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TCEP Treatment Reduces Proteolytic Activity of BoNT/B in Human Neuronal SHSY-5Y Cells

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ABSTRACT

The light chain (LC) of botulinum neurotoxin B (BoNT/B) is unable to enter target neuronal cells by itself. It is brought into the cell in association with the BoNT/B heavy chain (HC) through endocytosis. The BoNT HC-LC subunits are held together by a single disulfide bond. Intracellular reduction of this bond and separation of the two subunits activates the endopeptidase activity of the LC. This requirement suggests a strategy to prevent uptake by prophylactic reduction to disrupt the disulfide bond prior to endocytosis of the complex. We examined the utility of tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP), a relatively non-toxic, non-sulfur containing disulfide bond reducing agent that lacks the undesirable properties of mercapto-containing reducing agents. We found that TCEP was as effective as DTT with maximal LC endopeptidase activation occurring at 1 mM, a concentration not toxic to the human neuronal cell line, SHSY-5Y. In these cells, 1 mM TCEP maximally protected against BoNT/B inhibition of [^{3}H]-NA release, achieving 72% of the release from un-intoxicated controls. This effect appears to be due to the sparing of SNARE proteins as the levels of VAMP-2, the specific target of BoNT/B, were protected. These results show that TCEP disrupts the structure of BoNT/B by reduction of the LC and HC bridging disulfide bond and prevents neuronal intoxication. Since disulfide bond coupling between toxin subunits is a general motif for many toxins, e.g., ricin, snake venom, and all BoNT serotypes, this suggests that TCEP is a promising means to protect against these toxins by preventing cell penetration. J. Cell. Biochem. 107: 1021–1030, 2009. Published 2009 Wiley-Liss, Inc.[†]

KEY WORDS: TCEP; BoNT; BOTULINUM NEUROTOXIN; SYNAPTOBREVIN-2; VAMP-2

Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) is a non-odorous, oxygen-insensitive, non-toxic sulfhydryl reducing compound [Gray, 1993], which is used as a substitute for DTT (dithiothreitol) or 2-mercaptoethanol to reduce disulfide bonds of peptides [Cline et al., 2004]. TCEP is also desirable because it reduces disulfide bonds over a wider range of pH (pH 2–9) than DTT [Han and Han, 1994; Getz et al., 1999]. In addition, it is specific for disulfides since it does not react with other functional groups on proteins [Bingham et al., 2005]. Figure 1 shows the structures of reduced and oxidized TCEP and the expected mode of reduction of protein disulfides if the reaction were similar to that of Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid; DTNB), as described by Han and Han [1994]. TCEP in the rat has been reported to be relatively non-toxic with LD_{50} values of 3,500; 3,000; and 1,024 g/kg for oral, skin,

Abbreviations used: BoNT, botulinum neurotoxin; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; HPLC, high pressure liquid chromatography; HT, holotoxin; HU, hydroxyurea; NA, noradrenaline; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SNAP-25, 25 kDa synaptosomal-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; TCEP, tris-(2-carboxyethyl)-phosphine hydrochloride; TMB, tetramethyl benzidine; VAMP-2, vesicle-associated membrane protein-2/synaptobrevin-2; ANSA, 8-anilino-1-naphthalenesulfonic acid.

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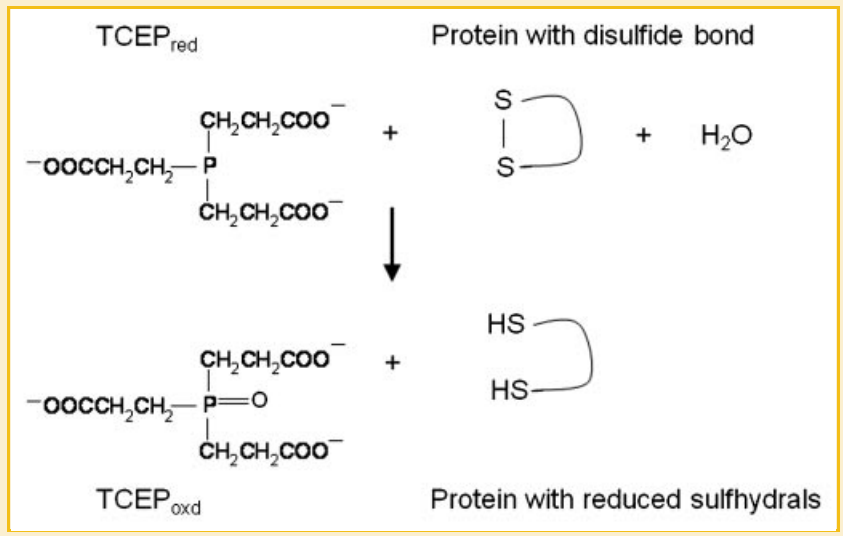

Fig. 1. Reduction of the disulfide bond by TCEP. The reduced form of TCEP becomes the oxide form following conversion of a disulfide bond on proteins into two sulfhydryl groups.

and *i.p.*, respectively (Sigma–Aldrich, Material Safety Data Sheet: http://www.sigmaaldrich.com/united_states.html and catalog # Ca706; last accessed on 6 February 2009).

