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A NOVEL STRATEGY TO ACTIVATE CYTOPROTECTIVE GENES IN THE INJURED BRAIN

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Abstract

The transcription factor nuclear factor E2-related factor 2 (Nrf2) regulates the expression of multiple cytoprotective genes that have been shown to offer protection in response to a number of insults. The present study describes a novel strategy to increase expression of Nrf2-responsive genes in brain injured mice. Under normal conditions, the adapter protein Kelch-like ECH-associated protein 1 (Keap1) binds to Nrf2 and promotes its proteosomal degradation in the cytoplasm. The amino acid sequence DEETGE, located at amino acid 77–82 of Nrf2, is critical for Nrf2-Keap1 interaction, and synthetic peptides containing this sequence can be used to disrupt the complex *in vitro*. We observed that intracerebroventricular (i.c.v.) infusion of a peptide containing the DEETGE sequence along with the cell transduction domain of the HIV-TAT protein (TAT-DEETGE) into brain-injured mice did not increase the mRNA levels for Nrf2-driven genes. However, when a calpain cleavage sequence was introduced between the TAT sequence and the DEETGE sequence, the new peptide (TAT-CAL-DEETGE) increased the mRNA levels of these genes. Increased gene expression was not observed when the TAT-CAL-DEETGE peptide was injected into uninjured animals. Furthermore, injection of TAT-CAL-DEETGE peptides before or after brain injury reduced blood-brain barrier compromise, a prominent secondary pathology that negatively influences outcome. The present strategy to increase Nrf2-responsive gene expression can be adapted to treat other insults or diseases based on their underlying mechanism(s) of cellular damage.

Keywords

blood-brain barrier; cytoprotective genes; Keap1; Nrf2; peptide-based therapy; TBI

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Introduction

Most cells possess endogenous protective mechanism(s) as a defense against environmental insults. These cellular defenses often involve induction of cytoprotective genes whose protein products scavenge and/or neutralize environmental toxins. The transcription factor Nrf2 (nuclear factor E2-related factor 2) can enhance the expression of multiple cytoprotective proteins and is protective following insults such as stroke and traumatic brain injury (TBI) [1–5]. Under normal conditions, the adapter protein Kelch-like ECH-associated protein 1 (Keap1) binds to both actin cytoskeleton and Nrf2, thereby sequestering Nrf2 in cytoplasm where it is degraded by proteasomes [6,7]. Immunoprecipitation and structural analysis studies have shown that the DEETGE motif is critical for Nrf2-Keap1 interaction and that synthetic peptides containing the DEETGE sequence can disrupt Nrf2-Keap1 interaction *in vitro* [8]. However, an effective strategy for utilizing these peptides to reduce tissue damage following insults has not been developed.

In this study, we tested if synthetic peptides containing the DEETGE sequence, in tandem with the cell transduction domain of the HIV-TAT protein, can increase the expression of Nrf2-responsive genes following experimental TBI. Our results show that i.c.v. administration of TAT-DEETGE had no effect on Nrf2-regulated gene expression in the injured brain. However, when a calpain cleavage site was introduced between the TAT and DEETGE sequences (TAT-CAL-DEETGE), increased Nrf2-regulated gene expression and attenuated blood-brain barrier (BBB) compromise were observed following TBI. This mechanism-based strategy may be useful for activating Nrf2-regulated gene expression in the injured brain to reduce neurovascular dysfunction.

Materials and Methods

Materials

Male C57/Bl6 mice (25–30g) were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed under temperature-controlled conditions with a 12-hour light/dark cycle and *ad libitum* access to water and food. Animal protocols were approved by the Institutional Animal Welfare Committee and were in compliance with NIH's *Guide for Care and Use of Laboratory Animals*. The following peptides were synthesized by Genemed Synthesis (San Antonio, TX):

TAT: NH₂-YGRKKRRQRRR-CONH₂

TAT-DEETGE: NH₂-YGRKKRRQRRRPLQL**DEETGE**FLPIQ-CONH₂

TAT-CAL-DEETGE: NH₂-YGRKKRRQRRRPLFAERL**DEETGE**FLP-CONH₂

Amino acids listed in bold correspond to the DEETGE motif. Underlined amino acids indicate the calpain cleavage site. SuperScript II Reverse Transcriptase and AmpliTaq DNA Polymerase were purchased from Invitrogen (Carlsbad, CA). The following primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) for use in qPCR analysis:

