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Synergistic protective effect of caspase inhibitors and bFGF against brain injury induced by transient focal ischaemia

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1 We tested the hypothesis that combined use of trophic factors and caspase inhibitors increases brain resistance to ischaemia in mice.

2 Intracerebroventricular administration of bFGF (>10 ng) 30 min after MCA occlusion decreased infarct size and neurological deficit in a dose-dependent manner following 2 h ischemia and reperfusion (20 h).

3 Combined administration of the subthreshold doses of bFGF (3 ng) and caspase inhibitors (z-VAD.FMK, 27 ng or z-DEVD.FMK, 80 mg) reduced infarct volume by 60%, and reduced neurological deficit.

4 Treatment with a subthreshold dose of bFGF (3 ng) extended the therapeutic window for z-DEVD.FMK (480 ng) from 1 to 3 h after reperfusion.

5 Caspase-3 activity in the ischaemic brain was increased 30 min and 2 h after reperfusion but, was significantly reduced in bFGF-treated animals by 29 and 16%, respectively. Caspase-3 activity was not reduced by a direct bFGF effect because addition of bFGF (10 nM–2 μM) did not decrease recombinant caspase-3 activity, *in vitro*.

6 Our data show that combining caspase inhibitors and bFGF lengthens the treatment window for the second treatment, plus lowers the dosage requirements for neuroprotection. These findings are important because low doses of caspase inhibitors or bFGF reduce the possibility of side effects plus extend the short treatment window for ischaemic stroke.

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Abbreviations: bFGF, basic fibroblast factor; DMSO, dimethylsulphoxide; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; eNOS, endothelial nitric oxide synthase; MAPK, mitogen-actived protein kinase; MCA, middle cerebral artery; NF-κB, nuclear factor–kappa B; NMDA, N-methyl-D-aspartate; TTC, triphenyltetrazolium choloride; z-DEVD.FMK, N-benzyloxycarbonyl-Asp(Ome)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; z-VAD.FMK, N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoro-methylketone

Introduction

Apoptotic mechanisms promote cell death in brain ischaemia (see MacManus & Linnik 1997 for review). Cleavage and activation of caspase-3 (Namura *et al*., 1998) and caspase-1 (Hara *et al*., 1997a) as well as upregulation of Bax/Bcl-2 ratio have been documented (Gillardon *et al*., 1996; Isenmann *et al*., 1998). In line with these findings, caspase inhibitors significantly reduce infarct volume when administered even 9 h after 30-min MCA occlusion, suggesting that activation of execution caspases evolves slowly with delayed therapeutic opportunity in mild ischaemia (Fink *et al*., 1998). In more severe ischaemia (2 h of occlusion), caspase inhibitors block cell death up to 2 h post-ischaemia (Hara *et al*., 1997b, see also Rothwell *et al*., 1997). Although longer than for NMDA receptor antagonists, this time window is still shorter than desirable for clinical practice. Recently, Ma *et al*. (1998) reported that an NMDA receptor antagonist, MK-801 augments the efficacy of caspase inhibitors and *vice versa* in a mouse model of focal cerebral ischaemia. Not only did combination of subthreshold doses protect against ischaemia but also the therapeutic window was extended beyond 3 h. A similar synergy between MK-801 and caspase inhibitors has also been shown for malonate-induced striatal toxicity in the rat (Schulz *et al*., 1998).

