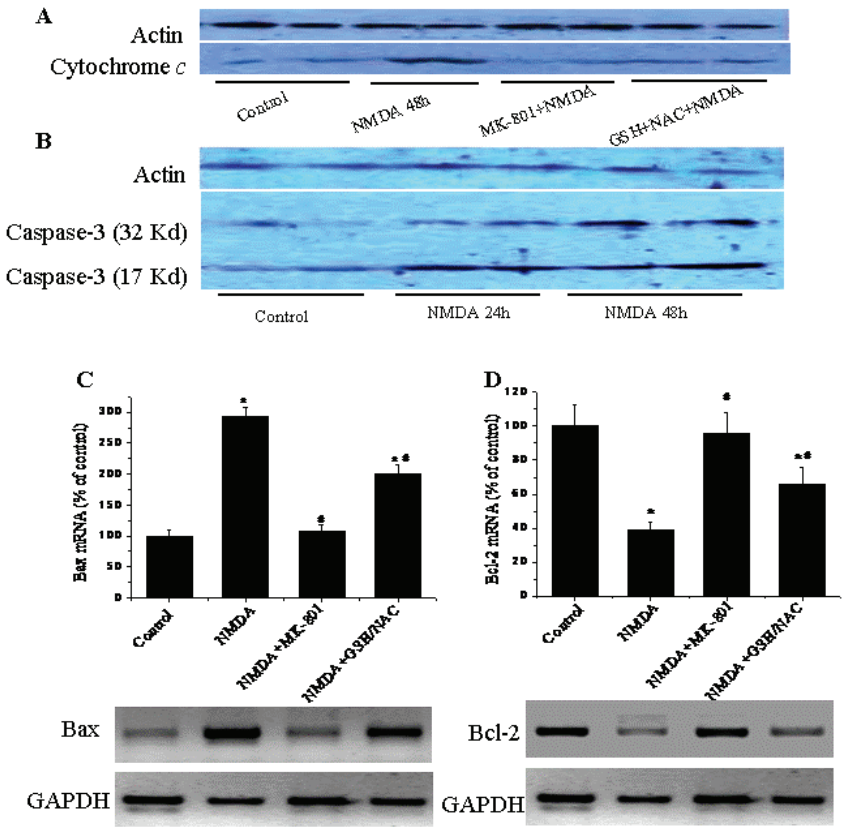

Fig. 3. A. Western blot analyses indicated the effects of NMDA on the cytosolic levels of cytochrome *c* and activated caspase-3 in cultured cardiomyocytes. NMDA pretreatment markedly increased the cytosolic levels of cytochrome *c*. Pretreatment with MK-801 or GSH/NAC inhibited the increase in cytosolic level of cytochrome *c* induced by NMDA. **B**. Western blot analyses indicated 17 kD caspase-3 protein expression in NMDA treated cells after 24 and 48 h were dramatically increased compared with control cells. **C** and **D**. Semiquantitative RT-PCR analyses showing the effects of NMDA on the mRNA levels of bax (C) and bcl-2 (D) in cultured neonatal rat cardiomyocytes. * P<0.05 *vs* control cells. # P<0.05 *vs* NMDA-treated cells (n=5 for each treatment). The GADPH mRNA level was used as an inner control.