<i>mouse β-actin:</i>	forward: 5'-GCTTCTTTGCAGCTCCTTCGT-3'
	reverse: 5'-ATATCGTCATCCATGGCGAAC-3'
<i>mouse GPx1:</i>	forward: 5'-GACTGGTGGTGCTCGGT-3'
	reverse: 5'-ACTTGAGGGAATTCAGAATCTCTTC-3'
<i>mouse Catalase:</i>	forward: 5'-AGGAGGCAGAACTTTCCCAT-3'
	reverse: 5'-TTTGCCAACTGGTATAAGAGGGTA-3'
<i>mouse GSTm1:</i>	forward: 5'-GAAGCCAGTGGCTGAATGAGA-3'
	reverse: 5'-GATGGCATTGCTCTGGGTG-3'

Brain Injury

An electric controlled cortical impact (CCI) device was used to cause brain injury as described previously [9–11]. Two hours following peptide infusion, mice were anesthetized, mounted on the stereotaxic frame and the wound clips removed. After re-exposing the skull, a 4 mm-diameter craniotomy was made midway between the bregma and the lambda on the right side, with the medial edge of the craniotomy 0.5 mm lateral to midline. The injury was caused by a single impact at 4 m/s velocity with 1.7 mm deformation using an impactor tip 3 mm in diameter at an angle of 10° from the vertical plane. Core body temperature was maintained at 37°C by use of a heating pad. After the surgery, all mice were allowed to completely recover from the anesthesia in a warm chamber before being sent back to their home cages.

Brain Infusion of Peptides

Peptides were infused into the brain either via the intracerebroventricular (for pre-injury infusions) or intrathecal (for post-injury infusions) route. Mice were anesthetized using 5% isoflurane with a 1:1 N₂O/O₂ mixture and then maintained with a 2.5% isoflurane with 1:1 N₂O/O₂ mixture via a face mask. Animals were mounted on the stereotaxic frame secured by ear bars and an incisor bar. The head was held in a horizontal plane with respect to the interaural line. A midline incision was made and the soft tissues reflected. For i.c.v. infusion, a small burr hole was prepared over the ipsilateral lateral ventricle (1.0 mm lateral, 0.34 mm posterior to bregma). A 30 gauge infusion needle was lowered via a stereotaxic arm to a depth of 2.25 mm from the skull surface. Mice were infused with the peptide (total volume of 1.5 μ l) at a rate 0.3 μ l/min. After infusion, the infusion needle was held in position for 1 min before being withdrawn from the ventricle. For intrathecal infusion, three sites were chosen for peptide infusion including the rostral pole, the epicenter and the caudal pole of the injury site. Ten minutes after injury, 0.5 μ l of peptide (at a rate 0.25 μ l/min) was injected into each of the three sites. The total amount of peptide of all three infusion sites equals to the total peptide amount delivered via a single i.c.v. infusion in other experiments. Core body temperature was maintained at 37°C during infusion using a heating pad. After infusion, the incision was closed with wound clips. After surgery, all animals were allowed to completely recover from the anesthesia in a warm chamber before being placed back in their home cages.

Quantitative RT-PCR

Twenty-four hours after the injury, mice were killed and parietal cortical tissues quickly dissected and frozen on dry ice. The frozen tissue was homogenized in 1 ml of TriZol (Invitrogen) per 100 mg tissue, followed by addition of chloroform (1/5 volume) and incubation on ice for 20 min. The homogenate was centrifuged at $14,000 \times g$ for 30 min, supernatant recovered and total RNA was precipitated by isopropanol. 1.0 μ g total RNA was reverse transcribed for 2 hours at 37°C in a 20 μ l mixture containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3.0 mM MgCl₂, 10 mM DTT, 2.5 μ M random hexamer, 1.0 mM each dNTP, 20 U RNasin, and 200 U Superscript II reverse transcriptase. The level of expression of each target gene was quantified using a BioRad iCycler real-time PCR system (BioRad, Hercules, CA). Amplification reactions were carried out in triplicate. Each 30 μ l reaction mixture consisted of 1.5 μ l of the cDNA in a reaction buffer containing 18 mM Tris-HCl pH 8.3, 55 mM KCl, 2.0 mM MgCl₂, 0.2 mM of each dNTP, 0.5 μ M of each primer, 10 nM fluorescein, 1:75,000 dilution of Sybr green I, and 2 U AmpliTaq DNA polymerase. The amplification protocol consisted of 1 cycle at 95°C for 3 min followed by 40 cycles at 95°C for 30 sec, 58°C for 30 sec, then 72°C for 30 sec. The specificity of the amplified product was confirmed by a melting point analysis performed at the end of the amplification, which consisted of 80 cycles beginning at 55°C for 10 sec, after which the temperature was increased by 0.5 °C/cycle. A standard curve for each target gene was generated to determine the linear range and amplification efficiency. The threshold cycle of each sample was fitted to the standard curve to calculate the relative abundance of the target mRNA. The resultant data was analyzed using the iCycler iQ Real-Time Detection System software [12]. β -actin was used as an internal control and against which the Nrf2-regulated gene expression data were normalized.

