J Huazhong Univ Sci Technol［Med Sci］
DOI 10.1007/s11596-008-0402-4 28(4):379-383,2008

Nerve Growth Factor Inhibits Gd^{3+}-sensitive Calcium Influx and Reduces Chemical Anoxic Neuronal Death*

Hui JIANG (江 慧), Shunlian TIAN (田舜莲), Yan ZENG (曾 艳), Jing SHI (施 静)#
Department of Neurobiology and Key Laboratory of Neurological Diseases of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Summary：To investigate whether glutamate and voltage-gated calcium channels-independent calcium influx exists during acute anoxic neuronal damage and its possible relationship to neuronal protective function of NGF. In *in vitro* model of acute anoxia, hippocampal cultures from newborn rats were exposed to 3 mmol/L KCN. Changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored by con-focal imaging and cell viability was assayed by PI and cFDA staining. The results showed that after treatment with primary hippocampal cultures with 3 mmol/L KCN for 15 min, $[Ca^{2+}]_i$ was significantly increased 6.27-fold compared to pre-anoxia level and 73.3% of the cells died. When combination of 20 μmol/L MK-801 (glutamate receptor antagonist), 40 μmol/L CNQX (AMPA receptor antagonist) and 5 μmol/L nimodipine (voltage-gated calcium channel antagonist) (hereafter denoted as MCN) were administrated to hippocampal cultures, levels of $[Ca^{2+}]_i$ and cell death rate induced by KCN were partially reduced by 35.9% and 47.5% respectively. However, Gd^{3+} (10 μmol/L) almost completely blocked KCN-mediated $[Ca^{2+}]_i$ elevation by 81.9% and reduced neuronal death by 88.8% in the presence of MCN. It is noteworthy that NGF, used in combination with MCN, inhibited KCN-induced $[Ca^{2+}]_i$ increase by 77.4% and reduced cell death by 87.1%. Only PLC inhibitor U73122 (10 μmol/L) abolished NGF effects. It is concluded that Gd^{3+}-sensitive calcium influx, which is NMDA (glutamate receptor) and voltage-gated calcium channels-independent, is responsible for acute anoxic neuronal death. NGF can inhibit Gd^{3+}-sensitive calcium influx and reduce anoxic neuronal death through activating PLC pathway.
Key words：nerve growth factor; chemical anoxia; protection

Intracellular calcium overload plays a critical role in ischemic neuronal injury[1]. Numerous previous studies showed that excitatory amino acids, such as glutamate, released by neurons during cerebral ischemia, acting on membrane receptor channels is a major route that contribute to a great deal of calcium influx and ultimate neuronal death. It is so-called excitotoxicity[2-6]. A recent research revealed that a Gd^{3+}-sensitive calcium influx plays an important role in neuronal calcium overload in the late stage of oxygen-glucose deprive (OGD) insult[7]. Given that anti-excitotoxic therapy was clinically ineffective[8-10], it is worthwhile to study if Gd^{3+}-sensitive calcium influx is involved in acute anoxia.

Nerve growth factor (NGF) is a well documented neuronal protector[11-13]. Although much attention has been paid to the protective role of NGF in cerebral ischemic damage for a long time, the inherent mechanism remains largely unknown and is worthy to explore. Studies have revealed that NGF can stabilize intracellular calcium level and promote neuronal survival[14, 15], but specific pathway is unclear.

In this study, using KCN as anoxic inducer and primary hippocampal cultures from neonate rat as target, to establish an *in vitro* model of anoxia, we explored whether Gd^{3+}-sensitive calcium influx exists during acute anoxic neuronal damage and its possible relationship to neuronal protective function of NGF.

1 MATERIALS AND METHODS

1.1 Experimental Animals

Postnatal Sprague-Dawley rats within 24 h after birth were supplied by the Center of Experimental Animal, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

1.2 Major Reagents and Drugs

DMEM high-glucose and 0.25% trypsin were from Hyclone, USA. Neurobasal and L-glutamine were products of Gibco, USA. MK-801, CNQX, nimodipine, $GdCl_3$ were made by Sigma, USA. NGF (2.5S) was produced by Promega, USA. U73122 and Sos-inhibitory-peptide were manufactured by Upstate, USA. Wortmannin was from Alexis, USA. cFDA and PI were products of Molecular Probes, USA. The other reagents were made in China and were of analytic purity.

