The Journal of Neuroscience, July 1993, 13(7): 3034–3040

Peptide Growth Factors Protect against Ischemia in Culture by Preventing Nitric Oxide Toxicity

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Reduction or elimination of nitric oxide (NO) production in cortical neurons by NO synthase (NOS) inhibitors during glutamate toxicity *in vitro* or during focal cerebral ischemia *in vivo* can prevent neuronal cell death. In contrast, growth factors can prevent neuronal degeneration induced by treatment with glutamate or potassium cyanide. We have determined whether NO mediates hippocampal cell death during anoxia *in vitro* and whether the peptide growth factors basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) can prevent hippocampal neuronal death during anoxia or NO exposure. Both bFGF and EGF increased hippocampal neuronal survival from about 35% in anoxic cultures to about 65% in treated cultures during an 8 hr period of anoxia. Inhibition of NOS by N^G-monomethyl-L-arginine, a competitive inhibitor of NOS, rescued 65–70% of the neurons that would normally die during an 8 hr anoxic incubation, and this effect was reversed by L-arginine, a precursor for NO. Thus, hippocampal neuronal death following anoxia is, at least in part, mediated by NO. NO, generated by either nitroprusside or 3-morpholino-sydnonimine, was toxic to hippocampal neurons. Pretreatment of cultures with either bFGF (10 ng/ml) or EGF (10 ng/ml) prior to NO exposure increased survival from approximately 40% in untreated cultures to 80% in treated cultures, yet the effect of combining bFGF and EGF was not greater than treatment with either of the growth factors alone. Knowledge that the growth factors bFGF and EGF are neuroprotective against NO toxicity provides insights into the mechanisms of ischemic neuronal death that may direct future therapeutic modalities for cerebrovascular disease and neurodegenerative disorders.

[Key words: anoxia, basic fibroblast growth factor, epidermal growth factor, hippocampal neurons, 3-morpholino-sydnonimine, nitric oxide, nitric oxide synthase, sodium nitroprusside]

Growth factors have gained increasing prominence as agents necessary not only for promotion of neuronal development but also for prevention of neuronal degeneration. During development of the nervous system, growth factors function to regulate cell division, neurite outgrowth, and cell survival (Morrison et al., 1988; Wagner and Kostyk, 1990; Deloulme et al., 1991). Growth factors have also been shown to prevent neuronal death in hippocampal cultures during glutamate toxicity (Mattson et al., 1989), potassium cyanide (KCN) administration (Pauwels et al., 1989), and hypoglycemia (Cheng and Mattson, 1991).

Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) are present throughout the brain and affect several cell types. Both growth factors have trophic effects on CNS neurons but may support cell survival through independent mechanisms (Kornblum et al., 1990). Recent interest in the ability of bFGF to promote neurite outgrowth and survival (Walicke et al., 1986; Morrison, 1987) has directed the application of this trophic factor to the treatment of neuronal injury and degeneration. During periods of neuronal injury, such as cerebral ischemia, the expression of bFGF is significantly increased (Finklestein et al., 1990; Kiyota et al., 1991). bFGF can prevent the death of septal cholinergic neurons following their transection (Anderson et al., 1988) and promote regeneration in severed optic nerves (Sievers et al., 1987). The agent has also been reported to protect striatal neurons from glutamate toxicity (Freese et al., 1992) and prevent retrograde degeneration of the thalamus following cortical infarction (Yamada et al., 1991) or ablation (Kohmura et al., 1991). Likewise, EGF is also neuroprotective during periods of anoxia and glutamate toxicity. Human recombinant EGF can increase cortical neuronal survival *in vitro* during a 4 hr period of anoxia when compared to control (Kinoshita et al., 1990). EGF also attenuates neurotoxicity in neuronal cultures following exposure to KCN or NMDA (Pauwels et al., 1989).

The mechanisms that lead to neuronal death during anoxia are not well defined. Several studies implicate the importance of preventing elevations in intracellular calcium following excitatory amino acid (EAA) transmitter release (Choi, 1985; Garthwaite et al., 1986; Rothman and Olney, 1986). An important insight into ischemic neuronal death has been provided by the demonstration that nitric oxide (NO) production is a key step in neuronal death during EAA toxicity. Inhibition of NO synthesis prevents the toxic effects of glutamate. NO is itself neurotoxic, suggesting that an overproduction of NO during EAA toxicity (and, by inference, during ischemia) leads directly to neurotoxicity (Dawson et al., 1991). Furthermore, in animal models of middle cerebral artery occlusion, cerebral infarction can be reduced by 72% following intraperitoneal administration of an inhibitor of NO synthesis (Nowicki et al., 1991).

NO is generated by the enzyme nitric oxide synthase (NOS) (Bredt and Snyder, 1992) and has a half-life of approximately

Received Sept. 16, 1992; revised Jan. 22, 1993; accepted Feb. 1, 1993.