Measurement of Blood-Brain Barrier (BBB) Permeability

BBB permeability was assessed by measuring the extravasation of Evans blue dye as described previously [13,14]. Twenty-four hours after injury, 3% Evans Blue dye in saline was injected slowly through the jugular vein (4 ml/kg) and allowed to circulate for 1.5 hr. Following the circulation time, animals were transcardially perfused with 1X PBS followed by 4% paraformaldehyde in PBS. The brains were removed and cerebral hemispheres separated. The hemispheres were sliced into 2 mm sections and then incubated in 1 ml formamide for 24 hr at 55 °C. After incubation, tissues were removed and the formamide solution was collected and centrifuged at 20,000Xg for 20 min. Absorbance of the supernatant at 620 nm was measured to determine the amount of Evans Blue dye in each sample.

Statistical Analysis

All data was analyzed by a Shapiro-Wilk normality test. Where appropriate, a Student's *t*-test for unpaired variables or a one-way ANOVA was used for evaluating mRNA levels and Evans Blue extravasation. Results were considered significant at $P < 0.05$. Data are presented as the mean $\pm$ standard error of the mean (S.E.M.).

Results

TAT-CAL-DEETGE but not TAT-DEETGE peptides increase the expression of Nrf2-regulated genes in brain-injured animals

Our previous studies using a chemical activator of Nrf2 have shown that activation of the Nrf2 system offers robust vascular protection following traumatic brain injury [1]. To test if DEETGE sequence-containing peptides can enhance Nrf2-driven gene expression in brain injured mice, this peptide was coupled to the protein transduction domain of the HIV-TAT protein to enhance cell penetration. A proline residue was included at the end of the TAT sequence in order to disrupt the continuation of the helical structure into the remainder of the peptide [15]. Mice (n=4/group) were i.c.v. injected with either the TAT-DEETGE (15 µg) peptide or an equimolar amount of the TAT alone peptide (7.2 µg). Saline injected mice (n=4) were used as controls. Two hours after peptide injection, mice were subjected to cortical impact injury. As a positive control, an additional group of mice (n=4) were injected 6 hr post-injury with 5 mg/kg sulforaphane. We have previously shown that this dose and time of sulforaphane administration significantly increases Nrf2-mediated gene expression [1]. Twenty-four hours after the injury, mice were euthanized for the preparation of total RNA from parietal cortex to examine the expression levels of the known Nrf2-responsive genes, glutathione peroxidase 1 (GPx1), catalase, and glutathione s-transferase mu-1 (GSTm1), by quantitative PCR (qPCR). Figure 1A shows that the relationship between the starting quantity of total RNA and number of PCR cycles that were required to detect a threshold level of amplified GPx1 using Sybr green fluorescence. The threshold signal for GPx1 was linear from at least 25 ng to 200 ng of input total RNA with an amplification efficiency of 107.3%. Similar standard curves were generated for catalase and GSTm1 (data not shown). Representative profiles for qPCR amplification of GPx1 using samples obtained from saline-, TAT peptide-, TAT-DEETGE peptide-, and sulforaphane-injected animals are shown in Figure 1B. The gray horizontal line indicates the fluorescence value used to determine the threshold cycle for each sample which was compared to a simultaneously amplified standard curve to calculate abundance. Although sulforaphane dramatically increased GPx1 expression level, neither the TAT nor the TAT-DEETGE peptides significantly changed GPx1 expression level compared to animals received vehicle (Figure 1D). Similar results were observed when the expression levels of catalase and GSTm1 were evaluated.

