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Anti-apoptotic therapy with a Tat fusion protein protects against excitotoxic insults *in vitro* and *in vivo*

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Abstract

A number of gene therapy approaches have been developed for protecting neurons from necrotic neurological insults. Such therapies are limited by the need for transcription and translation of the protective protein, delaying therapeutic impact. As an alternative, we explore the neuroprotective potential of protein therapy, using a fusion protein comprised of the death-suppressing BH4 domain of the Bcl-xL protein and the protein transduction domain of the human immunodeficiency virus Tat protein. This fusion protein decreased neurotoxicity caused by the excitotoxins glutamate and kainic acid in primary hippocampal cultures, and decreased hippocampal damage *in vivo* in an excitotoxic seizure model.

INTRODUCTION

There has been increasing use of gene therapy approaches, typically utilizing viral vectors (Verma et al., 2005) to protect neurons from *in vitro* and *in vivo* models of necrotic neurological insults (such as stroke and ischemia) (Sapolsky, 2003). Unfortunately, drawbacks to this approach include a) an invasive delivery method; b) the fact that viral vectors do not disperse well in target tissues; c) a time lag in the production of adequate amounts of functional protein, particularly given broad inhibition of protein synthesis in challenged neurons (Dwyer et al., 1986; Eck, 1999; Anderson, 1998; Bonadio, 2000; Weise et al., 2000).

An alternative would be protein therapy, using cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs), to deliver the biologically active therapeutic protein. The most commonly studied CPPs are Antennapedia (Antp) (Prochiantz, 2000; Joliot and Prochiantz, 2004), the herpes simplex virus (HSV) type 1 protein VP22 (Elliot and O'Hare, 1997), and the human immunodeficiency virus (HIV-1) transactivator Tat protein (Schwarze et al., 1999; Embury et al., 2001). CPPs generally have a net positive

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charge, are less than 30 amino acids long, and can transport cargoes, independent of their size, into cells.

The present study utilizes a CPP-protein therapy approach, using a fusion protein combining the Tat-PTD protein with a fragment of Bcl-xL, a well-characterized member of the anti-apoptotic Bcl-2 family (Gonzalez-Garcia et al., 1995; Motoyama et al., 1995; Parsadanian et al., 1998). Specifically, we have used the conserved N-terminal homology domain (BH4) of Bcl-xL, which appears essential to the neuroprotective effects of the entire protein (Shimizu et al., 2000). We examine its protective potential against models of excitotoxicity in primary hippocampal cultures, and the hippocampus *in vivo*.

MATERIALS AND METHODS

Preparation of Tat-MYC and Tat-Bcl-xL Fusion Proteins

A fusion protein containing the BH4 domain of Bcl-xL (amino acids 4–23) linked to the 10 amino acid Tat PTD (HIV-Tat₄₈₋₅₇) through a β -alanine spacer was obtained (Calbiochem, #197217); previous work has shown that this fusion protein enters target cells (Shimizu et al., 2000). A control construct, Tat-MYC (2.6 kDa), was graciously provided by Suorjit Paul and Paul Lombroso. For *in vitro* experiments, 1 mg of protein was dissolved in 653.5 μ L dimethyl sulfoxide (DMSO) yielding a concentration of 400 μ M Tat-Bcl-xL. For *in vivo* experiments, 1 mg was dissolved in 65 μ L DMSO and 588.5 μ L 1x PBS.

Primary Hippocampal Cultures

Mixed neuronal/glia hippocampal cultures were prepared from day 18 fetal rats by standard culturing techniques (Brooke et al., 1997). Cells were plated at a density of 1.2×10^5 cells/cm² on 96-well plates coated with poly-D-lysine (Sigma, St. Louis, MO), and were used on day 11. Under these conditions, cultures are approximately 20–30% neuronal.