This research was supported by the following grants: NIH NINDS KO8-NS-01599 (K.M.), National Stroke Association and Ciba–Geigy (K.M.), NIH Institutional Training Grant T32-NS-07141-13 (I.B.), NIH Training Grant for Health Professional Students (D.D.), and NIH NEI EY0654 (J.A.W.). We thank Dr. Fred Plum for his thoughtful review of the manuscript.

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30 sec (Palmer et al., 1987). NOS is regulated by the calcium/calmodulin-dependent protein kinase II (Bredt and Snyder, 1990). NOS oxidizes the substrate L-arginine (Palmer et al., 1988) to yield NO and citrulline (Moncada and Palmer, 1990). NO, in turn, activates the soluble form of guanylate cyclase resulting in an increase in guanosine 3′,5′-cyclic monophosphate (cGMP) (Miki et al., 1977). NO also has other effects such as increasing the activity of ADP-ribosyltransferase (Brune and Lapetina, 1990). In the brain, NOS is competitively inhibited by N^G-monomethyl-L-arginine (NMA), N^G-nitro-L-arginine, and N^G-iminoethyl-L-ornithine (Knowles et al., 1990). NOS activity has been documented in neurons (Bredt et al., 1991) and is present in the cerebellum, hypothalamus, midbrain, striatum, hippocampus, and medulla (Forstermann et al., 1990). During periods of ischemia, excessive NMDA receptor activation is thought to result in both calcium influx into cells and the activation of NOS to yield NO and cGMP (Bredt and Snyder, 1989; Garthwaite et al., 1989; Knowles et al., 1989) and subsequent neurodegeneration.

Given the apparent obligatory role of NO production in glutamate-induced neuronal cell death, it seems reasonable to propose that growth factors may ultimately determine neuronal survival during periods of anoxia through the regulation of the production and/or responses of neurons to NO. We examined the relationship between ischemic-induced NO toxicity and the neuroprotective role of peptide growth factors. Using dissociated hippocampal neuronal cultures, we demonstrate that prevention of NO production during anoxia is neuroprotective and that treatment with the growth factors bFGF and EGF is protective against NO toxicity.

Materials and Methods

Hippocampal cultures. The hippocampi were obtained from 1-d-old Sprague–Dawley rat pups and maintained by a modified method of Furshpan and Potter (1989). Hippocampal sections were then incubated in a solution of papain (10 U/ml), cysteine (3 mmol/liter), and dissociation medium for two 20 min periods. The hippocampi were then rinsed in dissociation medium and incubated in a solution of trypsin inhibitor (10–20 U/ml) and dissociation medium three times, for 5 min each. The cells were washed in growth medium three times, 2 min each, and triturated 50 times in 2 ml of growth medium. The trituration was repeated five times with fresh growth medium to yield a total of 10 ml of cell suspension. Forty milliliters of Opti-MEM-I (GIBCO–Bethesda Research Labs, Gaithersburg, MD) was added to this cell suspension and the dissociated cells were plated at a density of approximately 15 $\times$ 10^5 cells/mm^2 in 35 mm^2 polylysine-laminin–coated plates (Falcon Labware, Lincoln Park, NJ). Cells were maintained in growth medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% room air. Following a 2 hr incubation period to allow for cell adhesion, culture medium was replaced with 1.5 ml of growth medium and the medium was subsequently replaced weekly. All experiments were performed with cultured neurons that had been in culture for 2–3 weeks. Non-neuronal cells, which were identified by morphology, accounted for less than 20% of the total cell population.

Dissociation medium contained 90 mM Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 0.25 mM $CaCl_2$, 10 mM kynurenic acid, 100 mM $MgCl_2$, and 1 mM HEPES with pH adjusted to 7.4. Growth medium consisted of Leibovitz's L-15 Medium (GIBCO–Bethesda Research Labs, Gaithersburg, MD) with 6% sterile rat serum (Bioproducts for Science, Indianapolis, IN), 150 mM $NaHCO_3$, 2.25 mg/ml transferrin, 2.5 μg/ml insulin, 10 nM progesterone, 90 μM putrescine, 15 nM selenium, 35 mM glucose, 1 mM L-glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml), and vitamin mix. The vitamin mix contained L-proline (0.2 M), L-cystine (0.025 M), *p*-aminobenzoic acid (7.0 mM), vitamin B-12 (0.3 mM), inositol (11 mM), choline chloride (14.0 mM), fumaric acid (0.04 M), coenzyme A (0.1 mM), *d*-biotin (8 μM), and DL-6,8-thiotic acid (0.5 mM).