Botulinum neurotoxins (BoNTs) are believed to be the most toxic biological substances with mouse LD_{50} values less than a nanogram by *i.p.* administration [Gill, 1982]. BoNTs are grouped into seven serotypes: A, B, C, D, E, F and G based on their immunoreactivity [DasGupta, 1990]. BoNT/A, B, E, and F are mainly toxic to humans, while BoNT/C and D are the more neurotoxic agents to animals [Cherington, 1998; Collins and East, 1998]. BoNT holotoxins (HT) are 150 kDa consisting of a heavy chain (HC, size of approximately 100 kDa), and a light chain (LC, 50 kDa) [Simpson, 1979; Humeau et al., 2000]. The HC is responsible for the recognition of the neurotoxin with its cognate receptor on the surface of neuronal cells and penetration into cells through endocytosis. X-ray structure analysis has shown that a loop of the HC occludes the LC active site when the toxin is in the HT form [Lacy et al., 1998]. This arrangement presumably protects the LC from inactivation. In addition, the HC is coupled by a sulfhydryl bond to the LC and it is essential for the HC to translocate the LC into the neuronal cell; after penetrating the cell, the LC becomes dissociated by reduction of a sulfhydryl group coupling it to the HC [Schiavo et al., 1990; Simpson et al., 2004; Fischer and Montal, 2007]. The LC is an enzyme—a zinc-dependent peptidase. Each botulinum serotype has specificity for cleaving seven different SNARE protein sites after it enters the neuronal cell [Bandyopadhyay et al., 1987; Montecucco and Schiavo, 1994; Deloye et al., 1997; Pellizzari et al., 1999]. Cleavage of SNARE proteins leads to inhibition of neurotransmitter (acetylcholine) release from the synaptic vesicles, resulting in muscular paralysis and cellular damage [Schiavo et al., 2000; Simpson, 2004]. The SNARE proteins cleaved by the BoNTs are as follows: BoNT/A, C and E cleave SNAP-25; BoNT/B, D, F and G cleave synaptobrevin-2/VAMP-2. Unlike the other serotypes, BoNT/C can also cleave syntaxin [Barr et al., 2005].

To date, there are no therapeutic drugs for treatment of patients or victims who suffer from intoxication by BoNTs [Willis et al., 2008]. As BoNTs are similar in their dependence of the disulfide bond for the HC to translocate the LCs, treatments that induce the separation of the LC from the HC of the HT before it enters cells provide a potential prophylaxis for these toxins. In this study, we utilized TCEP for pretreatment of BoNT/B serotype HT and then measured the residual proteolytic activity, not only in vitro, but also in neuronal cells. We found that TCEP treatment could protect human neuronal cells from intoxication by BoNT/B.

MATERIALS AND METHODS

TCEP TREATMENT OF BoNT/B HT

BoNT/B HT (MetaBiologics, Madison, WI) was reduced with different concentrations of TCEP (Sigma, Saint Louis, MO) in 50 mM Tris–HCl, pH 7.5, at room temperature for 30 min. Typically, 1 μg of the toxin was treated with TCEP in a 15 μL reaction volume.

CELL CULTURE

SHSY-5Y cells, a human neuroblastoma cell line, were obtained from the American Type Culture Collection (Manassas, VA), and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in a 95% air and 5% CO_2 humidified incubator at 37°C. Typically, 2×10^5 cells were seeded in 12-well plates, while 2×10^6 cells were seeded in 10 cm dishes in various experiments. Cell monolayers were ~70% confluent when they were treated with BoNT/B.

ASSAY OF BoNT/B PROTEOLYTIC ACTIVITY

The endopeptidase activity of BoNT/B was measured using a modified FRET VAMPTide^{TM} assay as described by List Biological Laboratories (Campbell, CA) in a 96-well plate (Costar plate; Corning Life Science, Corning, NY) format with minor modifications [Shi

et al., 2008]. Briefly, 1 μg of BoNT/B HT or TCEP-treated BoNT/B HT in 15 μL of 50 mM Tris–HCl, pH 7.5 was added to wells containing 133.5 μL of 50 mM HEPES (pH 7.5) with 0.05% (v/v) Tween-20, 5 mM DTT, and 0.25 mM $ZnCl_2$. Recombinant BoNT/B LC (6 nM, MetaBiologics, Madison, WI) was used as a positive control. The plates were sealed with a plastic sealer and incubated at 37°C for 15 min after which 1.5 μL of 200 μM VAMPTide^{TM} was added to start the reaction. The plates were mixed for 30 s by the plate reader, and then read using the kinetic program of a Tecan Safire Microplate Reader (Tecan Systems, San Jose, CA). The wells were read in fluorescent mode at an excitation wavelength of 300 nm and emission wavelength of 415 nm for 30 min with mixing for 5 s every 30 s. The proteolytic activity of BoNT LC endopeptidase was represented as the slope of the linear portion of the kinetic curve determined with GraphPad Prism Ver. 4.1 software (GraphPad Software, San Diego, CA).