1.3 Primary Hippocampal Cultures

Primary culture of hippocampal neurons was prepared from postnatal Sprague-Dawley rats within 24 h according to procedure described previously[16] with minor modification. In brief, hippocampi were dissected in

Hui JIANG, female, born in 1978, Doctoral Candidate
E-mail: rosehui1218@yahoo.com.cn
#Corresponding author
*This work was supported in part by a grant of the National Natural Science Foundation of China (No. 30472234).

DMEM high-glucose and digested in 0.25% trypsin at 37°C for 15 min. Then hippocampus was triturated by a fire-polished Pasteur pipette and filtered through a Falcon cell strainer. Cells were plated at a density of 2×10^6 cells/mL onto poly-L-lysine coated dishes and maintained in DMEM high-glucose supplemented with 10% FBS. After 24 h, medium was completely replaced with maintenance medium containing 97% neurobasal, 2% B27, and 1% L-glutamine and was subsequently given half-changes every 3 days. Cultures were used for experiments at the 10th–14th day.

1.4 Experimental Groups

1.4.1 Anoxia Groups KCN group was treated with 3 mmol/L KCN for 15 min. KCN+MCN group was pre-incubated with 20 μmol/L MK-801, 40 μmol/L CNQX and 5 μmol/L nimodipine for 10 min, then treated with 3 mmol/L KCN for 15 min. KCN+MCN+Gd^{3+} group was pre-incubated with 20 μmol/L MK-801, 40 μmol/L CNQX, 5 μmol/L nimodipine and 10 μmol/L Gd^{3+} for 10 min, and then treated with 3 mmol/L KCN for 15 min). KCN+MCN+NGF group was pre-incubated 20 μmol/L MK-801, 40 μmol/L CNQX, 5 μmol/L Nimodipine and 100 ng/mL NGF for 10 min, and then treated with 3 mmol/L KCN for 15 min.

1.4.2 Inhibitor Treatment Groups NGF group was pre-incubated 100 ng/mL NGF for 10 min, and then treated with 3 mmol/L KCN for 15 min. NGF+U73122 group was first pre-incubated with 100 ng/mL NGF and 10 μmol/L U73122 for 10 min, and then treated with 3 mmol/L KCN for 15 min. NGF+wortmannin group was pre-incubated with 100 ng/mL NGF and 20 nmol/L wortmannin for 10 min, and then treated with 3 mmol/L KCN for 15 min. NGF+Sos-inhibitory-peptide group was pre-incubated with 100 ng/mL NGF and 10 μmol/L Sos-inhibitory-peptid for 10 min, and then treated with 3 mmol/L KCN for 15 min.

1.5 Measurements of Intracellular Calcium

Primary hippocampal cultures were incubated with Fluo-3 AM (10 μmol/L) in the dark for 2 h at room temperature and rinsed with PBS 3 times. Fluorescence intensity of Fluo-3 AM, representing intercellular calcium concentration ($[Ca^{2+}]_i$), was monitored by laser con-focal microscopy system. The curve of fluorescence intensity was obtained by evaluating fluorescence from three cells in three different areas. Florescence intensity before treatment (F0) was set as reference values, $[Ca^{2+}]_i$ was expressed as a ratio of real time fluorescence (F) to F0. Con-focal images were recorded every 20 s for 15 min.

1.6 Cell Viability and Death

Cell viability and death were assessed with amine-reactive probe (5, 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA) and propidium iodide (PI) staining. After 15-min treatment of KCN, cultures were maintained in fresh medium containing cFDA (5 μg/mL) and PI (5 μg/mL) at 37°C for 15 min and then rinsed with PBS 3 times. For each of the 5 cultures from each condition, cells were analyzed under con-focal microscope and 5 randomly selected fields were examined.