Experimental treatments. Cultures were deprived of oxygen by placing them in a humidified atmosphere at 37°C with 95% N_2 and 5% CO_2 for an 8 hr period or were made anoxic by the addition of KCN (Sigma Chemical Company, St. Louis, MO). In experiments involving NOS manipulation, either NMA (provided by Drs. Roberto Levi and Steven Gross, Cornell University Medical College) or L-arginine (Sigma Chemical Company, St. Louis, MO) was added directly to the cultures immediately prior to anoxic incubation. NO administration was performed by replacing the culture media with media containing sodium nitroprusside (SNP) (Sigma Chemical Company, St. Louis, MO) or 3-morpholino-sydnonimine (SIN-1) (provided by Drs. Roberto Levi and Steven Gross, Cornell University Medical College) for 5 min. Following treatment with oxygen deprivation, KCN, or NO, culture medium was replaced with fresh growth medium and the cultures were placed in a normoxic, humidified incubator at 37°C with 5% CO_2 for 24 hr prior to assessing neuronal cell death. The growth factors bFGF (recombinant human bFGF) and EGF (recombinant human EGF) were obtained from R&D Systems, Inc., Minneapolis, MN. The growth factors were prepared as stock solutions and diluted directly to the cultures at various time intervals per the experimental protocol. Unless otherwise specified, experimental cultures involving the use of either bFGF or EGF received replacement of fresh bFGF or EGF during any growth media changes.

Assessment of neuronal cell death. Hippocampal neuronal injury was determined by bright-field microscopy using a 0.4% trypan blue dye exclusion method 24 hr following oxygen deprivation or treatment with nitric oxide. Neurons were identified by morphology. The mean survival was determined by counting eight randomly selected nonoverlapping fields with approximately 10–20 neurons (viable + nonviable) in each 35 mm petri dish. The mean survival from each culture dish represents an $N = 1$ determination. Each experiment was replicated three to five times independently on separate occasions with different cultures.

Results

bFGF and EGF protect hippocampal neurons from anoxia. bFGF has been shown to prevent neuronal degeneration in hippocampal neurons during glutamate toxicity (Mattson et al., 1989) and EGF can increase hippocampal neuronal survival during both NMDA and KCN toxicity (Pauwels et al., 1989). We initially confirmed the neuroprotective role of bFGF and EGF during KCN administration. Application of KCN (1 mM) for 6, 20, and 24 hr or KCN (20 mM) for 20 min decreased neuronal survival to 15–30%, while pretreatment with bFGF (10 ng/ml) or EGF (10 ng/ml) for 24 hr prior to KCN application protected hippocampal neurons by maintaining survival of 60–70% of neurons (data not shown). To examine the neuroprotective role of the trophic factors during anoxia, hippocampal neurons that were pretreated with bFGF (10 ng/ml) or EGF (10 ng/ml) for 24 hr were placed in an oxygen-free environment for 8 hr. In the absence of growth factors, approximately 35% of hippocampal neurons survived after the oxygen deprivation period. In contrast, bFGF increased hippocampal neuronal cell survival to 64 ± 2%, and EGF increased neuronal survival to 63 ± 3% (Table 1). Thus, pretreatment with both bFGF and EGF protected hippocampal neurons from anoxia.

Inhibition of NOS protects hippocampal neurons from anoxia. To determine whether NO mediates hippocampal neuronal cell death during anoxia *in vitro*, we inhibited the production of NO in an oxygen-free environment with the potent NOS inhibitor NMA. Under conditions when anoxia was toxic to approximately 75% of the neurons, treatment with NMA (10 μM, 50 μM, 100 μM, 300 μM, and 1000 μM) increased hippocampal neuronal cell survival in a dose-dependent manner and allowed a maximum survival of 70% of neurons (Fig. 1). Thus, at approximately 90 μM of NMA, half the neurons were protected from NO toxicity. Addition of L-arginine (1 mM), a precursor for NO production, to the cultures during anoxia completely

Table 1. bFGF and EGF are protective against anoxia

Growth factor	Percentage survival of hippocampal neurons after anoxia	
	No growth factor	Added growth factor
bFGF (10 ng/ml)	37 ± 3	64 ± 2*
EGF (10 ng/ml)	36 ± 3	63 ± 3*

Cultures were pretreated with either bFGF or EGF 24 hr prior to placement in an anoxic chamber of 95% N_2/5% CO_2 at 37°C for an 8 hr period. Neuronal survival was assessed 24 hr following anoxia with trypan blue exclusion and expressed as a percentage of the total number of neurons (viable + nonviable). Values represent the mean and SEM of $N = 10$ determinations (culture plates) in five separate experiments per treatment group. In the absence of oxygen deprivation, 85 ± 3% of neurons were viable.

* The unpaired Student's t test was used for statistical analysis with df = 18 and $p < 0.001$ compared to anoxic cultures with no growth factor treatment.

reversed the effect of NMA (Fig. 1), supporting the idea that NMA protects against neuronal death by inhibiting NOS.