Unlike NMDA receptor antagonists, which decrease the intensity of ischaemic injury primarily by reducing Ca^{+2} influx, and as a consequence, delay caspase activation (Mattson *et al*., 1998; Budd *et al*., 2000), growth factors directly counteract cell death by promoting survival proteins which suppress apoptotic mechanisms (Datta *et al*., 1999). Promoting cell survival while inhibiting death effectors is a potentially promising neuroprotective approach, which may provide a longer treatment window for caspase inhibitors as well as insight into cellular mechanisms of death and survival after cerebral ischaemia. Basic fibroblast factor (bFGF) is a growth factor with neuro-, glio- and angio-trophic properties. bFGF protects the brain against transient as well as permanent focal ischaemia (Koketsu *et al*., 1994; Fisher *et al*., 1995; Jiang *et al*., 1996). Part of its protection may be due to eNOS-dependent

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vasodilation, however, knockout mice that do not express the gene for endothelial NOS, do not show cerebrovasodilation to bFGF administration but develop significantly smaller infarcts when subjected to middle cerebral artery (MCA) occlusion plus bFGF treatment (Huang *et al*., 1997).

We tested the hypothesis that combined use of trophic factors and caspase inhibitors increases brain resistance to ischaemia. Accordingly, we asked whether or not bFGF and caspase inhibitors enhance each other's activity when used in combination. Our data indicate that combining subthreshold doses protected against ischaemic cell death and, extended the therapeutic window for caspase inhibitors from 3 to 5 h post-ischaemia after 2 h MCA occlusion.

Methods

Physiology

Adult male 129/SV mice ($n=128$, Taconic Farms, Germantown, NY, U.S.A.), weighing 18–23 g, were allowed free access to food and water *ad libitum*. All animals were kept under diurnal lighting conditions. Anaesthesia was induced by 2% and was maintained by 1% halothane in 70% N_2O and 30% O_2 using a Fluotec 3 vaporizer (Colonial Medical, Amherst, NH, U.S.A.). Rectal temperature was maintained at approximately 36.5–37°C with a thermostatically controlled blanket (FHC, Brunswick, ME, U.S.A.) and a heating lamp during surgery. Since mice become hypothermic during the first 6 h after ischaemia, they were kept in an incubator (ThermoCare System, Incline Village, NV, U.S.A.) at 32°C and 45% humidity for 6 h after ischaemia.

Ischaemia model

Focal cerebral ischaemia was induced by occlusion of the left middle cerebral artery (MCA) with an 8-0 nylon monofilament (Ethicon, Somerville, NJ, U.S.A.) coated with a mixture of silicone resin (Xantopren, Bayer Dental, Osaka, Japan) and hardener (Elastomer Activator, Bayer Dental) as described previously (Hara *et al*., 1996). This coated filament was introduced into the external carotid artery up to the origin of the anterior cerebral artery through the internal carotid artery. For filament withdrawal, mice were briefly reanaesthetized with halothane after 2 h of ischaemia. Animals were sacrificed with an overdose of pentobarbitone (200 mg kg^{-1}, i.p.) 20 h after reperfusion.

Drugs

N-Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoro-methylketone (z-VAD.FMK) and N-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (z-DEVD.FMK) were obtained from Enzyme Systems Products (Dublin, CA, U.S.A.). Compounds were dissolved in 0.4% dimethylsulphoxide (DMSO, MC/B, Norwood, OH, U.S.A.) in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Recombinant human bFGF was obtained as a concentrated stock solution (2 mg ml^{-1}, Scios Inc., Mt. View, CA, U.S.A.) in 10 mM sodium citrate, 9% sucrose, 1 mM EDTA (pH 5.0) and, stored at −80°C before use. The bFGF stock solution or mock stock solution (vehicle) was diluted in 0.9% NaCl containing 100 μg ml^{-1} bovine serum albumin (Boehringer-Mannheim, catalogue No. 711454) to give a final bFGF concentration of 3–100 ng (pH = 7.4). Two μl of either bFGF or mock (vehicle) solution or z-VAD.FMK, z-DEVD.FMK or 0.4% DMSO solution (vehicle) were injected intracerebroventricularly (i.c.v.) using a Hamilton syringe (needle diameter: 0.508 μm; total volume 10 μl). The syringe was placed perpendicular to the skull at the following coordinates relative to the bregma; 0.9 mm lateral, 0.1 mm posterior and 3.1 mm deep and, free-handed injections were made 30 min after ischaemia while the animal was still anaesthetized.