Following brain injury, calcium release causes activation of calpain, leading to the degradation of structural proteins within cortical and hippocampal neurons [16,17]. We therefore hypothesized that the introduction of a calpain cleavage site between the TAT and DEETGE motifs may allow for the removal of the protein transduction domain and facilitate cytoplasmic accumulation of the DEETGE-containing peptide. Using degenerate peptide dendrimers, Cuerrier et al., found that the optimal cleavage sequence for μ -calpain is PLFAER [18]. We therefore synthesized a peptide (TAT-CAL-DEETGE) in which the TAT and DEETGE sequences were separated by the μ -calpain cleavage site PLFAER. Mice (n=4/group) were i.c.v. injected with either the TAT-CAL-DEETGE (15.6 µg) or TAT alone peptides (7.2 µg), then injured as described above. Figure 1C shows that, by comparison to the number of cycles required to amplify GPx1 from a TAT-infused, injured animal, fewer

cycles were required for detection of GPx1 in a TAT-CAL-DEETGE-treated mouse. The summary data in Figure 1D shows that pre-injury infusion of TAT-CAL-DEETGE, but not TAT-DEETGE, significantly increased the mRNA levels of Nrf2-dependent genes compared to TAT- and vehicle-treated controls (GPx1: one-way ANOVA on Ranks $H_{(3,12)}=8.824$, $p=0.032$; catalase: one way ANOVA $F_{(3,12)}=23.025$, $p<0.001$; GSTm1: one-way ANOVA $F_{(3,12)}=32.112$, $p<0.001$). There were no differences on the expression level of β -actin as a result of these treatments (data not shown).

TAT-CAL-DEETGE peptide has no effect on the expression of Nrf2-regulated genes in brain tissue of uninjured animals

Our qPCR data showed that DEETGE sequence-containing peptides could be used to stimulate Nrf2-regulated gene expression in the brain of injured mice only when a calpain cleavage site was introduced between the TAT and DEETGE motifs. This effect could have resulted from either cleavage and release of the DEETGE sequence as a result of TBI-induced calpain activity, or as a result of a change in the confirmation of the peptide that would allow for enhanced binding to Keap1. To help differentiate between these two possibilities, we tested if the TAT-CAL-DEETGE peptide could induce Nrf2-regulated gene expression in the absence of TBI-induced calpain activity. Uninjured mice ($n=4$ /group) were i.c.v. injected with either the TAT (7.2 μ g) or the TAT-CAL-DEETGE peptide (15.6 μ g), then killed 24 hr later for the preparation of total RNA. Sulforaphane-treated animals (5 mg/kg) were once again used as positive controls. Figure 2A shows representative amplification curves for GPx1 from a TAT, a TAT-CAL-DEETGE, and a sulforaphane-treated animal. No apparent influence of the TAT-CAL-DEETGE peptide was observed in uninjured animals compared to that observed in animals treated with the TAT peptide. Sulforaphane, by comparison, remained capable of stimulating GPx1 gene expression independent of the injury status of the animal. The summary data in Figure 2B shows that although sulforaphane significantly increased GPx1 mRNA levels, no significant difference was observed from uninjured mice treated with the TAT-CAL-DEETGE peptide. No significant difference was observed in any of the groups when the levels of β -actin mRNA were measured (data not shown).

Pre- or post-injury administration of TAT-CAL-DEETGE reduces BBB permeability following TBI

We have previously demonstrated that sulforaphane, when injected 6 hr post-injury, reduced TBI-associated disruption of the BBB [1]. To test if the TAT-CAL-DEETGE peptide can also reduce this secondary pathology, mice were i.c.v. infused with equimolar amounts of the TAT, TAT-DEETGE or TAT-CAL-DEETGE peptides, then injured 2 hours later. BBB permeability was assessed by Evans blue dye extravasation at 24 hr post-injury. Figure 3A shows that i.c.v. infusion of the TAT-CAL-DEETGE peptide, but not the TAT-DEETGE peptide effectively reduced Evans blue extravasation following injury as compared to TAT-infused, brain injured controls. In order to test the translational utility of the TAT-CAL-DEETGE peptide, another set of mice were brain injured and received intrathecal administrations of either the TAT-CAL-DEETGE or TAT peptide 10 min after injury. An intrathecal route was chosen because of the swelling of the injured brain made it difficult to accurately perform i.c.v. injections. Figure 3B shows that post-injury administration of the

TAT-CAL-DEETGE peptide also significantly reduced Evans blue extravasation as compared to the TAT peptide-infused group.