NO is toxic to hippocampal neurons in vitro. Since inhibition of NO production is neuroprotective during anoxia, we next determined whether NO was directly toxic to hippocampal neurons in culture. Hippocampal neurons were exposed to the NO generator SNP for 5, 30, or 60 min. In each case, increasing doses of SNP (1 μM, 10 μM, 25 μM, 50 μM, 100 μM, 300 μM, and 1000 μM) resulted in progressive neuronal degeneration with approximately 50% of neuronal cell death occurring at 50 μM (Fig. 2*a*). In accordance with NO's short half-life of 30 sec, SNP toxicity was not significantly different among the various time exposures (data not shown). Since SNP may be toxic to neurons through the production of cyanide (Bates et al., 1991), we examined the effects of NO through the application of the agent SIN-1, which also spontaneously releases NO. A 5 min exposure of SIN-1 (1 μM, 10 μM, 25 μM, 50 μM, 100 μM, 300 μM, and 1000 μM) elicited hippocampal neuronal cell death in a dose-dependent fashion and 50% of the neurons were killed at a dose of approximately 95 μM, supporting the idea that NO is the toxic agent (Fig. 2*b*). In contrast to SNP, SIN-1 was slightly less potent in producing neuronal toxicity at doses of 300 μM and 1000 μM.

bFGF and EGF protect hippocampal neurons from NO toxicity. Growth factors might protect against anoxia either by preventing NO generation or by protecting neurons against the deleterious effects of NO. To determine whether peptide growth factors could prevent NO toxicity, hippocampal cultures were pretreated with either bFGF or EGF 24 hr prior to NO generation with SNP or SIN-1. Pretreatment with bFGF (10 ng/ml) or EGF (10 ng/ml) significantly decreased NO-induced neuronal degeneration caused by a 5 min application of SNP. Compared to SNP administration alone, hippocampal survival following bFGF administration increased from approximately 50% to 80%. In a similar fashion, EGF improved neuronal survival from 50% to 75% following a 5 min exposure of SNP (Fig. 3*a*). bFGF and EGF were also protective against a 5 min exposure of SIN-1. Twenty-four hour pretreatment with 10 ng/ml of bFGF improved hippocampal neuronal cell survival from approximately 40% in untreated cultures to 75% in cultures receiving bFGF (Fig. 3*b*). EGF also decreased NO toxicity following SIN-1 exposure by increasing percentage of survival from 40% in untreated cultures to 75% in cultures receiving EGF (Fig. 3*b*).

To determine the concentration of growth factor required for neuroprotection, we pretreated hippocampal cultures 24 hr prior to exposure with SNP (300 μM) with increasing doses of bFGF and EGF (0.01 ng/ml, 0.1 ng/ml, 0.5 ng/ml, 1.0 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml, and 500 ng/ml). Both bFGF and EGF were protective beginning at a dose of 1.0 ng/ml, which also represented the EC_{50}, suggesting that the growth factors were acting at an appropriate cell surface receptor (Fig. 3*c*). Maximum protection was observed at doses of 10 ng/ml or greater, similar to studies of glucose deprivation toxicity (Cheng and Mattson, 1991).

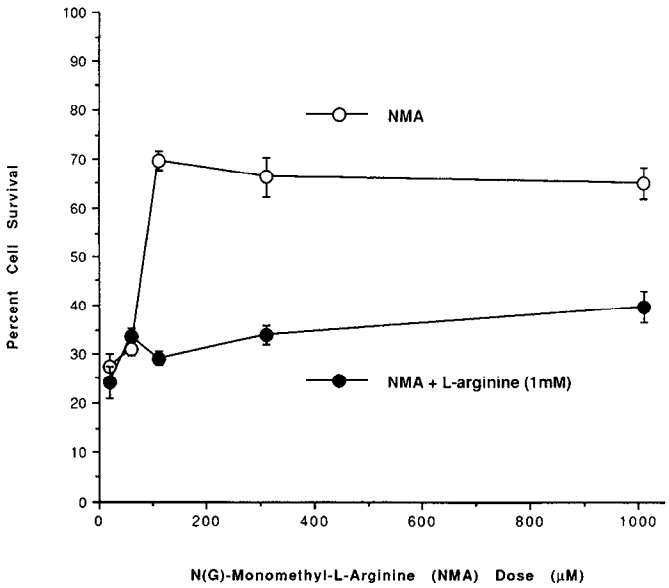

Figure 1. NO production contributes to cell death during anoxia. During an 8 hr period of anoxia, production of NO was inhibited with the NOS inhibitor NMA in the absence (*open symbols*) or presence (*closed symbols*) of L-arginine (1 mM). Neuronal cell viability was based on the percentage of the total number of neurons (viable + nonviable) and was determined by trypan blue exclusion 24 hr following the completion of anoxic period. Data represent the mean and SEM of $N = 6$ determinations (culture plates) from three separate experiments.