CHAOTROPE EFFECT ON BoNT ACTIVITY FOLLOWING REDUCTION WITH TCEP

Stock solutions of hydroxyurea (HU), a chaotrope, were prepared in water and filtered through 0.45 μm filters. BoNT/B holotoxin at a concentration of 100 μg/mL was reduced with 1 mM TCEP as described above. The reduced BoNT was then treated with increasing concentrations of hydroxyurea (0.5, 1, 2, 4, and 8 mM) for 30 min at 24°C.

CELL VIABILITY ASSAY

SHSY-5Y cells, 1×10^5, were seeded in 24-well plates and then treated with increasing concentrations of TCEP (0.5, 1, 2, 5, and 10 mM) for 48 h in the incubator. Viability of the cells was measured with an MTT assay (Sigma) as previously described [Mosmann, 1983].

[^{3}H]-NORADRENALINE (NA) RELEASE ASSAY

The release of [^{3}H]-NA was determined by using the method previously described by Murphy et al. [1991] with minor modifications [Shi et al., 2008]. Briefly, SHSY-5Y cultures were grown in 12-well plates to 70% confluency using RPMI 1640 media. Toxin was pretreated with different concentrations of TCEP and/or hydroxyurea for 30 min at 24°C immediately before use; typically the preincubation solution also contained 10 mM HEPES pH 7.0 and 5% bovine serum albumin (BSA). The treated toxin preparations were sterilized by filtration before addition to the cultures. Cultures were incubated with BoNT/B HT for 48 h. Untreated control cultures were also prepared. The cells were washed twice with HEPES buffered saline (HBS) buffer (135 mM NaCl, 5 mM KCl, 0.6 mM $MgSO_4 \cdot 7H_2O$, 2.5 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, 6 mM D-glucose, 0.2 mM ascorbic acid, 0.2 mM pargyline). Cells were then labeled by incubation with HBS containing 50 nM [^{3}H]-NA (10.9 Ci/mmol, PerkinElmer). After 1 h., the isotope-supplemented HBS was removed from the cells, and the cells were washed four times with HBS.

The cells were then evoked to release [^{3}H]-NA by stimulation with 100 mM K^+ for 5 min at 37°C. Following the evoked release, the cells were washed twice with HBS. Unreleased [^{3}H]-NA was released from the cells after lysis with 0.1% Triton X-100 (1 mL/well). Cell lysate, 0.5 mL, was added to 3 mL scintillation cocktail (Ultima Gold, PerkinElmer, Inc., Waltham, MA), and the radioactivity was measured with a 1450 LSC & Luminescence counter (PerkinElmer, Inc.). The [^{3}H]-NA released on stimulation was calculated as the difference of [^{3}H]-NA remaining in cells after K^+ exposure expressed as a percentage of unstimulated control cells.

VAMP-2 ELISA ASSAY

The effect of TCEP on BoNT/B cleavage of its specific intracellular substrate, VAMP-2, was measured in the SHSY-5Y cell line. Cleavage of VAMP-2 by BoNT/B LC endopeptidase was quantified by a modified sandwich ELISA previously described by Honer et al. [2002]. Thus, SHSY-5Y cell cultures were prepared in 10 cm plates and treated with either BoNT/B HT or TCEP-pre-treated BoNT/B HT (10 μg/mL) in RPMI 1640 culture medium for 48 h. After treatment, the cells were lysed in radio-immunoprecipitation assay (RIPA) buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% glycerol, and 1% Triton X-100, supplemented just-before-use with freshly prepared protease inhibitors (1 mM orthovanandate, 10 mM NaF, 10 μg/mL leupeptin, 25 μg/mL aprotinin and 50 μg/mL phenylmethylsulfonylfluoride). Control cultures exposed to untreated BoNT/B were processed identically. The ELISA was performed in 96-well microtiter plates coated with 1–2 μg/mL goat anti-human VAMP-2 antibody (Proteintech, Chicago, IL) at 4°C overnight. Lysates from cell cultures were added to antibody-coated wells and incubated at room temperature for 2 h. After six washes with 0.5% Tween-20 in PBS, a 1:1000 diluted mouse anti-human VAMP-2 antibody (QED Bioscience, San Diego, CA) was added in a volume of 0.1 mL. Following 1 h incubation at room temperature, a 1:1000 diluted anti-mouse antibody conjugated with horseradish peroxidase (Cell Signaling, Danvers, MA) was added in a volume of 0.1 mL, and incubated at room temperature for an additional 1 h. Immunoreactivity was measured following addition of the TMB (tetramethyl benzidine) reagent color reaction (KPL, Gaithersburg, MD) by development for 10 min. The plate was read in a Spectramax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, Spectramax software Ver. 5.1). The data was analyzed using Microsoft Excel. Protein levels for the cell lysate samples were determined using a Bradford Protein Assay kit (Bio-Rad, Inc., Hercules, CA). The ELISA plate results were normalized to protein content.