Discussion

Our results describe a novel strategy employing a cell-permeable, synthetic peptide containing the DEETGE sequence to induce Nrf2-regulated gene expression in the injured brain. Increased target gene expression in the injured brain was only observed when a calpain cleavage site separated the cell transduction domain (TAT) and the DEETGE sequence. This peptide had no detectable effect on target gene expression in normal brains, suggesting that this peptide has no influence on Nrf2-responsive gene expression in undamaged tissue. We further demonstrated that infusion of this peptide into the brain effectively attenuates brain injury-associated BBB compromise.

A number of studies have demonstrated that brain injury increases intracellular calcium resulting in the activation of calpain and proteolysis of substrate proteins [19–24]. Although not directly examined, cleavage of the TAT-CAL-DEETGE peptide by calpain would release the TAT sequence, allowing for the accumulation of the DEETGE peptide inside injured cells where it could disrupt Nrf2-Keap1 binding (Figure 4). We have previously demonstrated that the electrophilic compound sulforaphane effectively reduces TBI-associated BBB compromise. This influence of sulforaphane was dependent on the transcription factor Nrf2 as i.c.v. administration of decoy oligonucleotides containing the ARE sequence blunted this protective effect. In addition, sulforaphane showed no protective effect in *nrf2*^{−/−} mice [1]. Our results using the TAT-CAL-DEETGE peptide demonstrate that this peptide, presumably through disruption of Nrf2-Keap1 binding, also provides protection against TBI-associated BBB compromise. In support of this, we observed that there was a strong association between the ability of this peptide to induce Nrf2-regulated gene expression and its ability to preserve the integrity of BBB after injury. Peptides which failed to induce Nrf2-mediated gene expression (e.g. TAT alone or TAT-DEETGE) did not offer similar BBB protection.

The peptide used in the current study may have therapeutic potential as demonstrated by its ability to increase the expression of cytoprotective genes only in the injured brain. This strategy could be adapted for use to treat other conditions, using a disease-dependent molecular mechanism. For example, Nrf2-regulated gene expression could be enhanced in cells undergoing apoptosis by replacing the calpain cleavage site with a caspase-3 cleavage sequence. Similarly, the neuronal damage associated with Alzheimer's Disease might be reduced by generating a peptide containing a BACE1 (β -site of APP cleaving enzyme) cleavage site. Future studies will be required to demonstrate the general applicability of this strategy for treating other insults/diseases.

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Abbreviations

BBB	blood-brain barrier
CAL	calpain cleavage sequence
CCI	controlled cortical impact injury
GPx1	glutathione peroxidase
GSTm1	glutathione s-transferase mu-1
i.c.v	intracerebroventricular
Keap1	kelch-like ECH associated protein 1
Nrf2	nuclear factor E2-related factor 2
TBI	traumatic brain injury