In Vitro excitotoxicity

Fusion proteins (Tat-MYC or Tat-Bcl-xL) were first diluted to a concentration of 40 μ M using warm (37°C) minimal essential medium (MEM; Gibco). 10 μ L of the appropriate protein or MEM was added to each well (containing 200 μ L of media), yielding a concentration of the protein of 2 μ M. Two hours later, media were replaced with media containing indicated concentrations of glutamate or kainic acid (Sigma), or media alone. In other experiments, fusion protein was added either at the time of addition of the excitotoxins, or at indicated times thereafter. Other studies using Tat fusion proteins, including Tat-Bcl-xL, have shown that the fusion protein is present at least 24 hours after transduction, both *in vitro* and *in vivo* (Lai et al., 2005; Yin et al., 2006). Neuron death was quantified 24 hours after addition of excitotoxin.

Quantification of neuron loss

Neuron loss was quantified immunohistochemically with an ABTS assay (Brooke et al., 1999), an ELISA-based method, making use of MAP2 monoclonal antibodies (Sigma), secondary antibodies (Vectastain ABC kit), and the ABTS kit (Vector, Burlingame, CA, USA). As part of the assay, some wells were treated with a high concentration of excitotoxin

(5 mM) known to kill all the neurons (Brooke et al., 1999), but spare some glia. These wells were used as blanks in the assay.

For visualization of MAP2 stain, ABTS solution was removed from each plate, followed by three washes with 1x PBS. Peroxidase labeling was resolved using FAST-DAB solution as a substrate for 6–8 min (Vector Labs Burlingame, CA) according to the manufacturer's guidelines. Bright field digital images were captured with an Olympus IX70 microscope and Hamamatsu camera (model C4742-95).

In vivo excitotoxicity

Fusion protein (0.03 µg), either Tat-MYC or Tat-Bcl-xL, and kainic acid (0.06 µg) in PBS was injected stereotactically into the dentate gyrus of the left hemisphere and kainic acid alone was injected into the dentate gyrus of the right hemisphere of adult male Sprague-Dawley rats (275–350g; Charles Rivers) (coordinates from bregma: AP –4.8 mm, ML +/–3.0 mm, and DV –3.0 mm from the surface of the dura). Protein was delivered in 1 µL delivered at a rate of 0.2 µL/min. After delivery, syringes were left in tissue for 5 minutes to allow for diffusion. A second, age-matched group of animals received vehicle alone as positive control.

Twenty-four hours later, rats were perfused, brains removed and post-fixed in 4% paraformaldehyde/20% sucrose solution for approximately 24 hours, sliced into 30 µm sections, and cresyl violet stained. METAMORPH software (Universal Imaging) was used to quantify pixels of intact versus damaged/missing CA3 neurons in 40 coronal sections through the entire hippocampus at ~0.1 mm increments, and performed blind to treatment.

Data analysis

Data analysis was done with SigmaStat, (Jandel Scientific). *In vitro* data were analysed by one-way ANOVA followed by Tukey post hoc tests (SigmaStat, Jandel Scientific). Cell death is expressed as a percentage of the intrinsic cell death from changing media in control wells incubated without the respective excitotoxin. *In vivo* data were analyzed by paired t-tests performed comparing hemispheres receiving and not receiving Tat-Bcl-xL or Tat-MYC. A $p < 0.05$ is considered statistically significant. Data are presented as mean +/- SEM.

RESULTS

Pilot studies indicated that Tat-fusion proteins were not toxic in this system; neither 2 µM Tat-Bcl-xL nor the control protein Tat-MYC were toxic (data not shown). Representative images from *in vitro* experiments with glutamate illustrate neuronal morphology (Figure 1; neuronal morphology was similar in the subsequent studies utilizing KA; data not shown). Incubation with 50µM glutamate for 24 hours, after a prior two hour incubation with either vehicle or 2 µM Tat-MYC, caused approximately 50% loss of neurons in both treatments and did not differ significantly. However two hour pre-incubation with 2 µM Tat-Bcl-xL prior to glutamate exposure significantly decreased neuron loss (Figure 2).

Incubation with 50 μ M KA for 24 hours, after a prior two hour incubation with either vehicle or 2 μ M Tat-MYC, caused approximately 50–55% loss of neurons in both treatments and did not differ significantly (Figure 3A), while 100 μ M KA caused an approximate 80% loss in both treatments (Figure 3B). However pre-incubation with Tat-Bcl-xL was markedly protective against both KA concentrations.