SDS-PAGE METHODS

BoNT toxin proteins were analyzed by SDS-PAGE as described by Schägger and von Jagow [1987]. Briefly, 500 ng of BoNT toxin proteins were prepared in a non-reducing loading buffer, and then denatured by heating at 95°C for 7 min. The samples were then fractionated by electrophoresis through 10% non-reducing gels (Pre Cast PAGE gels, Life Therapeutics, Clarkston, GA) in a Bio-Rad Mini-Protean3 Electrophoresis Module. Proteins were visualized by silver-staining utilizing the SilverSNAP Stain kit II (Pierce Biotechnology, Rockford, IL) following manufactures instructions. Densitometry images of the stained gels were captured with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). Protein bands were quantified using ImageQuant software Ver. 5.1 (Molecular Dynamics, CA).

FLUORIMETRIC MEASUREMENTS

TCEP-induced conformational change of BoNT/B was measured with the fluorescent hydrophobicity marker 8-anilino-1-naphthalenesulfonic acid (ANSA, 50 μM) as described previously [Puhar et al., 2004]. Spectra were recorded at room temperature in a Fluorolog Spectrometer (HORIBA Jobin Yvon Inc., Edison, NJ) at an excitation slit of 12 nm and emission slit of 10 nm; the excitation wavelength was 380 nm, and the emission spectra was recorded from 400 to 650 nm. Fluorescence intensities were estimated by averaging three spectra. The final toxin and TCEP concentrations were 0.8 μM and 1 mM, respectively, in 1 M HEPES buffer. The change in fluorescence of ANSA was used to indicate the conformational change of the BoNT/B HT after TCEP addition.

RESULTS

1 mM TCEP MAXIMALLY DISSOCIATES BoNT/B LIGHT AND HEAVY CHAINS

To investigate the effect of TCEP on the dissociation of BoNT/B LC and HC, a commercial preparation of BoNT/B HT (native source) was treated with increasing concentrations of TCEP (0.5, 1, 2, 4, and 8 mM) for 30 min at room temperature. Then the proteolytic activity of the light chain peptidase was determined. TCEP dose-dependently activated the proteolytic activity of BoNT/B up to 1 mM, but then the activity gradually decreased at the higher concentrations of TCEP as shown in Figure 2. At 1 mM TCEP, the proteolytic activity was increased about 8-fold of the untreated toxin. In contrast to TCEP-treated BoNT/B HT, TCEP treatment of BoNT/B LC did not significantly change the proteolytic activity of the LC compared to the untreated LC (data not shown). Since BoNT/B HT exhibited little ability to cleave its substrate, VAMP-2, when its LC and HC were held together by a disulfide bond (Fig. 2A, 0 mM TCEP), the data suggests that TCEP was able to reduce the disulfide bond and allow the separation of the BoNT/B LC from the HC.

To verify these findings, TCEP-treated BoNT/B samples were subjected to non-reducing SDS-PAGE separation. As shown in Figure 3A, the dissociation of BoNT/B light and heavy chains with TCEP was dose-dependent. Densitometry of the LC band demonstrated that treatment with 1 mM TCEP was optimal (Fig. 3B). This is direct evidence that TCEP reduces the disulfide bond of the HT, yielding the individual light and heavy toxin chains. SDS-PAGE analysis of toxin treated with TCEP concentrations higher than 1 mM was not possible as the higher concentrations of TCEP interfered with the protein band resolution (data not shown).

HYDROXYUREA ENHANCES EFFECT OF TCEP DISSOCIATION OF BoNT/B LIGHT AND HEAVY CHAINS IN VITRO

To address the possibility that disruption of the disulfide bond linking BoNT LC and HC by TCEP might not be sufficient to separate the two chains because of non-covalent weak assembly forces, i.e. hydrogen bonds, ionic interactions, and Van Der Waals forces, a biocompatible chaotrope can be added to reduce the impact from these forces [Collins, 1997; Hallsworth et al., 2003; Moelbert et al., 2004]. Hydroxyurea (HU), a hydroxylated analogue of urea, is a biocompatible chaotropic often used for these purposes [Agnew et al., 2004; Doucette et al., 2004; Chinnasamy and Rampitsch, 2006; Vincent et al., 2006]. Thus, BoNT/B activity to cleave VAMP-2 in the presence of the sulfhydryl reducer TCEP (1 mM) with increasing concentrations of HU was determined. As shown in Figure 4, the peptidase activity was concentration dependent up to about 1–2 mM HU, with a greater than 30% increase of the activity. At concentrations greater than 2 mM, however, there was a reduction of peptidase activity. This data shows that the biocompatible chaotropic HU potentiated toxin peptidase in vitro by disrupting forces that would tend to keep the LC and HC together.