References

1. Zhao J, Moore AN, Redell JB, Dash PK. Enhancing expression of Nrf2-driven genes protects the blood brain barrier after brain injury. *J Neurosci.* 2007; 27:10240–10248. [PubMed: 17881530]
2. Ping Z, Liu W, Kang Z, Cai J, Wang Q, Cheng N, Wang S, Wang S, Zhang JH, Sun X. Sulforaphane protects brains against hypoxic-ischemic injury through induction of Nrf2-dependent phase 2 enzyme. *Brain Res.* 2010; 1343:178–185. [PubMed: 20417626]
3. Son TG, Camandola S, Arumugam TV, Cutler RG, Telljohann RS, Mughal MR, Moore TA, Luo W, Yu QS, Johnson DA, Johnson JA, Greig NH, Mattson MP. Plumbagin, a novel Nrf2/ARE activator, protects against cerebral ischemia. *J Neurochem.* 2010; 112:1316–1326. [PubMed: 20028456]
4. Yang C, Zhang X, Fan H, Liu Y. Curcumin upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia. *Brain Res.* 2009; 1282:133–141. [PubMed: 19445907]
5. Zhao X, Sun G, Zhang J, Strong R, Dash PK, Kan YW, Grotta JC, Aronowski J. Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. *Stroke.* 2007; 38:3280–3286. [PubMed: 17962605]
6. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 1999; 13:76–86. [PubMed: 9887101]
7. Kang MI, Kobayashi A, Wakabayashi N, Kim SG, Yamamoto M. Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. *Proc Natl Acad Sci USA.* 2004; 101:2046–2051. [PubMed: 14764898]
8. Lo SC, Li X, Henzl MT, Beamer LJ, Hannink M. Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. *EMBO J.* 2006; 25:3605–3617. [PubMed: 16888629]
9. Dixon CE, Clifton GL, Lighthall JW, Yaghmai AA, Hayes RL. A controlled cortical impact model of traumatic brain injury in the rat. *J Neurosci Methods.* 1991; 39:253–262. [PubMed: 1787745]
10. Meaney DF, Ross DT, Winkelstein BA, Brasko J, Goldstein D, Bilston LB, Thibault LE, Gennarelli TA. Modification of the cortical impact model to produce axonal injury in the rat cerebral cortex. *J Neurotrauma.* 1994; 11:599–612. [PubMed: 7861451]
11. Dash PK, Moore AN, Dixon CE. Spatial memory deficits, increased phosphorylation of the transcription factor CREB, and induction of the AP-1 complex following experimental brain injury. *J Neurosci.* 1995; 15:2030–2039. [PubMed: 7891150]
12. Redell JB, Moore AN, Dash PK. Expression of the prodynorphin gene after experimental brain injury and its role in behavioral dysfunction. *Exp Biol Med (Maywood).* 2003; 228:261–269. [PubMed: 12626770]

13. Shapira Y, Setton D, Artru AA, Shohami E. Blood-brain barrier permeability, cerebral edema, and neurologic function after closed head injury in rats. *Anesth Analg*. 1993; 77:141–148. [PubMed: 8317722]
14. Shohami E, Novikov M, Horowitz M. Long term exposure to heat reduces edema formation after closed head injury in the rat. *Acta Neurochir Suppl (Wien)*. 1994; 60:443–445. [PubMed: 7976614]
15. Ho A, Schwarze SR, Mermelstein SJ, Waksman G, Dowdy SF. Synthetic protein transduction domains: enhanced transduction potential in vitro and in vivo. *Cancer Res*. 2001; 61:474–477. [PubMed: 11212234]
16. Pike BR, Zhao X, Newcomb JK, Posmantur RM, Wang KK, Hayes RL. Regional calpain and caspase-3 proteolysis of alpha-spectrin after traumatic brain injury. *Neuroreport*. 1998; 9:2437–2442. [PubMed: 9721910]
17. Buki A, Siman R, Trojanowski JQ, Povlishock JT. The role of calpain-mediated spectrin proteolysis in traumatically induced axonal injury. *J Neuropathol Exp Neurol*. 1999; 58:365–375. [PubMed: 10218632]
18. Cuerrier D, Moldoveanu T, Davies PL. Determination of peptide substrate specificity for mu-calpain by a peptide library-based approach: the importance of primed side interactions. *J Biol Chem*. 2005; 280:40632–40641. [PubMed: 16216885]
19. Kampfl A, Posmantur R, Nixon R, Grynspan F, Zhao X, Liu SJ, Newcomb JK, Clifton GL, Hayes RL. mu-calpain activation and calpain-mediated cytoskeletal proteolysis following traumatic brain injury. *J Neurochem*. 1996; 67:1575–1583. [PubMed: 8858942]
20. Kampfl A, Posmantur RM, Zhao X, Schmutzhard E, Clifton GL, Hayes RL. Mechanisms of calpain proteolysis following traumatic brain injury: implications for pathology and therapy: implications for pathology and therapy: a review and update. *J Neurotrauma*. 1997; 14:121–134. [PubMed: 9104930]
21. Okonkwo DO, Buki A, Siman R, Povlishock JT. Cyclosporin A limits calcium-induced axonal damage following traumatic brain injury. *Neuroreport*. 1999; 10:353–358. [PubMed: 10203334]
22. Buki A, Farkas O, Doczi T, Povlishock JT. Preinjury administration of the calpain inhibitor MDL-28170 attenuates traumatically induced axonal injury. *J Neurotrauma*. 2003; 20:261–268. [PubMed: 12820680]
23. Ray SK, Banik NL. Calpain and its involvement in the pathophysiology of CNS injuries and diseases: therapeutic potential of calpain inhibitors for prevention of neurodegeneration. *Curr Drug Targets CNS Neurol Disord*. 2003; 2:173–189. [PubMed: 12769798]
24. Saatman KE, Creed J, Raghupathi R. Calpain as a therapeutic target in traumatic brain injury. *Neurotherapeutics*. 2010; 7:31–42. [PubMed: 20129495]