In the setting of clinical neurology, treatments are typically administered post-injury. Thus, we explored the time window for Tat-Bcl-xL's protective effects, relative to excitotoxin exposure. Tat-Bcl-xL was protective, whether administered two hours before KA exposure (neurotoxicity in Tat-Bcl-xL/KA wells: 52 $\pm$ 5% that of KA alone; $p < 0.01$), at the time of KA exposure (Tat-Bcl-xL/KA: 57 $\pm$ 7% of KA alone; $p < 0.05$), or two hours after KA exposure (43 $\pm$ 8% of KA alone; $p < 0.05$).

We next tested whether Tat-Bcl-xL would protect against KA-induced status epilepticus neurotoxicity *in vivo*. Saline, Tat-MYC or Tat-Bcl-xL was stereotactically infused into the dentate gyrus and, 30 minutes later, KA was infused at the same site. The Tat-MYC fusion protein did not show any protection, as the area of intact neurons in the CA3 area is almost equal to that of the contralateral hemisphere receiving only KA. Tat-Bcl-xL, on the other hand, was protective, resulting in approximately 50% more intact neurons in the CA3 area on the hemisphere receiving Tat and KA compared to the contralateral hemisphere that received KA alone (Figure 4).

DISCUSSION

Various gene therapy studies expressing an array of transgenes have demonstrated protection against the neuron loss caused by necrotic neurological insults (Sapolsky, 2003); despite such positive findings, the general approach suffers from the limitation of the lag time between introduction of the vector and the production of functional protein. For example, initial gene expression with HSV vectors takes approximately 2 hours, with latencies even longer for other viral vectors (Oehmig et al., 2004). In exploring the alternative approach of protein therapy, we observe that Tat-Bcl-xL protects against excitotoxins both *in vitro* and *in vivo*, even when administered two hours post-insult.

The Bcl-xL gene was chosen for this study because of its anti-apoptotic properties. The protein is a member of the Bcl-2 family, is expressed in adult neurons of the CNS, and can play an essential role in preventing neuron cell death (Gonzalez-Garcia et al., 1995; Motoyama et al., 1995; Kharbanda et al., 1997; Parsadanian et al., 1998; Sastry and Rao, 2000). Bcl-xL, specifically the conserved N-terminal homology domain (BH4), is localized primarily in the mitochondrial membrane and the nuclear envelope (Tsujimoto and Shimizu, 2000), and prevents cytochrome *c* release and the decline of mitochondrial potential by directly modulating the activity of the voltage-dependent anion channels (Kluck et al., 1997; Yang et al., 1997; Susin et al., 1999; Shimizu et al., 2000); in addition, there are reports of Bcl-xL interacting with caspases (Hu et al., 1998; Pan et al., 1998).

The mechanisms underlying the efficacy of cell penetrating peptides remain unclear, with uncertainty as to the temperature or energy dependency of cell penetration, and whether it

involves endocytosis (Derossi et al., 1996; Vives et al., 1997; Futaki et al., 2001; Tung et al., 2002). Amid this uncertainty, the HIV-1-derived Tat peptide (specifically its N-terminal domain) is well characterized for its cell penetrating capacity. It is capable of delivering functional fusion proteins to a variety of cell types (Zhao et al., 2004), including neurons (Schwarze et al., 1999). The fusion of anti-apoptotic Bcl-xL to cell-penetrating Tat holds great promise for being neuroprotective, a property that has been supported in neurodegeneration studies (Dietz et al., 2002; Kilic et al., 2002; Dietz & Bähr, 2005; Diem et al., 2005; Ebert et al., 2005; Dietz et al., 2006) as well as in several other models (Cantara et al., 2004; Klein et al., 2004; Ono et al., 2005; Pizzi et al., 2005; Hotchkiss et al., 2006).