1.7 Statistical Analysis

All values were expressed as $\bar{x}\pm s$, and statistical significance was determined by one-way analysis of variance combined with SNK test using SPSS12.0 software package. A $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Inhibition of Gd^{3+}-sensitive Calcium Influx and Reduction of Chemical Anoxic Neuronal Death by NGF

As shown in fig. 1, after treatment with KCN, $[Ca^{2+}]_i$ in primary hippocampal cultures was rapidly increased within 3 min and reached a peak after 15 min, where $[Ca^{2+}]_i$ was 6.27-times pre-anoxia level and cell death rate was 73.3%. Pre-incubation with MCN not only delayed elevation of $[Ca^{2+}]_i$ but also decreased $[Ca^{2+}]_i$ level induced by KCN. Nevertheless, $[Ca^{2+}]_i$ was still at a high level 15 min after exposure to KCN. MCN only partially reduced $[Ca^{2+}]_i$ by 35.9% and cell death by 47.5%. Gd^{3+}, applied with MCN, further decreased KCN-mediated $[Ca^{2+}]_i$ elevation and neuronal death rate by 81.9% and 88.8% respectively, a level that was not different from that of normal neurons. It indicated that non-NMDA, non-voltage-gated calcium channels-dependent and Gd^{3+}-sensitive calcium influx was implicated in KCN-mediated acute anoxia. In addition, it might participate in anoxic neuronal damage. It's worthy noting that, similar to Gd^{3+}, NGF significantly attenuated MCN-independent and Gd^{3+}-sensitive calcium influx. It inhibited KCN-induced $[Ca^{2+}]_i$ increase by 77.4% and reduced cell death by 87.1%, suggesting that NGF may promote anoxic neuronal survival by inhibiting Gd^{3+}-sensitive calcium influx.

2.2 Role of PLC Pathway in the Effect of NGF

Furthermore, wortmannin, sos-inhibitory-peptide and U73122, the inhibitors of PI3-K, MEK and PLC pathways respectively, were separately used to investigate which signaling pathway(s) were responsible for the effect of NGF. It showed that $[Ca^{2+}]_i$ and neuronal death in U73122+NGF group was significantly higher than NGF group. In contrast, treatment with wortmannin or sos-inhibitory-peptide did not change $[Ca^{2+}]_i$ and neuronal death in the presence of NGF (fig. 2), indicating that NGF inhibited Gd^{3+}-sensitive calcium influx and reduced acute anoxic neuronal death through PLC pathway.

3 DISCUSSION

In 2003, Arts *et al* reported that MCN eliminated calcium influx of cortical neurons exposed to NaCN (3 mmol/L) for 1 h. However, MCN failed to block calcium influx triggered by prolonged treatment with NaCN (for over 1.5 h), which unmasked a dominant death mechanism—Gd^{3+}-sensitive calcium influx. Their further study confirmed that this Gd^{3+}-sensitive calcium influx was mediated by TRPM7, a newly found member of the transient receptor potential cation channel superfamily[17,18]. TRPM7 plays an important role in prolonged (up to 2 h) periods of OGD challenge[7,19]. In the present study, we reproduced the cyanide-induced anoxic model used by Arts *et al*. Our results revealed that 15-min exposure to 3 mmol/L KCN was sufficient to trigger Gd^{3+}-sensitive calcium influx in hippocampal neurons and resulted in neuronal death, suggesting TRPM7 may be involved in acute anoxic neuronal death. Our finding seems to be