Neurological deficits

Mice were rated for behavioural changes by an observer naïve to the treatment group as described before (Hara *et al*., 1996): 0: no observable neurological deficits (normal), 1: failure to extend right forepaw (mild), 2: circling to the contralateral side (moderate); 3: loss of walking or righting reflex (severe).

Infarct measurement

The brains were removed and sectioned coronally into five 2-mm sections in a mouse brain matrix (RBM-2000C, Activational System, MI, U.S.A.). Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO, U.S.A.) in PBS, followed by 10% formalin overnight. The infarction area on each slice was measured by an image-analysis system (M4, Imaging Research, St. Catherines, Ontario, Canada) on the posterior surface of each section, and the total infarction volume was calculated by summing the volume of each slice as described before (Huang *et al*., 1994).

Experimental protocols

For dose-escalation study, mice were injected with bFGF (3, 10, 30 and 100 ng) or an equivalent volume of mock solution 30 min after MCA occlusion.

To study the effect of combined administration of subthreshold doses of caspase inhibitors and bFGF, z-VAD.FMK (27 ng) or z-DEVD.FMK (80 ng) or their vehicle (0.4% DMSO) were injected together with bFGF (3 ng) or its vehicle (mock solution) 30 min post-ischaemia.

For combination of a subthreshold dose of bFGF with delayed administration of a caspase inhibitor, bFGF (3 ng) or mock solution was injected 30 min after occlusion and, z-DEVD.FMK (480 ng) or its vehicle (0.4% DMSO) was injected 3 h after reperfusion.

Caspase-3 like protease activity assay

Mice were sacrificed 30 min and 2 h after reperfusion. Forebrains, after separating the frontal pole (2 mm), occipital pole (1 mm), hippocampus and brain stem, were immersed into ice-cold caspase-3 lysis buffer [10 mM HEPES-KOH (pH 7.4), 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate, 1 mM phenylmethylsulphonyl fluoride and 5 mM dithiothreitol (DTT)] and, were homogenized with a Dounce homogenizer (Qiu *et al*., 2000).

After centrifuging at 13,000 × *g* and 4°C, the supernatant was removed and the protein concentration was detected. A total 100 μg of protein was incubated with the assay buffer [20 mM HEPES-KOH (pH 7.5), 2 mM DTT, 10% glycerol, and 40 μM Ac-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (DEVD-MCA, Biomol, Plymouth Meeting, PA, U.S.A.)] for 1 h at 37°C. The reaction was stopped by adding 0.9 ml of cold water and placing the reaction mixtures on ice for at least 10 min. The intensity of fluorescence of each solution was measured by fluorescence spectrophotometry (Hitachi F-2000; Hitachi Instruments, Tokyo, Japan) at 380 nm excitation and 460 nm emission wavelengths. All readings were standardized using the fluorescence intensity of an equal volume of free 7-amino-4-methyl-coumarin (AMC) solution.

To test whether bFGF directly inhibited caspase-3 activity, bFGF (10 nM- 2 μM) was incubated with 1 μg of recombinant caspase-3 (Biomol, Plymouth Meeting, PA, U.S.A.) in assay buffer for 1 h, and then caspase-3 like protease activity was determined as described above. Complete inhibition of enzyme activity by 100 μM of DEVD-CHO was used to compare.

Statistical analysis

Data are expressed as mean ± s.e.mean. Statistical analysis was performed by one-way (dose escalation study) or two-way (combination treatments) ANOVA followed by Bonferroni's test. For evaluation of neurological deficits, Kruskal-Wallis nonparametric variance analysis test was used. The softwares INSTAT 2.0 (GraphPad Software, San Diego, CA, U.S.A.) or super ANOVA (Abacus, Berkeley, CA, U.S.A.) was used for statistical analysis. $P<0.05$ was considered of statistical significance.