We observed Tat-Bcl-xL to protect against glutamate and KA *in vitro*, and against KA *in vivo*. Previous studies with a Tat fusion protein containing the identical BH4 sequence fused to Tat at the same amino acid as in the present case showed that the fusion protein does indeed enter cells and localize to the mitochondria (Shimizu et al., 2000). Our results are in agreement with a report that systemic delivery of Tat-Bcl-xL protects neurons in a stroke model (Cao et al., 2002). Many gene therapy studies utilize viral vectors that are neurotrophic (e.g., HSV-1), with a pronounced preference for infecting neurons. In contrast, peptides such as Tat are not neurotrophic, and presumably penetrate both neurons and glia. However, the insults used were classically neurotoxic, without being gliotoxic; thus, the protection afforded by Tat-Bcl-xL would overwhelmingly be due to decreasing neuron, rather than glial, death.

Tat-Bcl-xL halved the extent of *in vitro* neurotoxicity induced by approximate LD50 concentrations of glutamate and KA; this is in the range of the extent of protection afforded by many of the anti-apoptotic viral vectors (Sapolsky, 2003). There was also a marked decrease in lesion size in the *in vivo* KA study, although this capacity for protection may not generalize to other degrees of insult-induced toxicity. Whether a decrease in the extent of neuron death (with either gene or protein therapy) is of sufficient magnitude to translate into sparing of function is often unclear. However, the extent of protection produced by Tat-Bcl-xL in the hippocampus *in vivo* is of a magnitude previously shown (in gene therapy studies) to be associated with sparing of hippocampal-dependent cognition (McLaughlin et al., 2000; Phillips et al., 2001).

As a final point, there are circumstances where there is a high probability of a necrotic neurological insult occurring (for example, in a person amid a period of repeated transient ischemic attacks), raising the possibility of prophylactic protein or gene therapy. Nonetheless, most insults preclude such intervention, and therapies would have to be carried out following an insult. Thus, the protective effects of Tat-Bcl-xL, even when delivered two hours post KA, is notable; in agreement, systemic administration of Tat-Bcl-xL protects against ischemic damage even when delivered 45 minutes after reperfusion (Cao et al., 2002). Whether there will be differences in the post-insult time window for protection when Bcl-xL is delivered as a fusion protein versus as a gene in a viral vector remains to be studied.

In conclusion, while there are many domains of neurological damage where a gene therapy approach is particularly auspicious, protein therapy may be efficacious in other circumstances. The present report strengthens the feasibility of the latter approach.

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Figure 1. Representative bright field images of hippocampal neurons exposed to glutamate *in vitro*.

A – F represent neurons incubated under various conditions: A) untreated neurons; B) 2 μ M Tat-MYC; C) 2 μ M Tat-Bcl-xL; D) 50 μ M glutamate; E) 2 μ M Tat-MYC followed by 50 μ M glutamate; F) 2 μ M Tat-Bcl-xL followed by 50 μ M glutamate. Scale bar = 100 μ m.

Figure 2. Tat-Bcl-xL protects cultured hippocampal neurons against glutamate insult
Cultures were incubated with indicated combinations of 50 μ M glutamate (Glut), control fusion protein – Tat-MYC (MYC), or experimental Tat-Bcl-xL fusion protein (Bcl-xL). Neuron death is expressed as a percentage of cells killed relative to glutamate-free cultures receiving the same vector treatment. ** $p < 0.01$, $n = 12$ /group.

Figure 3. Tat-Bcl-xL protects cultured hippocampal neurons against kainic acid insult
 A) Cultures were incubated with indicated combinations of 50 μ M kainic acid (KA), 2 μ M Tat-MYC (MYC), and 2 μ M Tat-Bcl-xL (Bcl-xL). B) Cultures were incubated with indicated combinations of 100 μ M kainic acid (KA), 2 μ M Tat-MYC (MYC), and 2 μ M Tat-Bcl-xL (Bcl-xL). Neuron death is expressed as a percentage of cells killed relative to KA-free cultures receiving the same vector treatment. * p < 0.05, ** p < 0.01, *** p < 0.001, n = 24/group.

Figure 4. Neuroprotective effects of Tat-Bcl-xL in a rat model of status epilepticus

Either 0.03 μ g Tat-MYC or Tat-Bcl-xL was infused unilaterally in the dentate gyrus of rats (six each), 30-minutes before infusion of 0.06 μ g KA. The control, contralateral hemisphere was infused with KA alone. Vehicle was injected into a second group of animals as a measure of intact CA3 (No KA). *** $p < 0.001$, $n = 6$ /group.