

Antagonistic effect of nerve growth factor on neuronal injury induced by hypoxia in cultured cerebral cortical neurons of rats

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KEY WORDS nerve growth factors; anoxia; cerebral cortex; free radicals; cultured cells; neurons

ABSTRACT

AIM: To observe the effects of nerve growth factor (NGF) on neuronal hypoxic injury induced by sodium dithionite in primary cultures from gestation of 17-d rat fetal cerebral cortex. **METHODS:** Neuronal death and lactate dehydrogenase (LDH) efflux in the bathing medium were measured. The homogenate of cortical cells was used to determine malonyldialdehyde (MDA) content and superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. **RESULTS:** When cultures were incubated for 24 h with sodium dithionite, efflux of LDH and MDA content increased while the number of surviving neurons decreased. NGF $100 \mu\text{g} \cdot \text{L}^{-1}$ increased the number of surviving neurons to $85 \% \pm 9 \%$ of normoxia level under hypoxia. NGF $1 - 100 \mu\text{g} \cdot \text{L}^{-1}$ concentration-dependently attenuated hypoxia-induced increase of efflux of LDH and MDA content, with IC_{50} of 27 and 49 (95 % confidence limits: 18 - 49 and 29 - 110) $\mu\text{g} \cdot \text{L}^{-1}$. NGF $30 \mu\text{g} \cdot \text{L}^{-1}$ induced a 3-fold increase in SOD and GSH-Px activities. The levels of SOD and GSH-Px activities in hypoxia group were increased 2.7-fold by NGF $100 \mu\text{g} \cdot \text{L}^{-1}$. **CONCLUSION:** NGF prevented hypoxic insults in cultured cerebral cortical

neurons by suppressing the generation of lipid peroxides and increasing the activities of antioxidant enzymes.

INTRODUCTION

Nerve growth factor (NGF), a neurotrophic factor, protected cultured hippocampal and cortical neurons against glucose deprivation-induced injury^[1] and protected hippocampal neurons against ischemic injury *in vivo*^[2]. However, the mechanisms of action of NGF against cerebral ischemic injury are not well understood. It was recognized that the free radical theory was one of the more persuasive hypotheses to explain the delayed neuronal death during cerebral ischemia. NGF increased glutathione and catalase levels in PC12 cells^[3], and NGF, infused into rat brain, increased the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)^[4]. In this study, the effects of NGF on neuronal injury induced by hypoxia in cultured cerebral cortical neurons of rats were studied to understand the protective mechanism of NGF on ischemic brain.

MATERIALS AND METHODS

Reagents and drugs NGF was obtained from Academy of Military Medical Sciences, the purity >95 %. The assay kit of lactate dehydrogenase (LDH) and sodium dithionite were products of Beijing Chemical Factory. 2-Thiobarbituric acid (TBA), SOD, glutathione (GSH), and malondialdehyde (MDA) were purchased from Sigma. Other chemicals were of AR.

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Neuronal cultures Cortical neurons were isolated from embryonic (gestation of 16 – 18-d-old Sprague-Dawley rats, Grade II, Certificate No 94-30) fetuses according to the method of reference⁵, with some modifications. The dissected hemispheres were rinsed with ice-cold Ca^{2+} - and Mg^{2+} - free Hanks' solution (pH 7.2 – 7.4) with the following compositions ($\text{mmol} \cdot \text{L}^{-1}$): NaCl 137, KCl 5, glucose 5.6, and HEPES 10. Meninges and blood vessels were meticulously removed. Following a wash step with Ca^{2+} - and Mg^{2+} - free Hanks' solution, the brain was mechanically dissociated by gentle triturate 10 – 15 times with a polished pipette. The isolated brain cells were filtered through nylon sieve (200 mesh, hole width 95 μm) and collected in a flask. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % bovine serum albumin, then were seeded onto *L*-polylysine-coated 24-well plates and incubated at 37 °C in 5 % CO_2 atmosphere. The cell density was 10^8 cells $\cdot \text{L}^{-1}$. The culture medium was renewed every 2 – 3 d.

Experiments with hypoxic injury

Experiments were performed in 7 – 9-d-old cultures when the neurons were vulnerable to glutamate toxicity and oxidative insults and could be protected against excitotoxic and metabolic insults by neurotrophic factors¹¹. The cells were incubated with sodium dithionite 0.5 $\text{mmol} \cdot \text{L}^{-1}$, an O_2 scavenger, in the DMEM at 37 °C for 24 h⁶. NGF was added to the medium prior to sodium dithionite and incubated at 37 °C for 30 min.

Measurement of surviving neurons and extracellular LDH Neuronal survival was quantified by previously reported methods¹⁷. LDH efflux assay was determined^{8j}. Protein content of samples was measured⁹ⁱ.