Results

bFGF dose-dependently decreases infarct size

Intracerebroventricular administration of bFGF decreased infarct size and neurological deficit in a dose-dependent manner (Figure 1). A dose of 3 ng was ineffective but 10 ng of bFGF or greater reduced the infarct volume to 60 ± 5 mm^3 (10 ng, $n=5$), 35 ± 8 mm^3 (30 ng, $n=4$) and 31 ± 8 mm^3 (100 ng, $n=5$). However, only protection obtained with doses greater than 10 ng was significantly ($P<0.05$) different from the vehicle group (94 ± 13 mm^3, $n=5$). Neurological deficit scores 20 h after recirculation were 2.0 ± 0, 2.0 ± 0, 1.3 ± 0.2, 1.3 ± 0.3 and 1.4 ± 0.2 for control, 3, 10, 30 and 100 ng bFGF-treated mice, respectively. All but the 3 ng bFGF group were significantly different than the vehicle-treated mice ($P<0.05$).

Combined subthreshold doses of caspase inhibitors and bFGF

The above data show that 3 ng bFGF does not protect against infarct volume after 2 h of transient MCA occlusion. Based on previously reported results, 27 ng of z-VAD.FMK or 80 ng of z-DEVD did not reduce infarct size in this same model, whereas higher doses did (Hara *et al*., 1997b). Combined administration of the subthreshold doses of caspase inhibitors with bFGF (3 ng) 30 min after MCA occlusion reduced infarct volume by 60% (Figure 2). Neurological deficits were also significantly improved compared to their controls (Table 1).

A subthreshold dose of bFGF prolongs the therapeutic window for caspase inhibitors

Treatment with a subthreshold dose of bFGF (3 ng) given 30 min after ischaemia extended the therapeutic window for

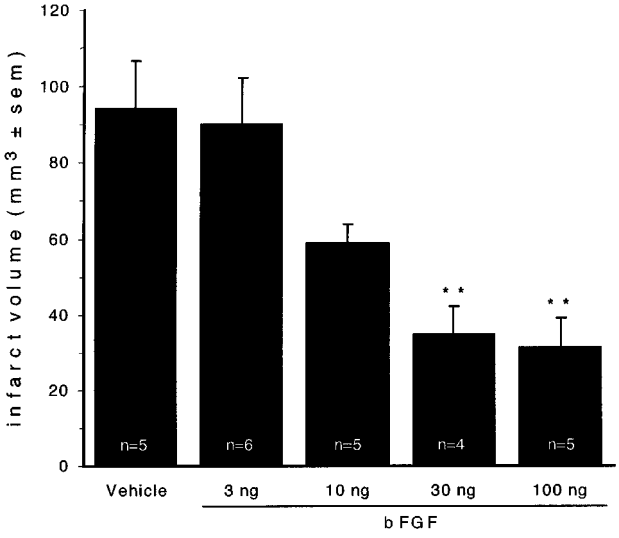

Figure 1 Intracerebroventricular administration of bFGF 30 min after MCA occlusion decreased infarct size in mice subjected to 2 h of ischaemia and 20 h of reperfusion in a dose-dependent manner. Vehicle: an equivalent volume of mock solution. $^{**}P<0.05$, compared to vehicle-treated control group.