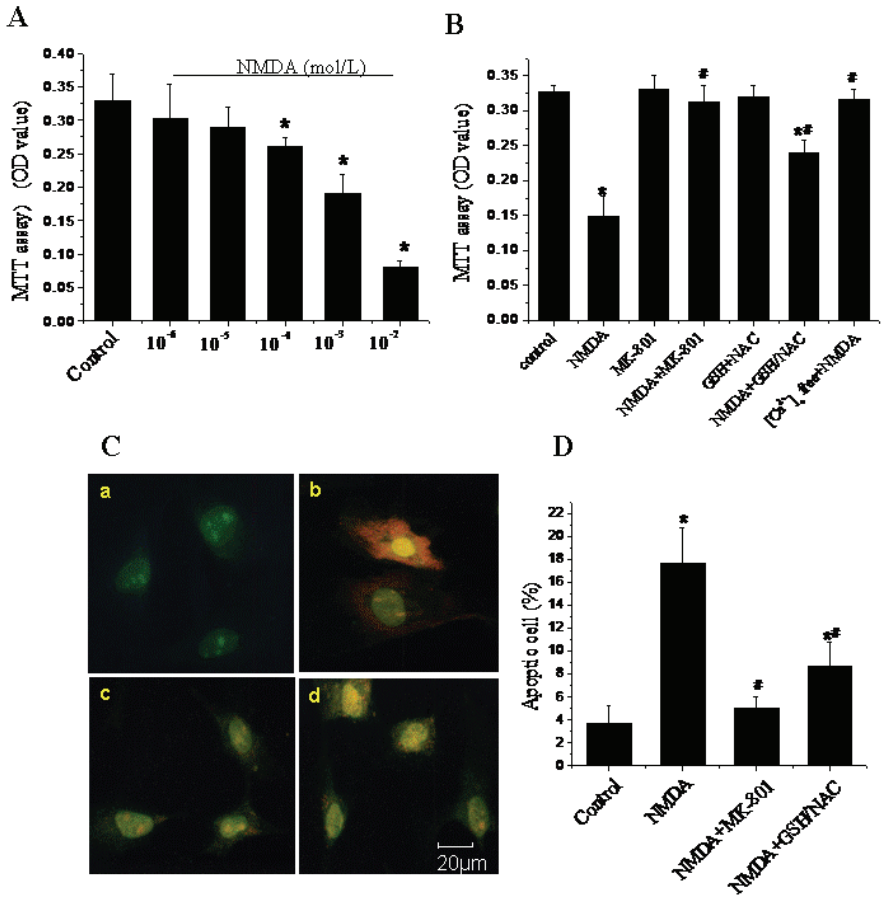

Fig. 4. A. NMDA dose-dependently decreased the cell viability. **B.** Cells treated with MK-801 (30 μM), GSH (10 mM)/NAC (5 mM) or deprivation of extracellular free calcium significantly restored the cell viability. Note that MK-801, GSH and NAC themselves did not affect the cell viability. * P<0.05 *vs* control. # P<0.05 *vs* NMDA. **C.** Representative acridine orange (AO) staining pictures. a, normal control cells, no apoptotic features was observed, b, NMDA (10^{-4} M) treatment, cells became retracted and had condensed cytoplasm or fragmented nuclei, c and d, NMDA+MK-801 and NMDA+GSH/NAC treatment, respectively, the apoptotic features of the cells were obviously reduced. Original magnification: 40×. **D.** the percentage of apoptotic cells in different groups. *P<0.05 *vs* control, # P<0.05 *vs* NMDA.

