Nerve growth factor prevents nitric oxide release and nitric oxide synthase gene expression induced by glutamate in primary cortical cultures of fetal rats

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Aim To study the effects of nerve growth factor (NGF) on nitric oxide (NO) release and constitutive nitric oxide synthase (cNOS) gene expression induced by glutamate (Glu) in cortical neuronal cultures. **Methods** Neuron viability was measured to assay NGF effect. NO content and the expression of cNOS mRNA were determined by fluorometric method and Northern blotting respectively. **Results** Glu (0.5~2.0 mmol/L) induced a marked increase of neuronal death and NO content in cultured cerebral cortical neurons. L-NAME (100 μmol/L) and NGF (100 μg/L) significantly increased neuronal surviving and decreased NO release. Hemolglobin (Hb), which binds NO, completely prevented Glu-induced cell death and NO release at 500 μmol/L. NGF (50, 100 μg/L) significantly inhibited cNOS mRNA expression induced by Glu (0.5 mmol/L). **Conclusion** NO mediates Glu neurotoxicity in cortical neuronal cultures and NGF protects the neurons against Glu neurotoxicity via inhibiting the activity of cNOS and the release of NO.

Key words nerve growth factor; cerebral cortex; cell; culture; glutamic acid; nitric oxide; nitric oxide synthase

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Nitric oxide (NO) has recently been recognized as an important neuromodulator and neurotransmitter in the central nervous system [1, 2]. But excessive NO release may lead to the meurotoxicity, and then neuronal death. N-methyl-D-aspartate (NMDA) causes release of NO from rat spinal cord in vitro and NO mediates glutamate neurotoxicity in primary cortical cultures [3,4]. Recent data indicated that nerve growth factor (NGF) prevents glutamate toxicity in hippocampal and cortical neuronal cultures [5, 6]. Up to now, the molecular mechanisms of NGF against glutamate neurotoxicity have not been elucidated. In the present study, rat primary cortical cultures were used to explore the relationship between NO and NGF against glutamate toxicity.

1 Materials and Methods

1.1 Reagents NGF, the purity >95%, was obtained from Chinese Academy of Military Medical Sciences. L-Glutamic acid (Glu), L-Arginine (L-Arg), NG-nitro-Larginine methyl ester (L-NAME) and hemoglobin (Hb) were purchased from Sigma Dulbecco's Modified

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Eagle Medium (DMEM) was a Gibco product. The probe of constitutive nitric oxide synthase (cNOS) cDNA was obtained from Beijing Medical University. α-^{32}P-dCTP (111×10^{6} GBq/mol) was purchased from Beijing Furei Radioactive Isotope Co.. ^{3}H Cu R (27×10^{-9}/Bq) was a product of Shanghai Radiomedicine Institute. All other reagents were of purity of AR.

1.2 Cell culture[7] Primary dissociated cell cultures were prepared from cerebral cortex of fetal rats (16~18 d gestation). The tissue was dissected, incubated in 0.125% trypsin for 15 min, and then transferred to DMEM containing 10% horse serum and 10% fetal bovine serum. The cortex was mechanically dissociated by gentle triturate 20~30 times with a polished pipette. The isolated brain cells were filtered through nylon (200 mesh, hole width 95 μm) and plated at a density of 1×10^{6}/ml in L-polylysine-coated 24-well plates or 250 ml bottles. Cells were maintained in growth medium consisting of 90% DMEM, 5% horse serum and 5% fetal bovine serum at 37℃ in 5% CO_2/ humidified atmosphere. The medium was changed twice weekly.

1.3 Experiments with glutamate and NGF Experiments were performed in 8~10 day-old cultures because previous studies showed that at this time in culture the neurons are vulnerable to Glu toxicity and can be protected against excitotoxic and metabolic insults by neurotrophic factors[8]. Before application of Glu, the cells were washed two times with Locke's solution without Mg^{2+} (NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, $CaCl_2$ 2.3, glucose 5.6, HEPES 5 mmol/L, pH 7.4), and then incubated with Glu for 30 min. L-NAME, Hb, or NGF were added prior to Glu in the Locke's solution and incubated at 37 ℃ for 24 h. After exposure to Glu, the medium was replaced by Locke's solution with 0.4% trypan blue, which stains nonviable cells. Two to four photographs (10~20×) were made of each well, and viable versus nonviable cells were counted.

1.4 Measurement of NO and cNOS NO content in culture supernatant was determined by spectrofluorometric method[9]. The cells were harested, total RNA was extracted with single-step method[10] and hybridized with cNOS cDNA probe by the method of Northern blot hybridization[11].

2 Results

2.1 Effects of NGF on glutamate neurotxicity Glu (0.5 mmol/L) decreased the number of surviving neurons to 31.3±4.2% (n=6 wells). L-NAME (100 μmol/L), a potent NOS inhibitor, inhibited the decreasing of the number of surviving neurons induced by Glu. Addition 1 mmol/L L-Arg to the exposure solution completely reversed the effect of L-NAME. Hb, which binds NO, Completely prevented Glu-induced cell death at 500 μmol/L. NGF 100 μg/L suppressed the death of cortical neurons induced by Glu, the action is similar to that of L-NAME (Tab 1).