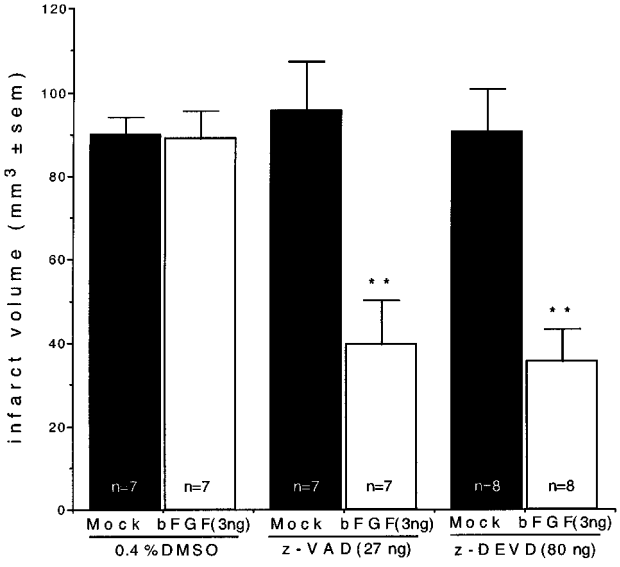

Figure 2 Combined administration of subthreshold doses of caspase inhibitors (27 ng of z-VAD.FMK or 80 ng of z-DEVD.FMK) plus a subthreshold dose of bFGF (3 ng) 30 min after MCA occlusion reduced infarct volume in mice subjected to 2 h of ischaemia and 20 h of reperfusion. As sham controls, equivalent volume of vehicles (mock or DMSO) were injected intraventricularly with bFGF or caspase inhibitors. $^{**}P<0.05$, compared to vehicle-treated control groups.

Table 1 Neurological deficit scores after 2 h of ischaemia and 20 h of recirculation in groups treated with a subthreshold dose of bFGF and a caspase inhibitor

	bFGF 3 ng and caspase inhibitor	*mock and caspase inhibitor*	*bFGF and DMSO*	*mock and DMSO*
z-VAD 27 ng	1.1±0.3*	1.9±0.1	1.7±0.2	
z-DEVD 80 ng	1.3±0.3*	1.9±0.1	1.7±0.2	
z-DEVD 480 ng	0.8±0.2*	2.1±0.1	2.0±0.0	2.1±0.1

First and second rows: bFGF (3 ng) was coadministered with either z-VAD (27 ng) or z-DEVD (80 ng) or their vehicle (DMSO). Third row; bFGF (3 ng) was administered 30 min and, z-DEVD (480 ng), 5 h after MCA occlusion. For sham controls, the vehicles (mock or DMSO) were injected with bFGF or caspase inhibitors or both together. *$P<0.05$, compared to vehicle-treated control groups.

z-DEVD.FMK (480 ng) from 1 to 3 h after reperfusion. z-DEVD.FMK (480 ng) administered 3 h after reperfusion without bFGF did not decrease infarct size except after pretreatment with a subthreshold dose of bFGF. In the latter experiment, infarct size was reduced by 48.5% (Figure 3) and neurological deficits were significantly less (Table 1).

bFGF pretreatment inhibits caspase-3 activity

To gain insight into mechanisms of synergy, we measured caspase-3 like protease activity in homogenates prepared from the ischaemic hemispheres of mice treated with bFGF or its vehicle. bFGF (3 ng) was injected intraventricularly on the 30th min of a 2-h MCA occlusion as in the other protocols used to show synergy between compounds. Since the activity detected in sham operated brains was not reduced by adding 100 μM zDEVD-fmk, it was subtracted from measurements of zDEVD-inhibitable enzyme activity obtained from ischaemic brains, as non-specific activity (Namura *et al*., 1998). Caspase-3 activity was above baseline at 30 min and 2 h after reperfusion as reported previously (Namura *et al*., 1998), but, was significantly reduced in bFGF-treated animals by 29 and 16%, respectively ($P<0.05$, Figure 4). This inhibition was not caused by a direct effect of bFGF on caspase-3 activity because we found that bFGF concentrations varying from 10 nM to 2 μM, had no effect on recombinant caspase-3 activity, *in vitro*.