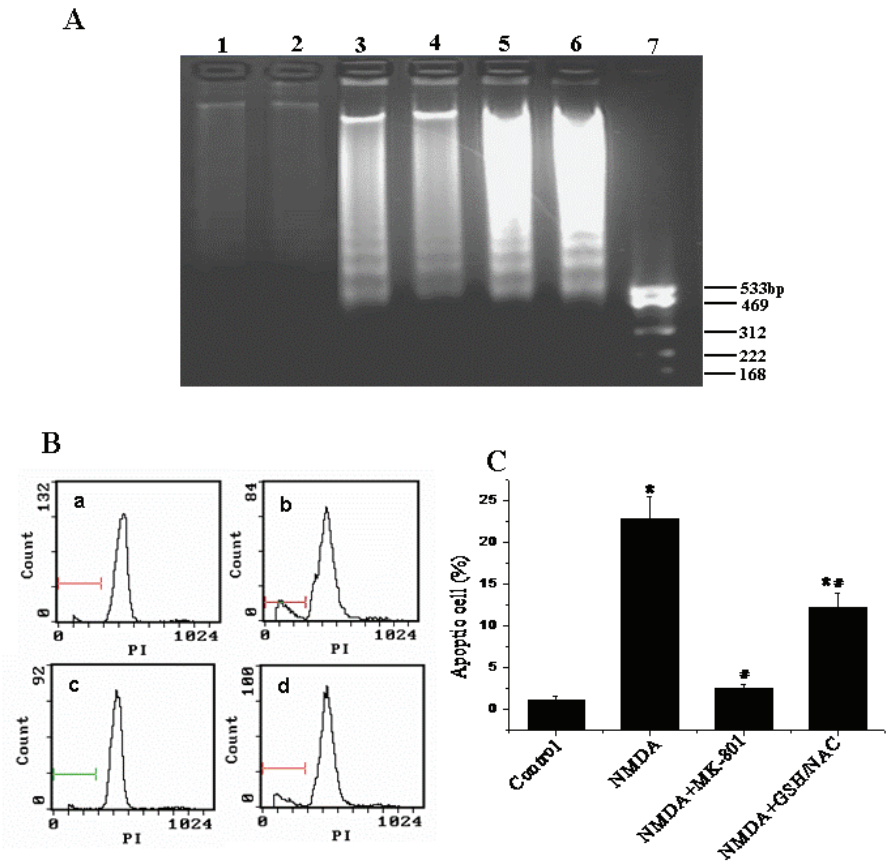

Fig. 5. A. Electrophoresis of genomic DNA showing the NMDA-induced DNA fragmentation in cultured cardiomyocytes. *Lane 1 and 2*: normal control cells. *Lane 3 and 4*: cardiocytes incubated with NMDA 10^{-4} M for 24 h. *Lane 5 and 6*: cardiocytes incubated with NMDA 10^{-4} M for 48 h. *Lane 7*: DNA marker. Note that cardiocytes exposed to 10^{-4} M NMDA for 24 h and 48 h showed a distinct DNA laddering characteristic of apoptosis compared with normal cells. **B** and **C.** Flow cytometric analysis of hypodiploid DNA contents in different groups. Cells undergoing apoptosis exhibited a greater peak in the hypodiploid region. Panel B shows the original recordings. a: control group. b: 10^{-4} M NMDA. c: 10^{-4} M NMDA+30 μM MK-801. d: 10^{-4} M NMDA+GSH/NAC. Panel C shows the percentages of cells with hypodiploid DNA, they were 1.11 ±0.44 %, 22.8±2.65 %, 2.57±0.37 % and 12.16±1.81 % in control cells, cells treated with NMDA, MK-801+NMDA and NMDA+GSH/NAC, respectively. Data are depicted from 3-6 experiments in each group, each performed in duplicate. **P*<0.05 *vs* control, # *P*<0.05 *vs* NMDA.

Discussion

This study provides the first evidence that NMDA-R overstimulation induces Ca^{2+} overload, ROS generation, ΔΨm depolarization, mitochondrial cytochrome *c* release, caspase-3 activation, alteration of bcl-2/bax mRNA expression and apoptosis in cultured cardiomyocytes. These cytotoxic effects of NMDA could be abolished by NMDA-R blockade, and by free radical scavengers, suggesting that Ca^{2+} overload, ROS generation and mitochondrial dysfunction are involved in the signaling cascade of NMDA-R overstimulation-induced cardiocyte apoptotic death.

Possible signaling pathways by which NMDA-R induces cardiomyocyte apoptosis

Although excitotoxicity initiated by overstimulation of NMDA-R has been associated with excessive Ca^{2+} influx and Ca^{2+} overload, the subsequent biochemical events leading to cell death are mostly understood in neuronal cell lines. Little is known about peripheral cells. In the present study, we found that NMDA induced an increase of $[Ca^{2+}]_i$ in cardiomyocytes. This result is compatible with a previous observation that NMDA-R stimulation leads to an increase in intracellular Ca^{2+} oscillation frequency in rat cardiomyocyte (Winter and Baker 1995).

The maintenance of intracellular Ca^{2+} homeostasis is crucial for cell survival, and its disruption may be involved in many pathological disorders. Increasing of intracellular free calcium has been shown to affect ΔΨm and induce apoptosis in neurons. Increased $[Ca^{2+}]_i$ can signal a variety of second messenger systems that play a role in cell death. On the other hand, intracellular Ca^{2+} overload can lead to the excessive uptake of Ca^{2+} into mitochondria. Mitochondrial Ca^{2+} overloading may activate cell death by mechanisms such as release of pro-apoptotic factors and increased generation of ROS. It is thought that Ca^{2+} loading by the mitochondria beyond its buffering capacity reduces the ΔΨm and disrupts electron transport, resulting in an increased production of the reactive free radical

superoxide anion (O_2^-) in neurons (Luetjens *et al.* 2000). ROS generation and high intramitochondrial Ca^{2+} may act together to trigger mitochondrial permeable transition pore (PTP) opening. Mitochondrial PTP opening results in disruption of the outer membrane and release of different apoptogenic factors into the cytosol (Kroemer *et al.* 1998). These factors, which include cytochrome *c* and apoptosis inducing factor (AIF), then evoke subsequent activation of caspases (Zoratti and Szabo 1995), which promote the execution of apoptosis. These phenomena are presumed to occur in cardiomyocytes but remain unproven. We demonstrated that NMDA-R activation induced Ca^{2+} mobilization and Ca^{2+} overload, $\Delta\Psi m$ depolarization, ROS generation and apoptosis in cardiomyocytes. Calcium mobilization may act as the key initial signal in the induction of cell toxicity mediated by NMDA-R, because deprivation of extracellular free Ca^{2+} totally abolished the cell toxicity induced by NMDA.

The generation of ROS is one of the common responses to injuries and contributes to cellular damage and apoptotic death (Fiskum 2000, Yuan and Yankner, 2000). ROS generation has been documented to be involved in activation of both NMDA receptors and non-NMDA excitatory amino acid receptors in some neuronal cells (McInnis *et al.* 2002, Boldyrev *et al.* 2003, Li *et al.* 2004). Whether NMDA-R activation induces ROS generation in cardiomyocytes is not exactly known. In the present study, we found that NMDA-R oversti-mulation significantly increased the cytosolic level of ROS in cultured cardiomyocytes *via* a Ca^{2+} influx-dependent signaling pathway, because deprivation of extracellular Ca^{2+} prevented the production of ROS.