Tab 1 Surviving neurons in primary cultures treated for 30 min with glutamate (0.5 mmol/L) and pretreated with NAME (100 μmol/L), Hb (500 μmol/L), or NGF (100 μg/L). NAME, Hb, or NGF was added 30 min before glutamate respertively (n=6 wells, $\bar{x}\pm s$)

Group	Surviving neurons (% of total)
Control	92.4±18.3
Glu (0.5 mmol/L)	31.3±4.2$^{\triangle\triangle}$
Glu+NAME (100 μmol/L)	85.2±16.4**
Glu+Hb (500 μmol/L)	90.7±19.5**
Glu+NAME+L-Arg (1 mmol/L)	27.5±3.7
Glu+NGF (100 μg/L)	84.6±18.5**

Compared with control $^{\triangle\triangle}P<0.01$. Compared with Glu group $^{**}P<0.01$

2.2 Effects of NGF on NO release induced by Glu Glu (0.5~2.0 mmol/L) stimulated the release of NO in cortical cultures by 314%, 528%, and 695% compared with Glu-free, respectively. Increase of NO content in cultures by Glu was antagonized by L-NAME. Hb (500 μmol/L) completely inhibited Glu-induced NO release. NGF 100 μg/L attenuated Glu (0.5~2.0 mmol/L)-induced release of NO (Fig 1).

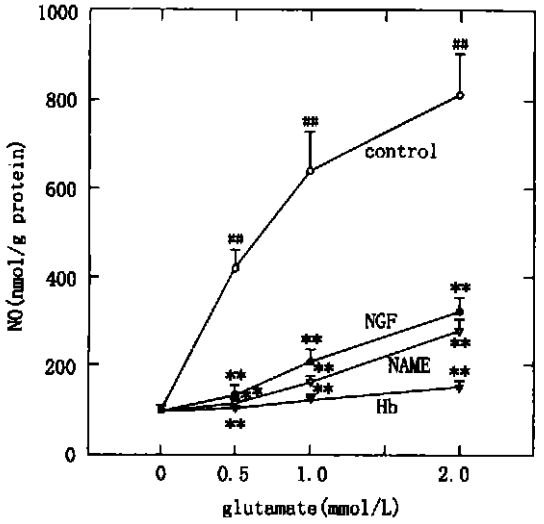

Fig 1 Effects of NGF (100 μg/L), NAME(100 μmol/L) and Hb (500 μmol/L) on release of NO induced by glutamate (0.5~2.0 mmol/L) in cerebral cortical neuronal cultures (n =6 wells, $\bar{x} \pm s$)

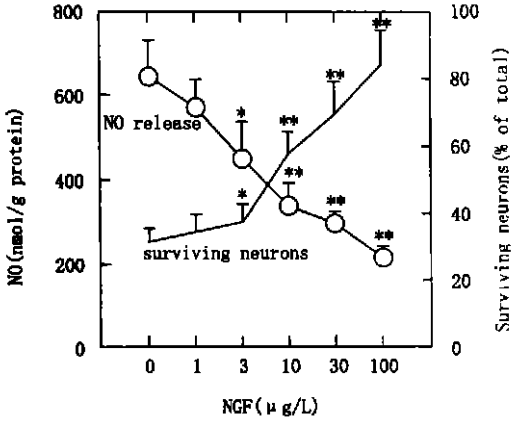

Fig 2 Effects of NGF (1~100 μg/L) against glutamate(0.5 mmol/L) neurotoxicity in cultured cortical neurons (n =6 wells, $\bar{x} \pm s$)

NGF (3~100 μg/L) dose-dependently attenuated Glu (0.5 mmol/L)-induced release of NO. All the four concentrations of NGF elevated the number of surviving neurons in a concentration-dependent manner (Fig 2).

2.3 Effects of NGF on cNOS mRNA levels induced by Glu Fig 3 showed that Glu 0.5 mmol/L stimulated cNos mRNA expression in cultured cerebral cortical neurons NGF (50, 100 μg/L) significantly inhibited this cNOS gene expression (Fig 3).

Fig 3 Effects of NGF (50, 100 μg/L) on cNOS mRNA expressions induced by glutamate (0.5 mmol/L) in cultured crebral cortical neurons. Total RNA of cells was extracted and hybridized with cDNA probe and undertaken autoradiograph for 10 days

A: control; B: glutamate (0.5 mmol/L); C and D: glutamate (0.5 mmol/L)+NGF (50, 100 μg/L)

3 Discussion

Glutamate may mediate the neurotoxicity in hypoxic-ischemic brain injury, selective antagonist of the NMDA subtype of glutamate receptor may prevent neuronal cell death in animal models of hypoxic-ischemic brain injury. Glutamate neurotoxicity has also been implicated in neurodegenerative disorders such as Alzheimer and Huntington diseases. The present results showed that glutamate stimulated the release of NO, indication that NO mediates glutamate neurotoxicity in neuronal cultures. L-NAME, an inhibitor of NOS, and Hb, which binds NO, prevent glutamate mediated cell death.