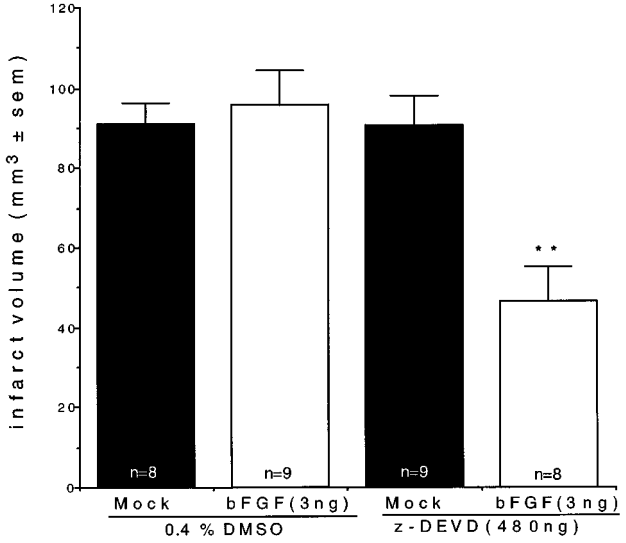

Figure 3 Treatment with a subthreshold dose of bFGF (3 ng) injected 30 min after MCA occlusion extended the therapeutic window for a maximally effective dose of z-DEVD.FMK (480 ng) from 1 to 3 h after reperfusion. Mice were subjected to 2 h of MCA occlusion and 20 h of reperfusion. As sham controls, the vehicles (mock or DMSO) were injected intraventricularly with bFGF or caspase inhibitors or both together. **$P<0.05$, compared to vehicle-treated control groups.

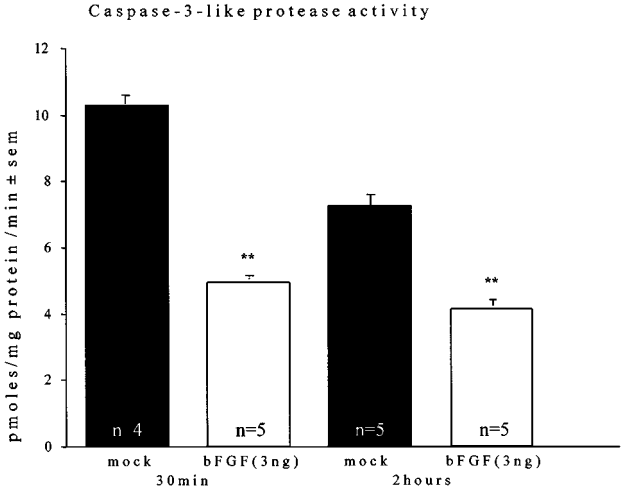

Figure 4 bFGF inhibited the increase in caspase-3-like protease activity in the ischaemic hemisphere. bFGF (3 ng) was injected intraventricularly on the 30th min of a 2-h MCA occlusion and, caspase-3-like activity (DEVD-MCA cleavage) was detected by spectrophotometry 30 min and 2 h after reperfusion. Values are mean (±s.e.m.) of 4 (30 min) and 5 (2 h) vehicle-(filled columns), and 5 (30 min) and 5 (2 h) bFGF-treated (open columns) mouse brains.

Discussion

Intracerebroventricular administration of bFGF decreases infarct volume in rats subjected to MCA occlusion (Koketsu *et al*., 1994; Fisher *et al*., 1995; Jiang *et al*., 1996). Using a similar model in mice, we found that bFGF (>3 ng) decreased infarct size and neurological deficit in a dose-dependent manner. Although 3 ng bFGF was not effective alone, combined with subthreshold doses of caspase inhibitors infarct size was reduced by 60% (approximately the maximal protection obtained in this model) and improved neurological deficit. Moreover, the subthreshold dose of bFGF prolonged the therapeutic window for caspase inhibitors given at maximally effective doses from 1 to 3 h after reperfusion. Caspase activity in ischaemic brain homogenate was attenuated after bFGF treatment, although bFGF did not suppress DEVD cleaving activity directly, *in vitro*. Taken together, these data indicate that bFGF given 30 min after the induction of ischaemia was able to activate cell survival pathways and inhibit caspase-mediated cell

death. Understanding the mechanisms of synergy may provide further insight to ischaemic cell death and help devise novel strategies for neuroprotection.