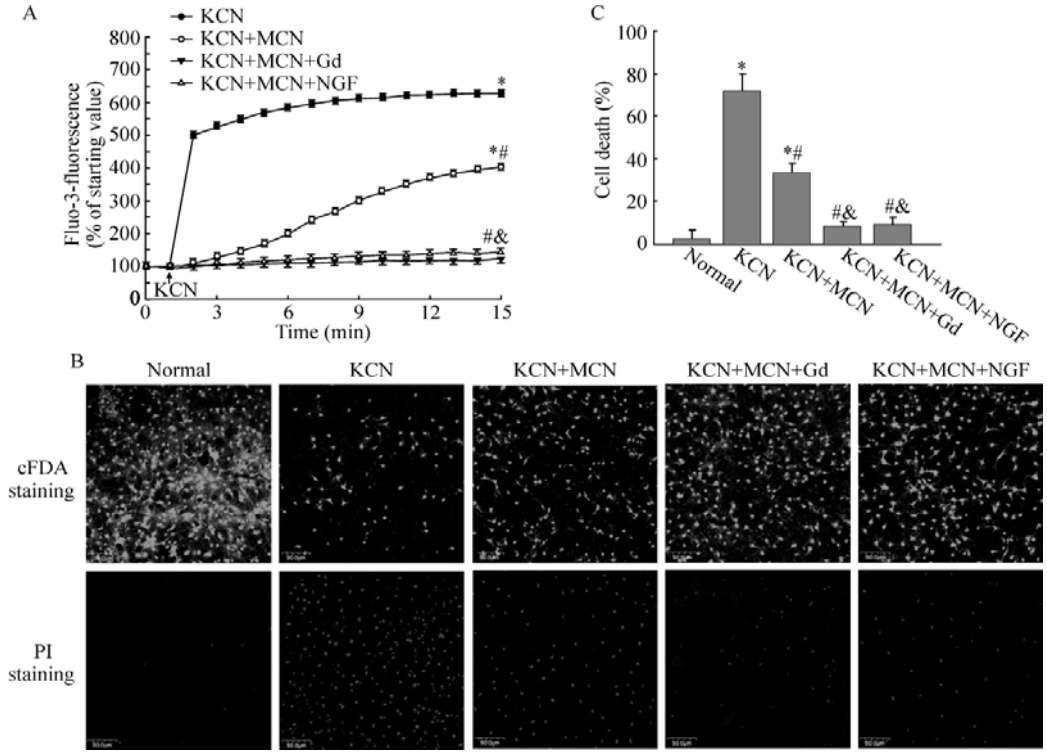

Fig. 1 NGF inhibited Gd^{3+}-sensitive calcium influx and reduced acute anoxic neuronal death.

A: Changes of intracellular calcium concentration with time in each group; B: cFDA and PI staining in each group; C: Cell death rate in each group. *P<0.05 compared with normal; $^{\#}P$<0.05 compared with KCN; $^{\&}P$<0.05 compared with KCN+MCN.