Growth factor protection against NO toxicity is most efficacious when trophic factors are present prior to NO exposure. Trophic factors may prevent neuronal degeneration following exposure to NO by altering cellular functions, such as calcium flux, glucose metabolism, or gene regulation, that render the cell resistant to the toxic effects of NO. Alternatively, the growth factors may protect neurons by reversing a previously sustained insult induced by NO. To determine whether bFGF or EGF either prevents or reverses cellular injury following NO exposure, we examined the neuroprotective role of bFGF (10 ng/ml), EGF (10 ng/ml), and combined bFGF (10 ng/ml) and EGF (10 ng/ml) administered either 24 hr, 12 hr, or 6 hr prior to, at the time of, or 6 hr following the onset of SNP (300 μM) administration. All cultures except the 6 hr group received fresh bFGF and EGF following the 5 min SNP exposure and growth media change.

Pretreatment with either bFGF or EGF or the combination of bFGF and EGF at 24, 12, and 6 hr protected hippocampal neurons from NO toxicity and increased neuronal survival from approximately 40% to 75%. In contrast, addition of growth factor at the time of SNP application (time = 0 hr) provided only modest protection and treatment with the growth factors 6 hr after exposure to NO provided no significant protection from NO toxicity (Fig. 4*a*).

To determine whether continued exposure to bFGF or EGF after application of NO was required for neuroprotection, hip-

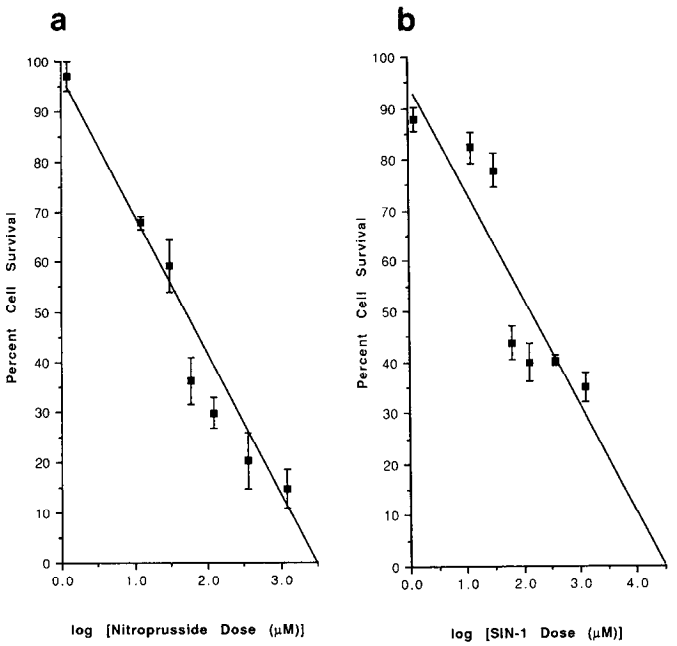

Figure 2. Neurotoxicity of NO. Hippocampal neuronal cultures were exposed to increasing SNP (a) or SIN-1 (*b*) concentrations for five min, and cell viability based on the percentage of the total number of neurons (viable + nonviable) was assessed 24 hr later by trypan blue exclusion. The values of percentage cell survival for the SNP and SIN-1 treatments are the means and SEM of $N = 4$–6 determinations (culture plates) from three separate experiments.

pocampal cultures were exposed to bFGF or EGF as described above, but cultures were not supplemented with fresh bFGF or EGF following the 5 min SNP exposure and growth media change. Since pretreatment with bFGF and EGF at 24, 12, and 6 hr protected hippocampal neurons from NO toxicity to the same degree afforded by continuous trophic factor application, we conclude that continuous treatment with the trophic factors is not essential for protection from NO toxicity. As expected, growth factor treatment at the time of SNP application or 6 hr post-SNP administration did not protect the hippocampal neurons (Fig. 4*b*).

Thus, it appears that the growth factors are capable of preventing NO toxicity rather than reversing a previously sustained cellular insult, suggesting that their effect on neuronal function is a long-term alteration rather than an ongoing process. This data may also suggest that NO, with an exceedingly short half-life, results in an *acute* cellular stress that the growth factors are preventing or reversing, yet during simultaneous application of SNP and the growth factors (Fig. 4*a,b*), the intracellular signaling by the trophic factors may not be rapid enough to elicit complete protection. In the case of growth factor removal following pretreatment with these agents (Fig. 4*b*), intracellular signaling may persist beyond the time of removal and would therefore provide acute protection upon treatment with an NO generator. In cases involving posttreatment of the growth factors following an NO insult, no protection would be expected following the acute cellular stress. Our data also show that combined bFGF and EGF treatment does not significantly decrease neuronal degeneration following NO exposure when compared to individual application of the growth factors. It is possible that bFGF interferes with the protective effect of EGF that has been demonstrated in non-neuronal cells (Huff and Schreier, 1990).