Ischaemia triggers a complex cascade of events leading to swelling and necrosis following collapse of mitochondrial functions; other less severely-injured cells die by a process involving caspase activation or by a combination of mechanisms including necrosis and apoptosis (MacMannus & Linnik, 1997; Chalmers-Redman *et al*., 1997). Caspase inhibitors decrease tissue injury in experimental stroke models (Hara *et al*., 1997a, b; Endres *et al*., 1998; Fink *et al*., 1998), however, the treatment window for these drugs tends to be rather short (a few hours) following 2-h reversible occlusion (Hara *et al*., 1997b). Caspase inhibitor plus MK-801 extended the treatment window for both drugs and also lowered the therapeutic dose (Ma *et al*., 1998), not unlike what was observed after bFGF plus inhibitors. Hence, the commitment point for cell death was delayed by combination therapy. Unlike NMDA antagonists, which reduce excitotoxic (mainly necrotic) cell death that develops rapidly at the onset of ischaemia, growth factors may have a more favourable treatment window perhaps because they act on downstream cell death pathways.

Treatment with bFGF protects cells by more than a single mechanism. Like the NMDA receptor antagonist, MK-801, bFGF decreases NMDA receptor-mediated Ca2+ influx and excitotoxicity in hippocampal neurons (Mattson *et al*., 1995) and upregulates the expression of free radical scavenging enzymes (Mattson & Scheff, 1994; 1995). By so doing, the stimulus for necrosis is diminished, as is the development of apoptosis for those cells less severely affected. bFGF decreases proapoptotic activity by modulation of upstream signalling mechanisms. For example, bFGF hinders the release of the proapoptotic BCL-2 family member BAD from cytoplasmic 14-3-3 protein (Datta *et al*., 1997). In addition, bFGF prevents BAD translocation to mitochrondria *via* neurotrophin receptor-phosphoinositide-3-kinase-Akt pathway and by mitogen-activated protein kinase (MAPK)/Ras-Raf pathway (Kaplan & Miller, 1997; Pettmann & Henderson, 1998; Tamatani *et al*., 1998a, b; Liu & Zhu, 1999a, b; Datta *et al*., 1999). bFGF has also been shown to stimulate *de novo* synthesis of BCL-XL through activation of MAPK possibly by phosphorylation of transcription factor CREB (Bryckaert *et al*., 1999; Finkbeiner, 2000). Moreover, phosphoinositide-3-kinase-Akt pathway can also promote cell survival by decreasing transcription of Fas receptor and activating NF-κB (Datta *et al*., 1999). Although more than a single mechanism seems likely, bFGF-induced phosphorylation of BAD and other kinase-promoting mechanisms appear more promising than mechanisms requiring transcription and protein synthesis because translational events are suppressed during ischaemia (Hossmann, 1993).

A subthreshold dose of MK-801 may decrease Ca^{2+} load (Choi, 1998), but is clearly not enough to prevent cell death. Nevertheless, reduced intracellular Ca^{2+} may slow down the death processes and allow caspase inhibition to reverse cell death before the commitment point or to be protective at lower doses. Pretreatment with subthreshold doses of bFGF and caspase inhibitors may protect cells in a similar way, although the precise mechanism remains for further study.

We did not determine whether combination therapy delays the onset of the initial treatment after stroke, a point of clinical relevance, or augments the absolute volume of protection in this model. Nevertheless, our studies did show that combining caspase inhibitors with bFGF lengthens the treatment window for the second treatment plus lowers the dosage requirements for neuroprotection. These findings are important because lower doses reduce the possibility of side effects, already reported for certain growth factors (Favoni & de Cupis, 2000), plus extend the short treatment window for ischaemic stroke.

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