

THE neuroprotective activity of basic fibroblast growth factor (bFGF) in combination with the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 was evaluated in organotypic hippocampal slice cultures. Oxygen/glucose deprivation produced neuronal damage which was assessed using propidium iodide fluorescence. Treatment with increasing doses of bFGF demonstrated significant neuroprotection that was optimal at 10 ng ml⁻¹. This effect was diminished at higher concentrations. MK-801, at the optimal concentration of 30 μM, demonstrated greater neuroprotective efficacy than bFGF. However, bFGF significantly enhanced the protection conferred by MK-801 alone. These results suggest that neurotrophic factors such as bFGF may augment the neuroprotective effects of NMDA antagonists against ischemic neuronal injury.

Key Words: Basic fibroblast growth factor; *N*-methyl-D-aspartate; NMDA antagonists; MK-801; Ischemia; Neuroprotection; Combination therapy; Organotypic hippocampal cultures; Neuronal cell death

bFGF enhances the protective effects of MK-801 against ischemic neuronal injury *in vitro*

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Introduction

Basic fibroblast growth factor belongs to a family of structurally related polypeptide growth factors. bFGF exhibits a diverse range of mitogenic and neurotrophic activities involved in the development of the nervous system. In addition, bFGF promotes neuronal survival in the mature nervous system and provides protection against a variety of insults such as exposure to excitotoxins, hypoglycemia or axotomy-induced cell death.^{1–3} bFGF is involved with many other growth factors in the repair mechanisms initiated by ischemic injury.⁴ Ischemia induces a strong and sustained release of bFGF which probably promotes reactive astrocytosis via autocrine signaling systems.^{5,6} Direct protective effects on neurons have been observed in dissociated neuronal cultures pretreated with bFGF and exposed to ischemic-like or anoxic conditions.^{7,8} bFGF has also been able to rescue neurons and decrease the infarct size in different animal models of cerebral ischemia.^{9,10}

The mechanism by which bFGF enhances neuronal survival in response to ischemia is not fully understood but may involve stabilization of intracellular calcium levels.⁴ bFGF may also activate additional intracellular pathways that culminate in enhanced resistance to noxious insults. The purpose of this study was to examine the neuroprotective efficacy of bFGF in organotypic hippocampal slice cultures and to test therapeutic combinations with

MK-801, a non-competitive NMDA receptor antagonist, for possible additive effects against ischemic neuronal injury.

Materials and Methods

Details of the methodology have been described elsewhere.¹¹ Organotypic hippocampal slice cultures were prepared from 5- to 7-day-old neonatal rats (Sprague-Dawley, Bantin and Kingman Inc., Fremont, CA) according to the method of Stoppini *et al*.¹² and grown on sterile transparent Anocel membranes (Whatman Inc., Clifton, NJ) for 10–14 days before experimentation. The growth medium consisted of 50% MEM (Gibco Laboratories, Grand Island, NY) supplemented with HEPES and sodium bicarbonate, 25% Hanks' balanced salt solution (HBSS), 25% horse serum and glucose to a final concentration of 6.5 mg ml⁻¹. The cultures were grown at 36.5°C, 90–100% humidity and 5% CO₂. Propidium iodide (PI) (Sigma Chemical, St. Louis, MO), which rapidly enters cells with damaged membranes and becomes brightly fluorescent after binding to nucleic acids, was added (0.5 μg ml⁻¹) to the culture medium as an indicator of neuronal death during the experiments. Animals were cared for according to the guidelines of the University of Washington animal care committee.

MK-801 was obtained from Research Biochemicals Inc., Natick, MA. Human recombinant bFGF was

generously supplied by Syngene Inc., Boulder, CO. Compounds were added to the medium immediately before exposure to ischemic-like conditions and subsequently maintained in the growth medium for an additional 48 h. For induction of neuronal damage, cultures were rinsed three times in glucose-free HBSS and then placed in HBSS without glucose. All HBSS solutions were adjusted to the same molarity as the growth medium with sucrose. The temperature of each individual culture well was maintained at 37°C with an electronic thermometer. The cultures were then transferred into an anaerobic chamber (Forma Scientific) pre-equilibrated to 37°C in an atmosphere of 0% O₂, 10% H₂, 5% CO₂, and 85% nitrogen. The hydrogen was present for interaction with a palladium catalyst that maintained the oxygen concentration at 0%. The severity of neuronal damage in the control cultures varied according to the duration of anoxic/hypoglycemic exposure. Upon removal from the anaerobic chamber, the cultures were transferred to pre-warmed growth medium containing PI and placed in a CO₂ incubator at 36.5°C for 48 h.