Excessive glutamate release may lead to the production of NO through the activation of the enzyme NOS. The previous data demonstrated that the constituent calcium-dependent NOS (cNOS) is mainly present in cortex[12,13]. Our results support this view that glutamate stimulated cNOS gene expression in cultured cortical neurons.

The mechanism by which NO kills cells is unknown. Free radical formation has been implicated in various forms of neurotoxicity, and NO itself is a reactive free radical. NGF inhibited the NO release and cNOS gene expression induced by glutamate in cortical cultures. In addition, Lin *et al*[14] reported that NGF protected cortical neurons against glutamate-induced toxicity via "stabilizing" intracellular free calcium ($[Ca^{2+}]_i$) level or suppressing the rising in $[Ca^{2+}]_i$. So that, loss of calcium homeostasis has been postulated to potentiate neuronal damage through the activation of NOS and the subsequent production of NO. The other findings have shown that NGF increase the activities of antioxidant enzymes[6]. All together, NGF may prevent glutamate neurotoxicity by modulating calcium homeostasis, inhibiting the activity of cNOS and the release of NO.

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神经生长因子在培养的大鼠脑皮质神经细胞抑制谷氨酸引起的一氧化氮合酶基因表达及一氧化氮释放

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目的 研究神经生长因子(NGF)对谷氨酸引起的原代培养的神经细胞一氧化氮(NO)释放和原生型一氧化氮合酶(cNOS)基因表达的影响。方法 测定神经细胞的生存力来分析NGF的作用。用荧光分析法测定细胞上清液NO含量,用Northern blot杂交法观察cNOS mRNA表达。结果 谷氨酸(0.5~2.0 mmol/L)引起神经元大量死亡,NO过量释放。NOS抑制剂L-NAME(100 μmol/L)及NGF(100 μg/L)显著抑制谷氨酸引起NO释放及细胞死亡。NGF(50, 100 μg/L)显著降低谷氨酸引起的cNOS mRNA的高表达。结论 NO介导了谷氨酸对皮质神经元的毒性,NGF通过抑制cNOS活性,降低NO释放来保护大脑皮质细胞对抗谷氨酸毒性。

关键词 神经生长因子 大脑皮质 细胞培养 谷氨酸 一氧化氮 一氧化氮合酶

奥曲肽、卡托普利及胰岛素对长期实验性糖尿病肾脏变化及尿白蛋白排泄的影响

Groabek H, *et al*. Kidney Int, 1998;53:173~180

1 目的 糖尿病早期,就会出现肾脏及肾小球增大,尿中白蛋白排泄(UAE)增多。已有资料证实,奥曲肽、卡托普利及胰岛素均能延缓糖尿病的肾脏变化。本研究的目的在于了解未经治疗的实验性糖尿病鼠给予奥曲肽、卡托普利单用或联用、或应用胰岛素,效果有何不同。

2 方法 成熟雌鼠随机分成6组。(1)非糖尿病对照组(n=24)。(2)安慰剂组(n=18)。(3)奥曲肽组(n=12)。(4)卡托普利组(n=12)。(5)奥曲肽及卡托普利联用组(n=12)。(6)胰岛素组(n=10)。上述糖尿病大鼠均为静注链脲霉素(50 mg/kg)诱导产生。链脲霉素注射后24小时测定血糖、尿糖、体重,头3个月,每月测1次上述指标,3个月后进行药物治疗,每周测2次。另外观察:肾脏生长及肾小球增生、UAE、血压、血清及局部肾皮质、肾髓质胰岛素样生长因子I(IGF-I)水平。

3 结果 3周治疗后,与对照组比,安慰剂组肾总重量及肾小球总容积(TGV)明显增加($P<0.001$)。奥曲肽、卡托普利单用或联用组,肾总重量与安慰剂组相比,明显减轻($P<0.05$),但与对照组相比,仍增加($P<0.05$),但TGV无下降。在研究期间,所有糖尿病大鼠UAE增加($P<0.001$),安慰剂组明显高于对照组($P<0.01$)。与安慰剂组相比,联合应用奥曲肽、卡托普利组UAE明显减少($P<0.05$),但单用上述任一种,UAE减少均不明显。胰岛素治疗组,肾重量、TGV、UAE与对照组基本相似,与其它对照组相比,明显下降($P<0.01$)。另外,除胰岛素治疗组血糖正常外,其余治疗组均与治疗前相似。

4 结论 实验性糖尿病鼠予以奥曲肽、卡托普利单用或联用3周,能减少肾脏生长及UAE排泄,但不能减轻肾小球增生,且对糖尿病代谢无影响。奥曲肽、卡托普利联用比单用更能有效减少UAE,但对抑制肾脏生长作用相似。而胰岛素治疗能使糖尿病血糖正常,代谢紊乱得到纠正,且使肾脏及肾小球增大得到完全控制,UAE正常。

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