Research Highlights

- A strategy to increase cytoprotective gene expression in injured tissue is outlined.
- A peptide containing a DEETGE motif can increase Nrf2 responsive genes in vivo.
- Gene expression in injured brains requires a calpain cleavage site.
- This peptide decreases BBB compromise when infused pre- or post-brain injury.
- Cleavage sites for disease-specific proteases could be used to treat that condition.

Figure 1. The TAT-CAL-DEETGE peptide induces the expression of Nrf2-regulated genes in the injured brain

A) Relationship between amount of input RNA and the threshold cycle required for detection of glutathione peroxidase 1 (GPx1) amplification. **B)** Representative amplification curves for GPx1 from saline-, TAT-, TAT-DEETGE, and sulforaphane-treated injured animals. **C)** Representative amplification curves for GPx1 from TAT-, and TAT-CAL-DEETGE-treated injured animals. The gray line represents the fluorescence level at which the cycle threshold was calculated. **D)** Summary data for GPx1, catalase, and glutathione S-transferase mu-1 (GSTm1) mRNA expression following peptide administration (n=4/group). *, P<0.05 by one-way ANOVA. Data is presented as the mean $\pm$ SEM relative to saline-treated controls.

Figure 2. The TAT-CAL-DEETGE peptide does not alter the expression of GPx1 in uninjured brains

A) Representative amplification curves for GPx1 mRNA from TAT-, TAT-CAL-DEETGE-, and sulforaphane- treated injured animals. The gray line represents the fluorescence level at which the cycle threshold was calculated. **B)** Summary data showing that i.c.v. administration of the TAT-CAL-DEETGE peptide (n=4) has no effect on GPx1 mRNA level in the cortex of uninjured mice by comparison to TAT-infused controls (n=4). In contrast, i.p. injection of sulforaphane (n=4) significantly induced GPx1 mRNA. *, $P < 0.05$ by one-way ANOVA. Data is presented as the mean $\pm$ SEM relative to TAT-treated controls.

Figure 3. TAT-CAL-DEETGE peptide attenuates BBB compromise following TBI

A) Summary data showing that pre-injury administration of TAT-CAL-DEETGE peptide, but not an equimolar amount of the TAT-DEETGE, reduced the extravasation of Evans blue dye compared to TAT-infused controls. **B)** Summary data showing that post-injury administration of TAT-CAL-DEETGE peptide significantly reduced the extravasation of Evans blue dye compared to injured animals receiving the TAT peptide. *, $P < 0.05$. Data is presented as the mean $\pm$ SEM.

Figure 4. Hypothetical cascade for induction of Nrf2-responsive genes by TAT-CAL-DEETGE in injured cells

(1) The cell transduction domain of the HIV-TAT protein (TAT) increases the cell penetrability of cargo peptides, allowing them access to the intracellular environment. The TAT-CAL-DEETGE peptide contains a calpain cleavage sequence (PLFAER) between the TAT and DEETGE motifs. (2) As a result of injury, calpain is activated and cleaves the TAT-CAL-DEETGE peptide, presumably allowing for cytoplasmic accumulation of the DEETGE peptide. Nrf2 is normally sequestered in the cytoplasm through its interaction with Kelch-like ECH-associated protein 1 (Keap-1) where it is ubiquitinated by Cullin 3 (Cul3) for degradation. (3) The DEETGE peptide binds to Keap-1, thereby disrupting the Nrf2-Keap1 complex. (4) In the absence of normal ubiquitin-mediated degradation, Nrf2 accumulates and can stimulate the expression of genes containing the antioxidant response element (ARE) sequence. The products of these genes convey protection after TBI, preserving the integrity of the blood-brain barrier (BBB).