For assessment of cell death, the cultures were examined using a Nikon Diaphot inverted fluorescent microscope. Fluorescent images were obtained using a Dage 72 CCD camera (Michigan City, IN) and were digitized using Optimas image analysis software (Bio-Scan Inc., Edmonds, WA). The intensity of PI fluorescence in the CA1 area was used as an index of cell death. The first measurement of fluorescent intensity was performed 40–48 h after initial exposure. The remaining neurons were killed by exposing the cultures to 3 h of anoxia. The fluorescent intensity obtained 24 h after 3 h of anoxia was set equal to 100% damage, and was then compared with the fluorescent intensity following the initial insult. The integrated gray values (fluorescent intensity) from CA1 following the initial insult were then expressed as a percentage of the value obtained following 3 h of anoxia (complete damage). The values were averaged for each group and expressed as mean \pm s.e.m. Statistical analysis of the results was performed using the Mann–Whitney test with Bonferroni correction for multiple comparisons. Differences were considered significant at $p < 0.05$.

Results

Marked propidium iodide fluorescence was observed in the organotypic hippocampal cultures following 35 min of oxygen/glucose deprivation. The fluorescence was predominantly located in the CA1 subfield and corresponded on average to 75% cell death in the control cultures (Fig. 1, upper panel). Treatment with increasing doses of bFGF

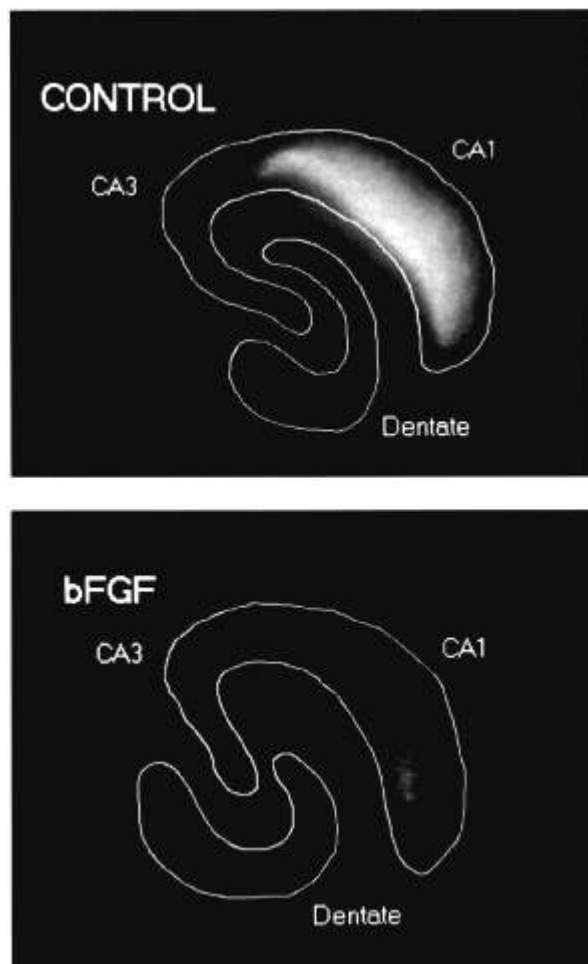


FIG. 1. (Upper panel) Propidium iodide fluorescence observed in a hippocampal culture exposed to 35 min of oxygen and glucose deprivation. The concentration of the fluorescence in the area CA1 reflects the particular vulnerability of this region to ischemia. (Lower panel) Marked reduction in propidium iodide fluorescence observed in a hippocampal culture exposed to 35 min of oxygen and glucose deprivation in the presence of 10 ng ml⁻¹ bFGF.

from 0.1 ng ml⁻¹ to 50 ng ml⁻¹ produced a dose-dependent reduction in the fluorescence intensity, corresponding to increased neuronal survival (Fig. 2). The peak effect was obtained at a concentration of 10 ng ml⁻¹, at which bFGF provided statistically significant protection against ischemic injury (Fig. 1, lower panel). The protective effects of bFGF were diminished at higher concentrations, which produced an inverse-bell shape to the dose-response curve (Fig. 2).