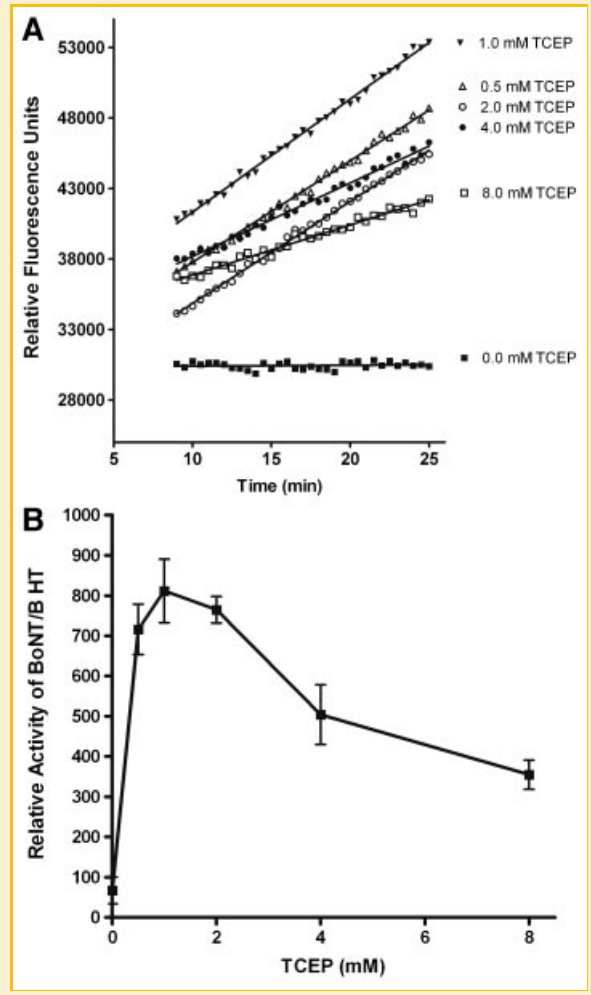

Fig. 2. Dose-dependent proteolytic activity of BoNT/B HT after TCEP treatment, as measured by FRET assay. A: Proteolytic activity of the dissociated BoNT/B light chain after treatment with varying concentrations of TCEP. B: Proteolytic activity of the dissociated peptidase (LC) represented as a mean rate (the slope of a linear line in A) and TCEP concentrations. Data shown were obtained in triplicate wells and are the means ± S.D. of three experiments.

TCEP PROTECTS AGAINST INHIBITION OF [^{3}H]-NA RELEASE BY BoNT/B IN HUMAN NEURONAL SHSY-5Y CELLS

The ability of TCEP to affect the BoNT/B HT structure – to separate the LC from the HC – may allow TCEP to be useful as a prophylactic agent against BoNT intoxication as dissociated LC cannot be

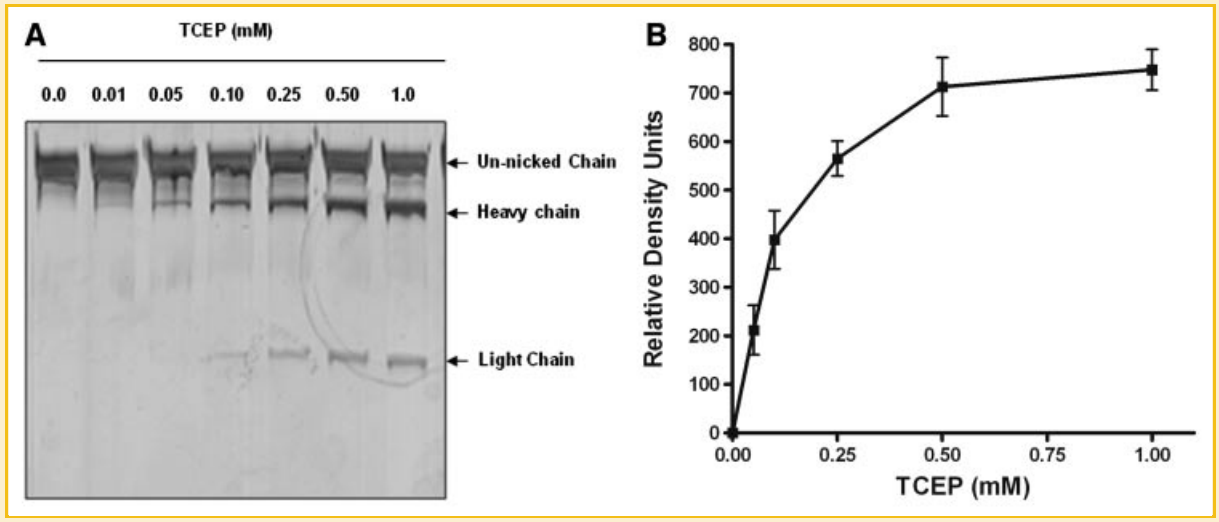

Fig. 3. Dissociation of BoNT/B light and heavy chains by TCEP. A: Silver-stained non-reducing SDS-PAGE. B: Densitometric analysis of the BoNT/B light chain SDS-PAGE. Data shown are the means ± S.D. of three experiments.