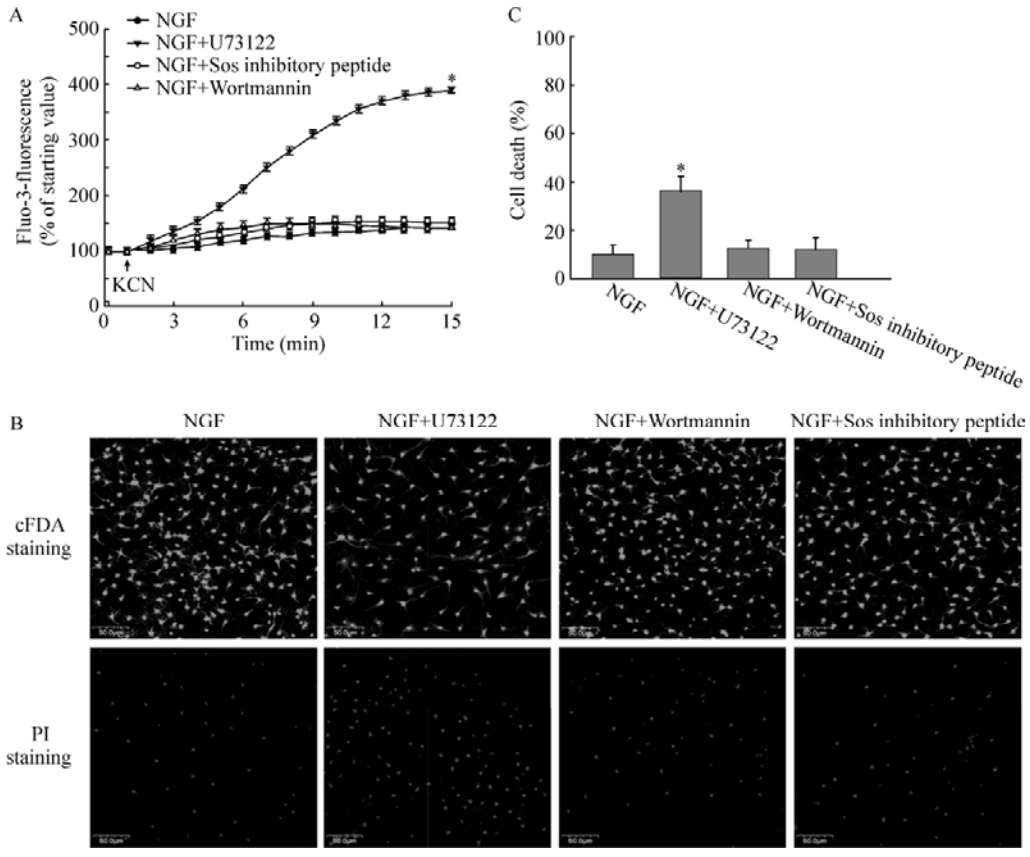

Fig. 2 PLC pathway was responsible for the role of NGF.

A: Changes of intracellular calcium concentration with time in each group; B: cFDA and PI staining in each group; C: Cell death rate in each group. $^{*}P$<0.05 compared with NGF.

inconsistent with findings of Arts *et al*. However, given two different neuronal types used in these two studies, the discrepancy is understandable. Primary hippocampal cultures from neonate rats were used in the present study while Arts *et al* employed primary cortical cultures from embryonic mouse. Firstly, hippocampal neurons are more vulnerable to anoxia than cortical neurons. Secondly, cultured embryonic neurons are relative insensitive to ischemia compared to cultured postnatal neurons. Therefore, different damage mechanism may be involved. Recently, using hippocampal slices from P18 to P42 rats as target, Lipski *et al* found that TRPM7 participated in neuronal calcium influx, cell membrane depolarization and cell swelling[20] at the early stage of OGD, which is consistent with our findings.

NGF, the first identified neurotrophic factor, supports the survival and maintenance of many neuronal populations of central and peripheral nervous system[21,22]. The protective effects of NGF are mediated by binding to its high affinity receptors TrkA and subsequently, activating appropriate signal transduction pathways[23]. Once activated by NGF, TrkA stimulates three main signal transduction pathways: phosphatidyl-inositol 3-kinase (PI-3K), ras/mitogen-activated protein kinase (MAPK), and phospholipase C-γ (PLC-γ) pathways[24]. Among them, the PI-3K/Akt and MAPK/ERK pathways have been reported to be implicated in the regulation of the gene expression of apoptotic and anti-apoptotic molecules against ischemic insults[25]. In this study, we found that NGF also protected acute anoxic neurons exposed to moderate dosage of KCN, although the regulation of gene expression did not happen. Our results showed that NGF inhibited Gd^{3+}-sensitive calcium influx and reduced neuronal death through PLC pathway. Two recent studies revealed that TrkA and TRPM7 co-existed in hippocampal neuronal membrane and that NGF inhibited TRPM7 inward current in hippocampal neuron through PLC pathway. On the basis of all evidence mentioned above and the fact that TRPM7 is the mediator of Gd^{3+}-sensitive calcium influx[7], we are led to conclude that NGF inhibits TRPM7-mediated calcium influx and protects acute anoxic neurons through activating PLC pathway.