Previous experiments with our organotypic culture preparation had shown that 30 μ M MK-801 afforded nearly complete protection to cultures exposed to 35 min of oxygen/glucose deprivation.¹¹ For that reason, the exposure time was increased to 40 min in the combination experiments in order to produce approximately 90% cell death in the controls. For such a severe injury, the results in cultures pretreated with bFGF alone were not different from the

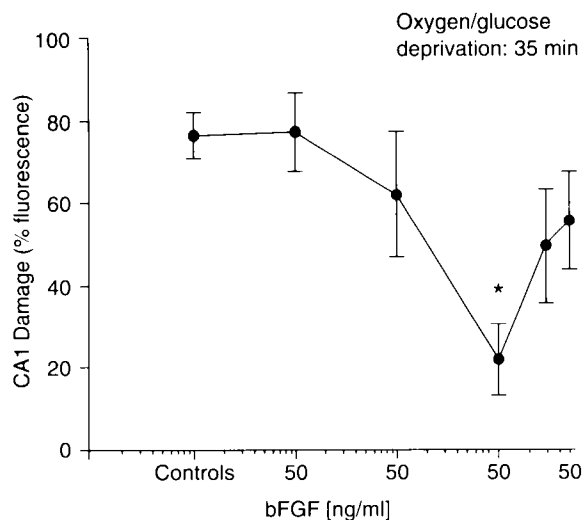


FIG. 2. Dose-response curve showing the effects of increasing concentrations of bFGF on reducing propidium iodide fluorescence in CA1 after 35 min of oxygen/glucose deprivation. Ischemic-like exposure produced $76.6 \pm 5.2\%$ cell death in the controls. Note that the neuroprotective effect of bFGF is dose-dependent with maximal and significant protection at a concentration of 10 ng ml^{-1} ($22.1 \pm 8.6\%$ cell death). $n = 10$ for each data point. * $p < 0.002$ compared with control using Mann-Whitney test with Bonferroni correction for multiple comparisons.

controls, whereas MK-801 was still able to provide statistically significant, although partial protection. However, the addition of bFGF to MK-801 enhanced the efficacy of the latter so that the combination was significantly more efficient than MK-801 alone (Fig. 3).

Discussion

Our results demonstrate that treatment with bFGF protects CA1 neurons against ischemic-like injury in hippocampal slice cultures. The neuroprotective effects of bFGF were dose-dependent in a manner similar to its other previously described mitogenic and neurotrophic effects.^{1,13,14} In our organotypic culture preparation, bFGF is active in the nanogram/milliliter range with optimal neuroprotection conferred at a concentration of 10 ng ml^{-1} . In addition, our results show that combination therapy associating bFGF with the NMDA receptor antagonist MK-801 can increase the efficacy of the latter against ischemic injury. The protective action of bFGF *in vitro* has been shown to counteract excitatory amino acid-mediated neurotoxicity by preventing the resultant rise in intracellular calcium.^{2,3} This action appears to be dependent upon new gene transcription and protein synthesis.² Fibroblast growth factors are known to exert their effects by binding to a family of receptors with intrinsic tyrosine kinase activity.¹⁵ Receptor binding and activation is thought to initiate a series of protein

phosphorylations which ultimately result in activation of transcription factors and new gene expression. Recent *in vitro* studies using hippocampal neuronal cultures have identified gene products which are possible effectors of the neuroprotective activities of bFGF. Mattson *et al*¹⁶ found that bFGF suppresses the expression of a 71 kDa NMDA receptor-related protein which mediates intracellular calcium influx. These authors have also observed that bFGF can antagonize the accumulation of oxygen reactive species following excitotoxic exposure by increasing the activity of antioxidant enzymes such as superoxide dismutase and glutathione reductase.¹⁷ Nitric oxide-mediated toxicity, which may be an important mechanism of neuronal cell death after ischemia, was prevented by administration of bFGF in neuronal cultures.⁷ bFGF has also been shown to exert a regulatory function on the protein kinase C phosphorylating system which resulted in increased survival of cultured hippocampal neurons subjected to excitotoxic and ischemic-like injury.¹⁸ Further putatively protective activities resulting from new gene transcription by bFGF in neurons include the synthesis of other calcium-regulating proteins and the initiation of genetic programs aiming to antagonize apoptotic cell death after ischemia.¹³

bFGF is also potent stimulator of glial cell proliferation and metabolic activation. As functional glia