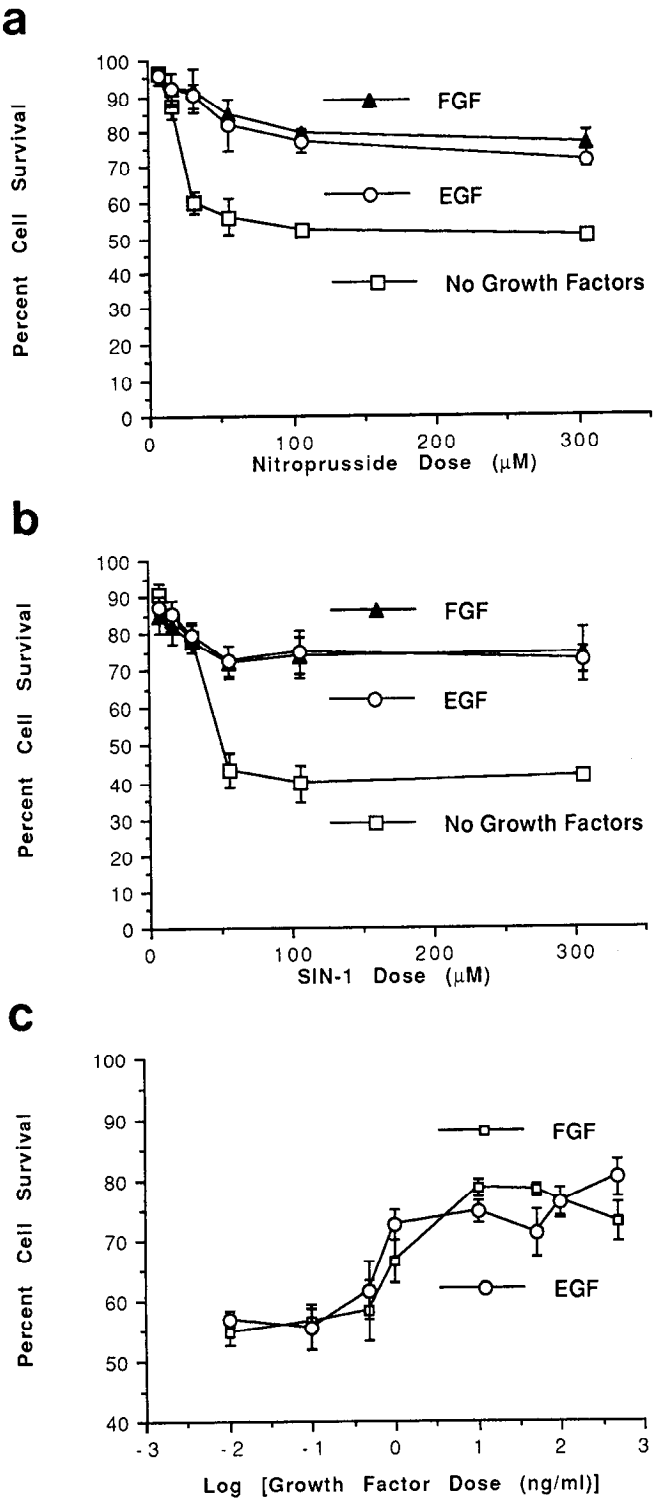

Figure 3. Growth factors prevent NO toxicity. Hippocampal cultures were pretreated with either bFGF (10 ng/ml) or EGF (10 ng/ml) 24 hr prior to a 5 min exposure to SNP (*a*) or SIN-1 (*b*). Neuronal survival was based on the percentage of the total number of neurons (viable + non-viable) and determined by trypan blue exclusion 24 hr following exposure to the NO generators and expressed as a percentage of the original number. Each data point represents the mean and SEM of $N = 6$ determinations (culture plates) from three separate experimental preparations. *c*, A dose–response curve for the neuroprotective effects of bFGF and EGF following a 5 min application of SNP (300 μM) was determined using the same procedure. Data points are the mean and SEM of $N = 6$ determinations (culture plates) from three separate experiments. Survival for cultures treated with SNP (300 μM) without either bFGF or EGF was 35 ± 2%.