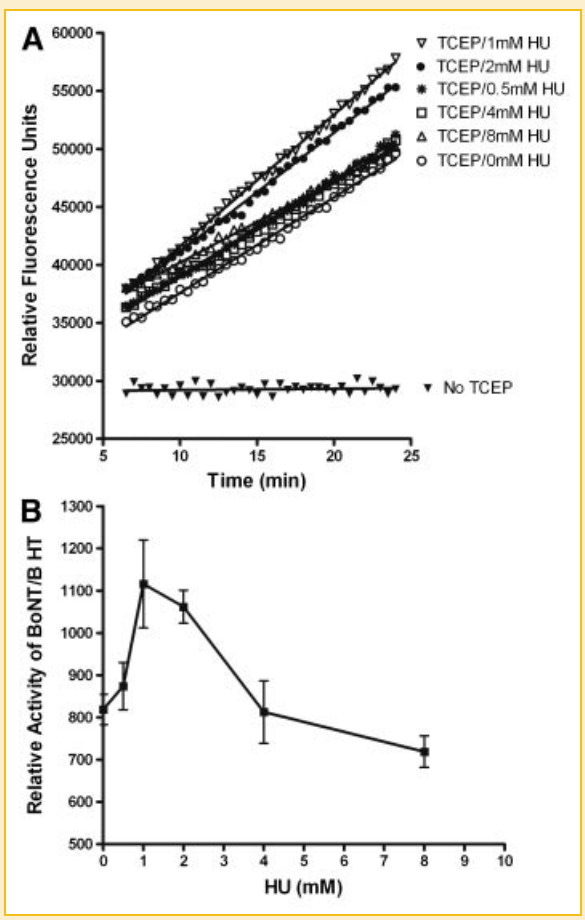

Fig. 4. Proteolytic activity of BoNT/B HT after co-treatment with hydroxyurea and TCEP, as measured by FRET assay. A: Proteolytic activity of the dissociated BoNT/B light chain peptidase with varying concentrations of hydroxyurea together with 1 mM TCEP. B: Proteolytic activity of the dissociated peptidase represented as a mean rate (the slope of a linear line in A) and hydroxyurea concentrations. Data shown were obtained in triplicate wells and are the means ± S.D. of three experiments.

translocated into cells. The utility of TCEP to prevent BoNT intoxication in the human neuronal cell line SHSY-5Y was evaluated. This cell line is susceptible to BoNT intoxication and can be tested by a noradrenaline release functional assay for content of VAMP-2, the native BoNT/B substrate. As shown in Figure 5A, 1 mM TCEP reduced BoNT inhibition by 70% while not significantly affecting cell viability as shown in Figure 5B. However, TCEP at 5 mM and greater significantly decreased cell viability (Fig. 5B). The decreased cell viability may result in low levels of noradrenaline release from those cells treated with higher doses of TCEP (Fig. 5A).

HU at 1 mM was found to improve TCEP-mediated dissociation of BoNT/B LC from the HC in a cell-free assay (Fig. 4). Therefore, the affect of the two agents together on the [^{3}H]-NA release assay was evaluated. As shown in Figure 6A, in contrast to the in vitro experiments, 1 mM HU reduced the TCEP effect; however, HU alone prevented [^{3}H]-NA release, as shown in Figure 6B. These data suggest that HU would likely not be useful in vivo. Although the mechanism of inhibition of NA release has not yet been established, HU caused apoptosis in susceptible cell lines at concentrations used in this study [Johnson et al., 1992].

TCEP REDUCES DEGRADATION OF VAMP-2 BY BoNT/B IN HUMAN NEURONAL CELLS

Since VAMP-2 is the native substrate of BoNT/B, toxin treated cells were evaluated to determine if the loss of vesicular transport was correlated with a loss of VAMP-2. As shown in Figure 7, there was a reduction of VAMP-2 in toxin treated SHSY-5Y cells. In contrast, VAMP-2 levels were almost the same as untreated cells if the BoNT/B HT was pretreated with 1 mM TCEP.

TCEP SIGNIFICANTLY CHANGES THE CONFORMATION OF BoNT/B HOLOTOXIN

8-Anilino-1-naphthalenesulfonic acid (ANSA) binds to hydrophobic regions of protein and is widely used to monitor conformational