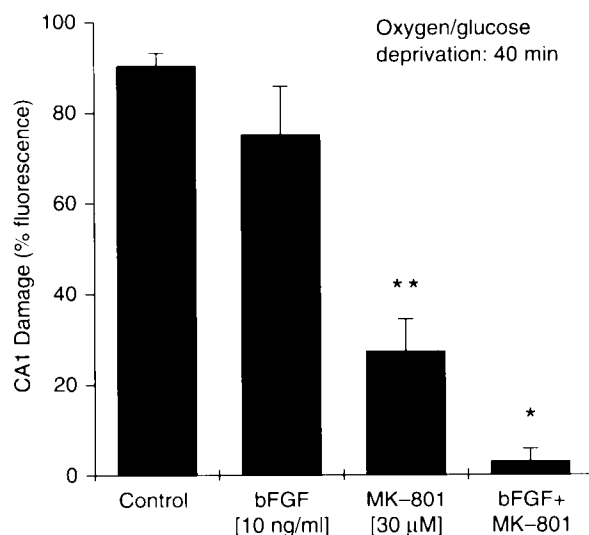


FIG. 3. Neuronal protection provided by bFGF (10 ng ml^{-1}), MK801 (30 μM), and the combination bFGF + MK-801 (same concentrations) against a severe ischemic insult in CA1. Forty minutes of oxygen/glucose deprivation produced $90.3 \pm 2.9\%$ cell death in control cultures. For such a severe insult, cultures pre-treated with bFGF alone were not significantly different from controls ($75.1 \pm 6.4\%$ cell death). MK-801 alone was still able to provide statistically significant, although partial neuroprotection ($27.5 \pm 10.9\%$ cell death). The combination bFGF + MK-801 was significantly more efficient than MK-801 alone and rescued the neurons almost completely ($3.1 \pm 3.0\%$ cell death). $n = 12$ for each group. * $p < 0.01$ (compared with MK-801 alone); ** $p < 0.002$ (compared with control) using Mann-Whitney test with Bonferroni correction for multiple comparisons.

and neuronal–glial interactions are largely preserved in organotypic hippocampal cultures, these effects could contribute to neuronal rescue after ischemia by activating glial cells to proliferate and to secrete other neurotrophic factors.^{13,19} In particular, acidic FGF (aFGF) and bFGF have been shown to stimulate nerve growth factor (NGF) synthesis and secretion by astrocytes *in vitro*.²⁰ Induction of NGF mRNA and secretion by aFGF has also been demonstrated *in vivo* in the injured neocortex of adult rats.²¹ In animal models of cerebral ischemia, bFGF may possibly have further protective effects by altering systemic and cerebral physiological parameters such as arterial blood pressure, vascular tone and cerebral blood flow.¹⁰

Application of combinations of pharmacological agents to block several steps of the cascade leading to cellular death after ischemia opens new promising possibilities of increasing the extent of neuroprotection.²² Our results support the conclusion that the association of a neurotrophic factor and an NMDA receptor antagonist constitutes a potentially useful therapeutic approach against ischemic neuronal damage. Competitive and non-competitive NMDA receptor antagonists have proved to possess strong neuroprotective effects against a variety of neuronal injuries and some of them are currently in advanced testing for clinical use against stroke and traumatic brain injury.²³ NMDA receptor blockade opposes an early and fundamental step of the excitotoxic cascade and results in marked neuroprotection in our model. Although it provided significant protection, bFGF in contrast, never showed such potent efficacy in our preparation, probably because it affects events subsequent to the activation of NMDA receptors and requires time-dependent processes such as new gene transcription and protein synthesis. However, based on its ability to regulate calcium homeostasis and initiate other putatively protective intracellular events, bFGF appears to ideally complement excitatory amino acid receptor antagonists because it counteracts further downstream steps of the excitotoxic cascade.

NMDA receptor antagonists have been tested in other *in vitro* and *in vivo* combinations with variable success. The most promising results have been obtained in combinations using non-NMDA glutamate receptor antagonists, GABAergic agonists, or

oxygen free radical scavengers.^{24,25} Our results indicate that polypeptide growth factors such as bFGF or pharmacological analogs with comparable intracellular effects could also be considered as potential candidates for inclusion in therapeutic combination protocols for brain ischemia.

Conclusion

Our results confirm the neuroprotective effects of bFGF in organotypic hippocampal slice cultures and show that bFGF can significantly enhance the efficacy of NMDA receptor blockade against ischemic neuronal damage. These results suggest that neurotrophic factors with proven efficacy could be included in the elaboration of a therapeutic cocktail aiming to protect the brain against ischemic injury.

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