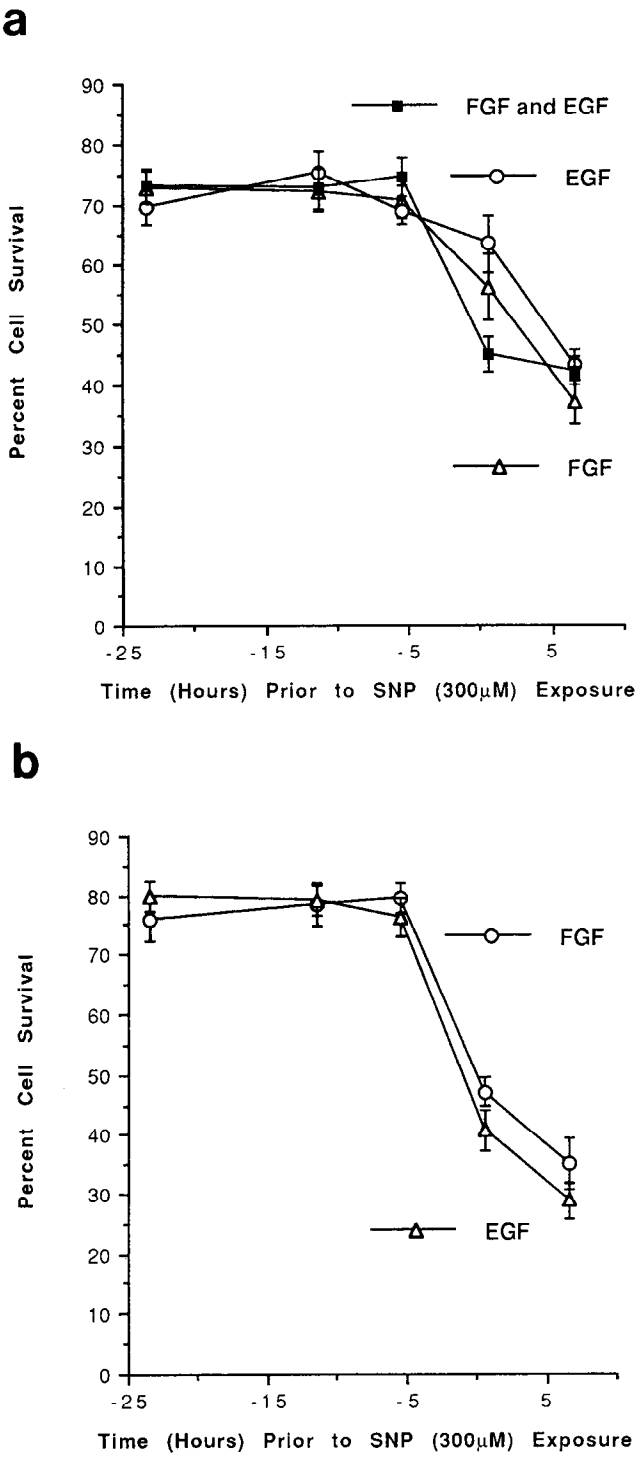

Figure 4. Growth factors are effective only if added prior to NO exposure. *a*, Hippocampal cultures received bFGF (10 ng/ml), EGF (10 ng/ml), or both bFGF (10 ng/ml) and EGF (10 ng/ml) 24 hr, 12 hr, and 6 hr prior to, at the time of, or 6 hr following the onset of a 5 min SNP (300 μM) exposure. All cultures except the 6 hr group received fresh bFGF and EGF following SNP administration and growth media change. *b*, Hippocampal cultures were exposed to bFGF (10 ng/ml) or EGF (10 ng/ml) as described above, but the cultures were not supplemented with fresh bFGF or EGF following the 5 min SNP (300 μM) exposure and growth media change to assess whether persistent growth factor treatment was necessary for protection against NO. Neuronal survival was based on the percentage of the total number of neurons (viable + nonviable) and determined by the trypan blue exclusion method. In the absence of NO, the percentage of viable cells averaged 80% (*a*) and 85% (*b*), while NO reduced viability to 41 ± 3% (*a* and *b*). The values are the means and SEM of $N = 6$ determinations (culture plates) from three separate experiments.

Discussion

Neuronal degeneration from anoxic insults is not fixed, but it can be modified by several pharmacological agents. During focal cerebral ischemia, the core of an ischemic insult suffers from loss of cerebral blood flow and metabolism (Sako et al., 1985), yet the penumbral zone, that is, the region surrounding the ischemic core, is characterized by decreased blood flow and patchy areas of hypermetabolism (Astrup et al., 1981). Pharmacological manipulation with agents such as glutamate receptor antagonists (Simon et al., 1984; Choi et al., 1988) and imidazole receptor binding agents (Maiese et al., 1992) can reduce the extent of ischemia within the penumbral zone. Neuroprotection from ischemia is often thought to be a result of preserving calcium homeostasis (Orrenius et al., 1988; Siesjo, 1988) and the modulation of neurotransmitter release (Gustafson et al., 1989).