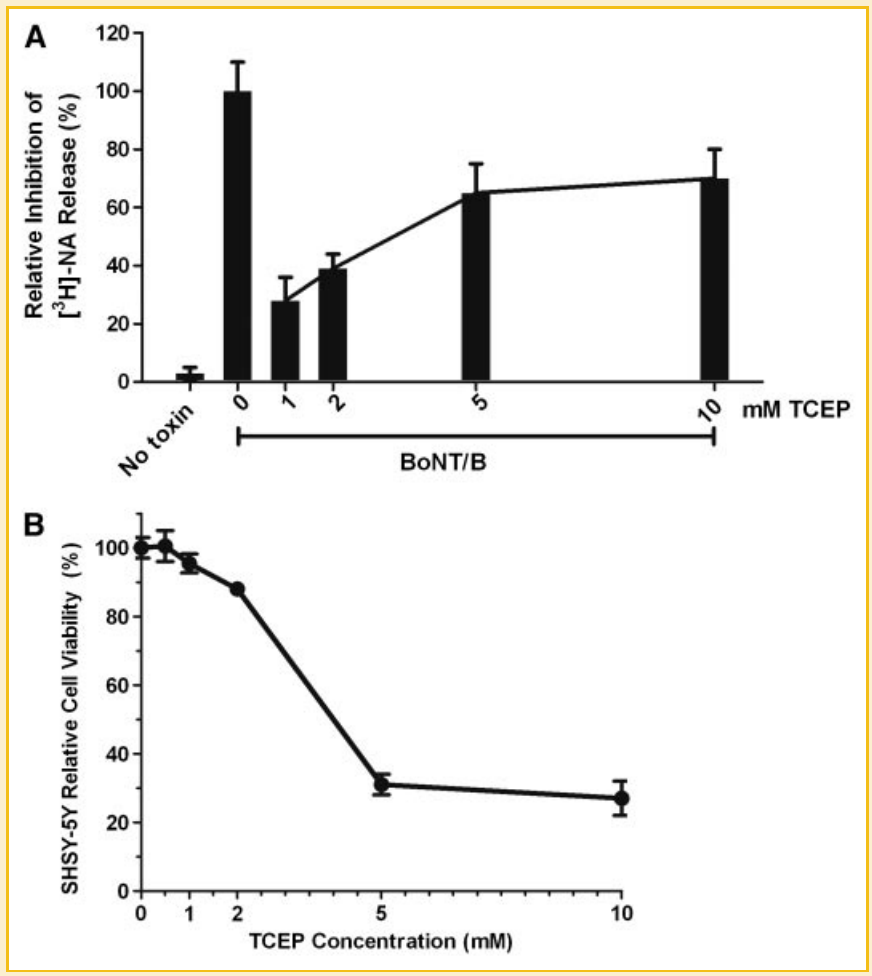

Fig. 5. TCEP effect on the cellular activity of BoNT/B HT in SHSY-5Y cells and its effect on cell viability. A: TCEP effect on the cellular activity of BoNT/B HT assessed by using a [^{3}H]-noradrenaline release assay. The percentage of [^{3}H]-NA released after K^+ stimulation compared to the total radioactivity in the cells prior to stimulation. [^{3}H]-NA released from control cells without TCEP treatment was set to 100%. B: TCEP effect on cell viability measured with a MTT assay. Cell viability of the control cells without treatment was set to 100%. Data shown were obtained in duplicate wells and are the means ± S.D. of three experiments.

changes of proteins [reviewed by Royer, 1995]. When protein structure changes, hydrophobic regions can be exposed to the aqueous solvent and become accessible to ANSA, leading to an increase in fluorescence. We used ANSA to monitor the conformational changes of BoNT/B HT after TCEP treatment. As shown in Figure 8, 1 mM TCEP (the concentration found to be optimal for BoNT activation in vitro) increased the fluorescence spectra. There was a significant increase in fluorescence intensity at a peak of approximately 505 nm compared to untreated toxin (Fig. 8A). The time course clearly showed that BoNT/B HT fluorescence, indicative of a conformation change, increased with time upon TCEP treatment (Fig. 8B). In a previous study, we reported that 1 mM TCEP did not fragment un-nicked BoNT/B HT [Shi et al., 2008]. Therefore, these results show that the increased toxin proteolytic activity observed after TCEP was due to reduction of the inter-chain disulfide bond, and that once the HC is separated from the LC, the LC is proteolytically active.

DISCUSSION

Although BoNT/A, B, and C are considered the most likely biological warfare agents (BWA) due to their toxicity, all seven toxin serotypes are potential BWA [Atlas, 1998; Arnon et al., 2001]. Therefore, universal treatments would be desirable as toxin serotype identification would take time and would not be necessary for therapy. This non-toxin specific approach drove our interest in TCEP as a potential therapeutic. TCEP is a relatively non-toxic, non-sulfur containing reducing agent that lacks the undesirable properties of those containing sulfur, such as 2-mercaptoethanol [Han and Han, 1994; Getz et al., 1999; Bingham et al., 2005]. As all seven BoNT seroptypes are similar in that they are di-chain constructs held together through a disulfide bond [Simpson, 1979; DasGupta, 1990], the reduction of the disulfide bond with TCEP would be useful across all the toxin serotypes since the heavy chain would be separated from the light chain, and then the light chain would be precluded