In order to determine whether apoptosis is an important mechanism for the cardiomyocyte death induced by NMDA as shown by the MTT assay in the present study, we used three independent assays to assess cardiomyocytes apoptosis, including acridine orange staining, DNA laddering and flow cytometry. All assays showed that cardiomyocytes stimulated with NMDA revealed features of apoptosis. The induction of apoptosis by NMDA-R activation has been found in a variety of neuronal cells (Inomata *et al.* 2003, Takai *et al.* 2003, Jing *et al.* 2004). We demonstrated for the first time that NMDA-R overactivation induced cardiocyte apoptotic death.

NMDA-R and mitochondrial function

Mitochondrion plays a crucial role in the development of apoptosis by releasing several apoptotic factors, such as cytochrome *c*. Cytochrome *c* release from mitochondria is a critical step in the cell apoptotic process. Cytochrome *c* normally localizes to the mitochondria and can diffusely distribute in the cytoplasm in response to some stress stimuli such as NMDA-R overactivation. Cytosol cytochrome *c* can initiate the caspase cascade (Nunez *et al.* 1998). Measurements of mitochondrial membrane potential (MMP) tell us more about the role of mitochondria in normal cell function and in processes leading to cell death. The maintenance of mitochondrial membrane potential is also important for the normal function of cardiac myocytes (Škárka and Ošťádal 2002). The present study demonstrated that NMDA induced a depolarization of the $\Delta\Psi m$ and increased cytochrome *c* release to cytosol. MK-801 and GSH/NAC inhibited cytochrome *c* release. Although the mechanism underlying cytochrome *c* release is still controversial, the involvement of Bcl-2 protein family in regulating cytochrome *c* release is well recognized (Finucane *et al.* 1999). Bcl-2 protein is antiapototic and is localized in the mitochondria, endoplasmic reticulum, and nuclear membranes where most of the ROS are generated to exert their apoptotic effects. Overexpression of Bcl-2 protein family such as Bcl-2 and Bcl-X_L prevents mitochodrial cytochrome *c* release after exposure to cytotoxic stimuli (Kluck *et al.* 1997). Bax, a pro-apoptotic member of the Bcl-2 family, can potentiate the mitochondrial release of cyto-*c* (Finucane *et al.* 1999). We observed here that NMDA downregulated the bcl-2 mRNA expression and upregulated the bax mRNA expression, leading to a marked reduction of bcl-2/bax ratio in favor of cell apoptosis. This indicates that bax and bcl-2 play important roles in cardiomyocytes apoptosis induced by NMDA. MK-801 and GSH/NAC could modify bcl-2/bax ratio, suggesting that blockade of NMDA-R or antioxidants may potentially prevent the apoptosis induced by glutamate. In addition, our study demonstrated that the inactive precursor and the active cleaved 17 Kd active subunits of caspase-3 proteases increased dramatically after incubation with NMDA, suggesting the involvement of caspase-3 in the induction of cardiomyocytes apoptosis by NMDA. Caspases play a central role in NMDA-mediated apoptosis of neurons. Individual members of the caspase family may be transiently activated and affect different stages of the signaling cascade leading to apoptosis. We show here for the first time that NMDA induced caspase-3 activation in cardiomyocytes, implicating that caspase-3-like enzymes

are involved with NMDA-induced cardiomyocytes apoptosis.

How does glutamate affect the heart?

Although some clinical trials show that glutamate monosodium as a food additive is basically safe, we cannot neglect a possibility that glutamate may be a neurotransmitter of sympathetic nerve (Zhou and Misler 1995). Therefore, it is highly probable that glutamate might induce adverse cardiac effect *via* iGluR not through a circulation but through a sympathetic nervous system. Serum concentration of glutamate was significantly higher in schizophrenic group compared to controls (598.8±574.5 vs. 196.2±171.3 nmol/ml) (Morshed *et al.* 2005) and the concentration of circulating glutamate may increase after a meal which contained larger dose of glutamate. Subjects with cardiac disease might have a higher sensitivity to glutamate because our unpublished data indicated that NMDA-R expression was upregulated in rats after myocardial infarction. Therefore, both circulating glutamate and the glutamate in the synaptic gaps of sympathetic nerve may affect the heart. Patients with cardiac diseases may have a higher sensitivity to glutamate.

In summary, the apoptosis induced by NMDA in cardiomyocytes can be visualized as a cascade of events that begins with overstimulation of NMDA receptors and excessive Ca^{2+} influx. This influx leads to mitochodrial Ca^{2+} overload, depolarization of $\Delta\Psi m$ and ROS generation. Mitochondrial dysfunction may lead to a release of numerous apoptosis factors into cytosol and cause the activation of downstream effector of caspase-3, resulting in cardiomyocytes apoptosis. These findings may provide further insight into a novel therapeutic target for heart disease associated with NMDA receptor activation.

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