During periods of cerebral ischemia, activation of NMDA receptors results in both calcium influx into cells and the production of NO and cGMP (Bredt and Snyder, 1989; Garthwaite et al., 1989; Knowles et al., 1989). Both loss of calcium homeostasis as well as the production of NO may result in neuronal degeneration. NO has been linked to cell death in neuronal cultures during glutamate toxicity (Dawson et al., 1991) and to cerebral infarction in animal models of focal stroke (Nowicki et al., 1991). The mechanism of NO toxicity in neurons has not been established. NO might cause neuronal death by facilitating calcium influx into cells. Alternatively, NO, a free radical, may be directly toxic. NO also reacts with superoxide to produce peroxynitrite ($\cdot ONNO^-$), an agent that leads to cell lipid peroxidation (Radi et al., 1991). Although the mechanism of NO toxicity is not well defined, our experiments provide pharmacological evidence that NO is a mediator of neuronal death during anoxia. NMA, a competitive inhibitor of NOS, prevented anoxic-induced neuronal cell death in a dose-dependent fashion. In addition, the neuroprotective effect of NMA was reversed with the addition of L-arginine, a substrate of NOS and a precursor for NO production.

The prevention of neuronal cell death during anoxia by the NOS inhibitor NMA strongly suggests that NO is necessary for neuronal degeneration; however, this observation does not directly address whether NO itself is neurotoxic. Increasing concentrations of the NO generators SNP and SIN-1 result in progressive neuronal cell death, providing persuasive evidence that NO is a toxic agent. These data suggest that NO, a subsequent NO by-product such as $\cdot ONOO^-$, or a compound such as cGMP that is increased in response to NO must be toxic during anoxia. Cyanide is a metabolite of SNP that may be toxic, but since SIN-1, which spontaneously releases NO but not cyanide, also results in neuronal death it is likely that NO is the toxic agent. Furthermore, we have shown that brief exposure (30 min) to cyanide is not toxic to hippocampal neurons (data not shown).

In light of the apparently essential role NO plays as a mediator of neuronal death during anoxia, it seems reasonable that growth factors may ultimately determine neuronal survival by modifying NO production or NO-dependent metabolism. A working model of the toxic role of NO and the protective role of peptide growth factors in ischemia involves the release of excessive EAA

following an anoxic insult. EAAs are known to induce NO synthesis via calcium influx through NMDA receptors (Garthwaite et al., 1989). Prevention of subsequent neuronal degeneration by trophic factors following NO release would result by decreasing the production or toxic effects of NO, or by reversing a previously sustained cellular insult. Prior studies have confirmed the neuroprotective role of growth factors against the toxic effects of glutamate (Mattson et al., 1989), KCN (Pauwels et al., 1989), and hypoglycemia (Cheng and Mattson, 1991). Our results demonstrate that growth factors can protect against NO toxicity.

Non-neuronal cells have long been known to provide trophic support to neurons (Ramon y Cajal, 1928). In particular, astrocytes can promote the survival of hippocampal neurons in culture (Banker, 1980; Muller et al., 1984). Astrocytes can also induce neurite outgrowth (Noble et al., 1984) and secrete growth factors such as bFGF (Hatten et al., 1988). Although some have reported that the neurotrophic effects of growth factors are not mediated through non-neuronal cells (Walicke and Baird, 1988; Unsicker et al., 1992), others have demonstrated a protective effect of astrocytes on neuronal cells exposed to anoxia (Vibulsreth et al., 1987) or EAA toxicity (Mattson and Rychlik, 1990). These studies suggest that astrocytes may ultimately mediate the protective effects of the trophic factors during NO toxicity. To help establish if the protective effects of bFGF and EGF are mediated by astrocytes in our hippocampal cultures, we prepared cytosine arabinoside (2.4 μM)-treated cultures that contained approximately 5% non-neuronal cells. bFGF ($N = 4$) and EGF ($N = 4$) protected neurons against NO toxicity in astrocyte-depleted cultures as effectively as cultures containing 20% astrocytes, suggesting that the neuroprotective actions of both bFGF and EGF are primarily a result of a direct effect on the hippocampal neurons rather than a secondary interaction with glial cells.

Dissecting the biological processes that determine neuronal degeneration is a clinically relevant issue. For example, the incidence of cerebrovascular disease is significant, with 150 events occurring per 100,000 individuals annually (Kurtzke, 1982). The therapeutic window for the treatment of ischemia is narrow, less than 10 hr, and requires rapid reversal of the toxic cellular events. Employing dissociated hippocampal neuronal cell cultures, we demonstrate that NO causes neuronal degeneration during periods of oxygen deprivation. In addition, NO toxicity can be prevented by application of the growth factors bFGF and EGF. Understanding the mechanisms of growth factor neuroprotection during anoxic and NO insults may direct future therapeutic modalities for cerebrovascular disease.

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