Determination of MDA, SOD, and GSH-Px Cells were pelleted by low-speed

centrifugation and homogenized in 2.5 mL of a nitrogen-purged buffer (pH 7.4) with the following composition ($\text{mmol} \cdot \text{L}^{-1}$): HEPES 10, NaCl 137, KCl 4.6, KH_2PO_4 1.1, MgSO_4 0.6. The homogenate was centrifuged at $100\,000 \times g$ at 4 °C for 1 h, and the supernatant was used for enzyme assays. The MDA, SOD, and GSH-Px assays were basically the same as the method of Mizuno^{10j}.

Statistics Results were expressed as $\bar{x} \pm s$. Statistical analysis was performed using unpaired *t* test.

RESULTS

Effect of NGF on hypoxic insult The hypoxia-induced injury was estimated by the number of surviving neurons and LDH released into media from damaged neurons. Hypoxia caused an efflux of LDH by 225 % compared with normoxia and surviving neurons decreased to $42 \% \pm 4 \%$ of total. A marked increase of cell number was observed when cultures were incubated with NGF. NGF (1 – 100 $\mu\text{g} \cdot \text{L}^{-1}$) concentration-dependently attenuated hypoxia-induced efflux of LDH by 15 %, 31 %, 41 %, 50 %, and 63 %, respectively, with an IC_{50} of 27 (95 % confidence limits: 18 – 49) $\mu\text{g} \cdot \text{L}^{-1}$. (Fig 1)

Effects of NGF on SOD and GSH-Px In control cortical cultures the basal level of SOD activity was (1.8 ± 0.4) $\text{kU} \cdot \text{g}^{-1}$ (protein). The level of SOD activity was increased approximately 3-fold in cultures after addition of NGF 30 $\mu\text{g} \cdot \text{L}^{-1}$. The basal level of GSH-Px activity was (15 ± 4) $\text{kU} \cdot \text{g}^{-1}$ (protein). NGF 30 $\mu\text{g} \cdot \text{L}^{-1}$ induced 3-fold increase in GSH-Px activity.

Effect of NGF on hypoxia-induced increase of MDA content There was a marked increase of MDA content when cultures were incubated with sodium dithionite for 24 h. NGF 1 – 100 $\mu\text{g} \cdot \text{L}^{-1}$ inhibited hypoxia-induced

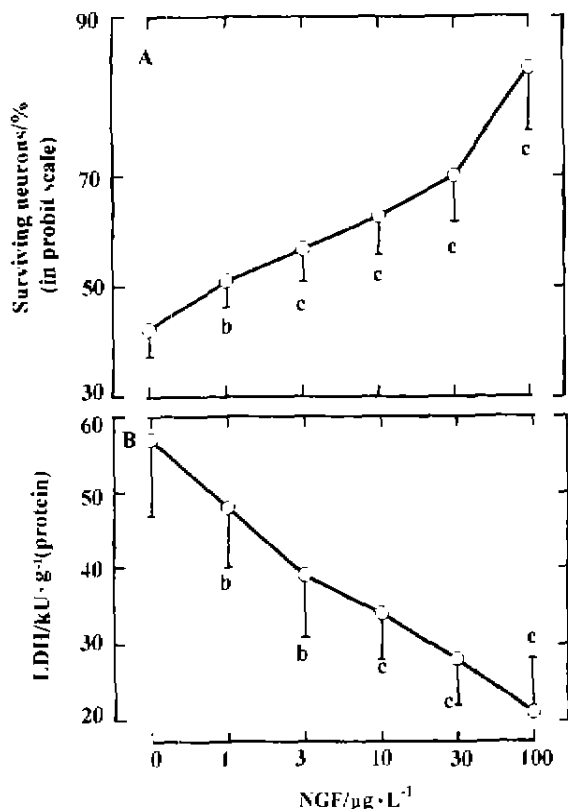


Fig 1. Effect of NGF against hypoxia-induced neuronal death (A) and on hypoxia-induced neurotoxicity (B). $n = 6$ wells from 3 rats. $\bar{x} \pm s$. ^b $P < 0.05$, ^c $P < 0.01$ vs NGF-free group.

generation of MDA by 15 %, 24 %, 37 %, 46 %, and 55 %, respectively, with an IC_{50} of 49 (95 % confidence limits: 29 - 110) $\mu\text{g}\cdot\text{L}^{-1}$. (Tab 1)

Effects of NGF on hypoxia-induced reduction of SOD and GSH-Px activities The activities of SOD and GSH-Px were inhibited by 50 % and 47 %, respectively, when cells were exposed to hypoxic bathing media for 24 h. A marked increase on activities of antioxidant enzymes was observed 24 h after hypoxic injury when NGF 1 $\mu\text{g}\cdot\text{L}^{-1}$ was added prior to sodium dithionite. NGF 100 $\mu\text{g}\cdot\text{L}^{-1}$ induced 2.7-fold increase in SOD and GSH-Px activities in hypoxic injury culture (Tab 1).

Tab 1. Effects of nerve growth factor (NGF) on hypoxia-induced cerebral cortical lipid peroxidation and antioxidant enzyme activity. $n = 6$ wells from 6 rats. $\bar{x} \pm s$. ^c $P < 0.01$ vs normoxia. ^e $P < 0.05$, ^f $P < 0.01$ vs 24-h hypoxia.