REFERENCES

1 Lipton P. Ischemic cell death in brain neurons. Physiol Rev, 1999,79:1431-1568
2 Goldberg M P, Weiss J H, Pham P C *et al*. N-methyl-D-aspartate receptors mediate hypoxic neuronal injury in cortical culture. J Pharmacol Exp Ther, 1987, 243:784-791
3 Choi D W, Maulucci-Gedde M, Kriegstein A R. Glutamate neurotoxicity in cortical cell culture. J Neurosci, 1987,7: 357-368
4 Sattler R, Charlton M P, Hafner M *et al*. Distinct influx pathways, not calcium load, determine neuronal vulnerability to calcium neurotoxicity. J. Neurochem. 1998,71: 2349-2364
5 Sattler R, Xiong Z, Lu W Y *et al*. Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science, 1999,284:1845-1848
6 Aarts M, Liu Y, Liu L *et al*. Treatment of ischemic brain damage by perturbing NMDA receptor-PSD-95 protein interactions. Science, 2002,298:846-850
7 Arts M, Iihara K, Wei W L *et al*. A key role for TRPM7 channels in anoxic neuronal death. Cell, 2003,115:863-77
8 Davis S M, Albers G W, Diener H C *et al*. Termination of acute stroke studies involving selfotel treatment. ASSIST Steering Committed. Lancet, 1997,349(9044):32
9 Morris G F, Bullock R, Marshall S B *et al*. Failure of the competitive N-methyl- D-aspartate antagonist selfotel (CGS 19755) in the treatment of severe head injury: results of two phase III clinical trials. J Neurosurg, 1999,91: 737-743
10 Lees K R, Asplund K, Carolei A *et al*. Glycine antagonist (gavestinel) in neuroprotection (GAIN International) in patients with acute stroke: a randomised controlled trial. Lancet, 2000,355:1949-1954
11 Shigeno T, Mima T, Takakura K *et al*. Amelioration of delayed neuronal death in the hippocampus by nerve growth factor. J Neurosci, 1991,11:2914-2919
12 Lindvall O, Kokaia Z, Bengzon J *et al*. Neurotrophins and brain insults. Trends Neurosci, 1994,17:490-496
13 Semkova I, Krieglstein J. Neuroprotection mediated via neurotrophic factors and induction of neurotrophic factors [Review]. Brain Res Brain Res Rev, 1999,30:176-88
14 Cheng B, Mattson M P. NGF and bFGF protect rat hippocampal and human cortical neurons against hypoglycemic damage by stabilizing calcium homeostasis. Neuron, 1991, 7(6):1031-41
15 Mattson M P, Lovell M A, Furukawa K *et al*. Neurotrophic factors attenuate glutamate-induced accumulation of peroxides, elevation of intracellular Ca^{2+} concentration, and neurotoxicity and increase antioxidant enzyme activities in hippocampal neurons. J Neurochem, 1995,65:1740-1751
16 Sombati S, DeLorenzo R J. Recurrent spontaneous seizure activity in hippocampal neuronal networks in culture. J Neurophysiol, 1995,73:1706-1711
17 Nadler M J, Hermosura M C, Inabe K *et al*. LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability. Nature, 2001,411:590-595
18 Montell C, Birnbaumer L, Flockerzi V. The TRP channels, a remarkably functional family. Cell, 2002,108:595-598
19 Aarts M M, Tymianski M. TRPM7 and ischemic CNS injury. Neuroscientist, 2005,11:116-123
20 Lipski J, Park T I, Li D *et al*. Involvement of TRP-like channels in the acute ischemic response of hippocampal CA1 neurons in brain slices. Brain Res, 2006, 1077:187-99
21 Crowder J, Freeman R S. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci, 1998,18:2933-2943
22 Culmsee C, Gerling N, Lehmann M *et a*l. Nerve growth factor survival signaling in cultured hippocampal neurons is mediated through TrkA and requires the common neurotrophin receptor P75. Neuroscience, 2002,115:1089-108
23 Wang W, Post J I, Dow K E *et al*. Zinc and Copper inhibit nerve growth factor-mediated protection from oxidative stress-induced apoptosis. Neurosci Lett, 1999,259:115-118
24 Kaplan D R, Miller F D. Signal transduction by the neurotrophin receptors. Curr Opin Cell Biol, 1997,9:213-221
25 Saito A, Narasimhan P, Hayashi T *et al*. Neuroprotective Role of a Proline-Rich Akt Substrate in Apoptotic Neuronal Cell Death after Stroke: Relationships with Nerve Growth Factor. J Neurosci. 2004,24(7):1584-1593

(Received May 18, 2008)