	MDA/ $\mu\text{mol}\cdot\text{g}^{-1}$ (protein)	SOD/ $\text{kU}\cdot\text{g}^{-1}$ (protein)	GSH-Px/ $\text{kU}\cdot\text{g}^{-1}$ (protein)
Normoxia	5.4 ± 0.8	1.8 ± 0.4	15 ± 4
24-h hypoxia	11 ± 4	0.88 ± 0.19	8.1 ± 2.2 ^e
NGF/ $\mu\text{g}\cdot\text{L}^{-1}$			
1	9.6 ± 1.8 ^c	1.15 ± 0.22 ^f	10.7 ± 1.9 ^d
3	8.6 ± 1.2 ^f	1.29 ± 0.24 ^f	11.6 ± 2.3 ^d
10	7.1 ± 2.4 ^f	1.6 ± 0.4 ^f	15 ± 4 ^d
30	6.1 ± 1.1 ^f	1.8 ± 0.4 ^f	17 ± 5 ^f
100	5.1 ± 1.0 ^f	2.4 ± 0.4 ^f	22 ± 5 ^f

DISCUSSION

The present results showed that hypoxia induced increase of both LDH efflux and cell death in cultured cortical neurons. NGF dose-dependently increased the number of surviving neurons and inhibited efflux of LDH induced by hypoxia, indicating that NGF could protect cortical neurons against hypoxic insults.

It is well known that free radicals are detrimental factors of traumatic and ischemic or anoxic lesions to neurons. Oxygen free radicals are toxic due to their oxidation of protein-containing thiol groups, lipid peroxidation, DNA or RNA fragmentation and mutations as well as destruction of extracellular matrix. Depletion of antioxidants or deficits in antioxidant defense mechanisms will increase susceptibility to oxidant stress³⁾. Our results suggested that NGF decreased hypoxia-induced lipid peroxide action.

Our study indicated that the basal level of SOD activity in cortical cultures 1.8 $\text{kU}\cdot\text{g}^{-1}$ (protein) was considerably lower than that for hippocampus from adult rat brain 16 $\text{kU}\cdot\text{g}^{-1}$ (protein) reported by Mizuno & Ohta¹¹⁾, which the basal GSH-Px activity in the cortical cultures 15.2 $\text{kU}\cdot\text{g}^{-1}$ (protein) was 10 times higher than

that for cultured PC12 cells reported by Jackson *et al*^[12] and approximately 3 times lower than the reported figures for adult rat hippocampus^[11]. In the present study antioxidant enzyme activity determination was performed in total cell homogenates which contained about 20 % - 30 % astrocytes although we did not know the basal levels of antioxidant enzyme activities in neurons and astrocytes. Makar *et al*^[13] reported that the levels of GSH-Px and GSSG-R in cultured cortical neurons and astrocytes were almost the same.

The results suggested that the decreases of SOD and GSH-Px activities induced by hypoxia were markedly increased by pretreatment with NGF. The finding that NGF protected at concentrations as low as $1 \mu\text{g}\cdot\text{L}^{-1}$ was consistent with published work regarding the effects of NGF on neurite outgrowth from chick dorsal root ganglia^[14]. However, the molecular mechanisms of NGF how to increase the antioxidant enzyme activities are not well understood. Based on the present data it is considered that antioxidant actions of NGF involve signaling through NF κ B, which is a transcription factor and could increase some antioxidant enzymes such as Mn-SOD expression^[15].

In conclusion, NGF prevented the injury of hypoxia in cultured cortical neurons by suppressing the generation of lipid peroxide and increasing the activities of antioxidant enzymes.

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神经生长因子对培养大鼠脑皮质神经元缺氧损伤的拮抗作用

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关键词 神经生长因子; 缺氧症; 大脑皮质; 自由基; 培养的细胞; 神经元

目的: 以连二亚硫酸钠造成原代培养的大鼠胎鼠脑皮质神经元缺氧损伤, 观察神经生长因子(NGF)对缺氧损伤神经元的影响. 方法: 测定神经元生存力及细胞外液乳酸脱氢酶(LDH)的活性来分析NGF的作用, 脑皮质细胞匀浆用于测定丙二醛(MDA)含量及超氧化物歧化酶(SOD)和谷胱甘肽过氧化物酶(GSH-Px)活性. 结果: 缺氧后, LDH释放及细胞生存力降低. NGF(1-100 $\mu\text{g}\cdot\text{L}^{-1}$)浓度依赖地减少LDH的释放及MDA的生成, NGF 100 $\mu\text{g}\cdot\text{L}^{-1}$ 显著提高细胞生存力及提高SOD和GSH-Px活性2.7倍. 结论: NGF通过减少脂质过氧化物生成及提高SOD和GSH-Px活性来保护大脑皮质细胞抗缺氧损伤.

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