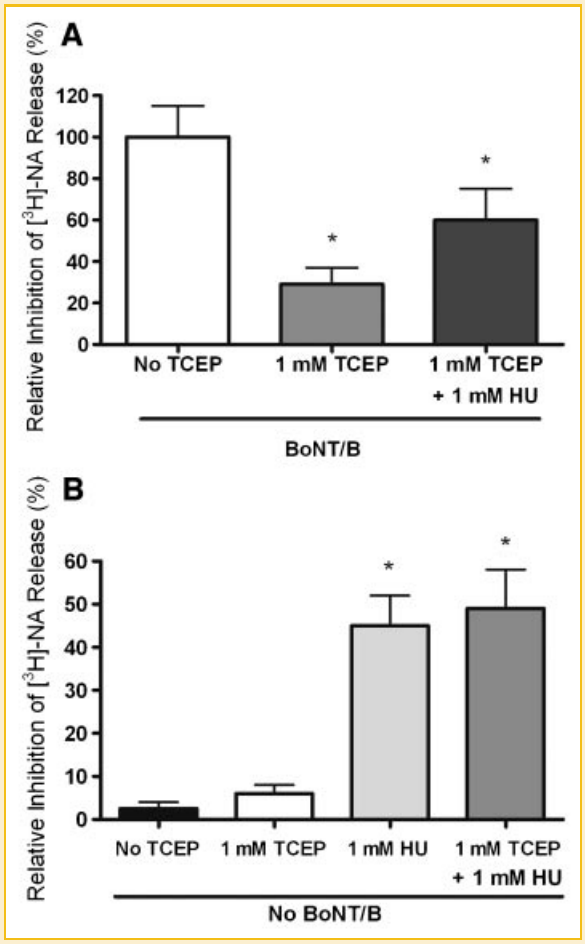

Fig. 6. Effect of TCEP and hydroxyurea co-treatment on the cellular activity of BoNT/B HT in SHSY-5Y cells and their effect on noradrenaline release. A: Effect of TCEP and hydroxyurea co-treatment on the cellular activity of BoNT/B HT assessed by using a [^{3}H]-noradrenaline release assay. [^{3}H]-NA released from control cells without TCEP treatment was set to 100%. B: Effect of TCEP and hydroxyurea on noradrenaline release from non-toxin treated cells. Data shown were obtained in duplicate wells and are the means ± S.D. of three experiments. $^*P < 0.05$ versus No TCEP treatment (Student's *t*-test, two-tailed).

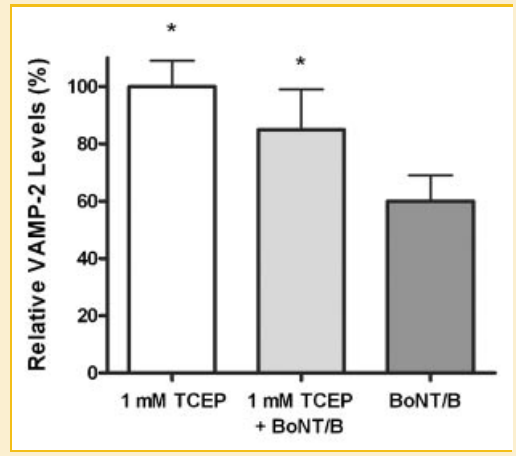

Fig. 7. TCEP effect on the proteolytic activity of BoNT/B HT to cleave VAMP-2 protein in SHSY-5Y cells. A capture ELISA was used to measure VAMP-2 in whole cell lysates following 48 h toxin treatment. The 100% level of VAMP-2 in the cell lysate was determined from BoNT untreated cells. Data shown were obtained in triplicate wells and are the means ± S.D. of three experiments. $^*P < 0.05$ versus BoNT/B treatment (Student's *t*-test, two-tailed).

from entering the neuronal cell. In this report, we show that TCEP is as effective as DTT [Cleland, 1964; Ruegg and Rudinger, 1977] for reducing the LC-HC disulfide bond, maximally activating the peptidase LC activity at 1 mM TCEP. The human neuronal cell line, SHSY-5Y, was our model for BoNT/A and B intoxication [Shi et al., 2008], and TCEP showed a little toxicity even at a 2 mM concentration (Fig. 5B). TCEP was effective in preventing intracellular intoxication by the BoNT/B LC endopeptidase activity when the toxin was pretreated with TCEP. Others have shown that low pH induced conformational changes of BoNTs (monitored by ANSA) and that the reduction of the essential disulfide bond took place after the penetration of the toxin into the membranes of the acidic endocytic compartments [Puhar et al., 2004]. Likewise, we observed similar conformational changes of BoNT/B HT with TCEP treatment (Fig. 8) showing that the reduction of the disulfide bond by TCEP occurs, allowing the separation of the HC and LC, activating the LC proteolytic activity in vitro (Fig. 2) and preventing penetration into cells. This latter point was shown by the near normal vesicular transport (attaining values greater than 70% of un-intoxicated controls) cellular function due to the sparing of VAMP-2 (Fig. 5A). This work demonstrates the feasibility of the approach where neuronal cells are protected from BoNT intoxication by holotoxin disruption. Use of a perturbant such as HU to improve on the separation of LC from HC heavy chain was not successful since the HU itself was toxic to the SHSY-5Y neuronal cells [Johnson et al., 1992; Adragna et al., 1994].

Commercial preparations of native BoNT/B HT are known to be mixtures of nicked and un-nicked forms [Sathyamoorthy and DasGupta, 1985]. The HC and LC of un-nicked toxin forms cannot be separated following disulfide reduction in vitro. In a previous report, we showed that purified un-nicked BoNT/B HT exhibited much weaker effects than nicked BoNT/B HT in SHSY-5Y cells [Shi et al., 2008]. As can also been seen in this report (Fig. 5A), a similar modest cellular activity of BoNT/B HT remained after treatment with TCEP. Because disulfide bond reducers such as TCEP were unable to separate the un-nicked HT into light and heavy chains even though it reduced its intra-disulfide bond, part of un-nicked BoNT/B HT still remained in the commercial toxin preparations, though this form was shown to be much less effective. Disulfide bonds coupling toxin subunits is a general motif for other toxins such as ricin; that is, two independent chains are held together by a disulfide bond [Wright and Robertus, 1987; Servent et al., 1997]. Therefore, the data presented here suggest that TCEP would be a potential pretreatment for other toxins by preventing cell penetration.

In summary, there is extensive investigation for post-exposure small molecule inhibitors of botulinum toxins, but they are serotype specific [Park et al., 2006; Willis et al., 2008; Zuniga et al., 2008]. Immunization as prophylaxis for BoNT is also serotype specific

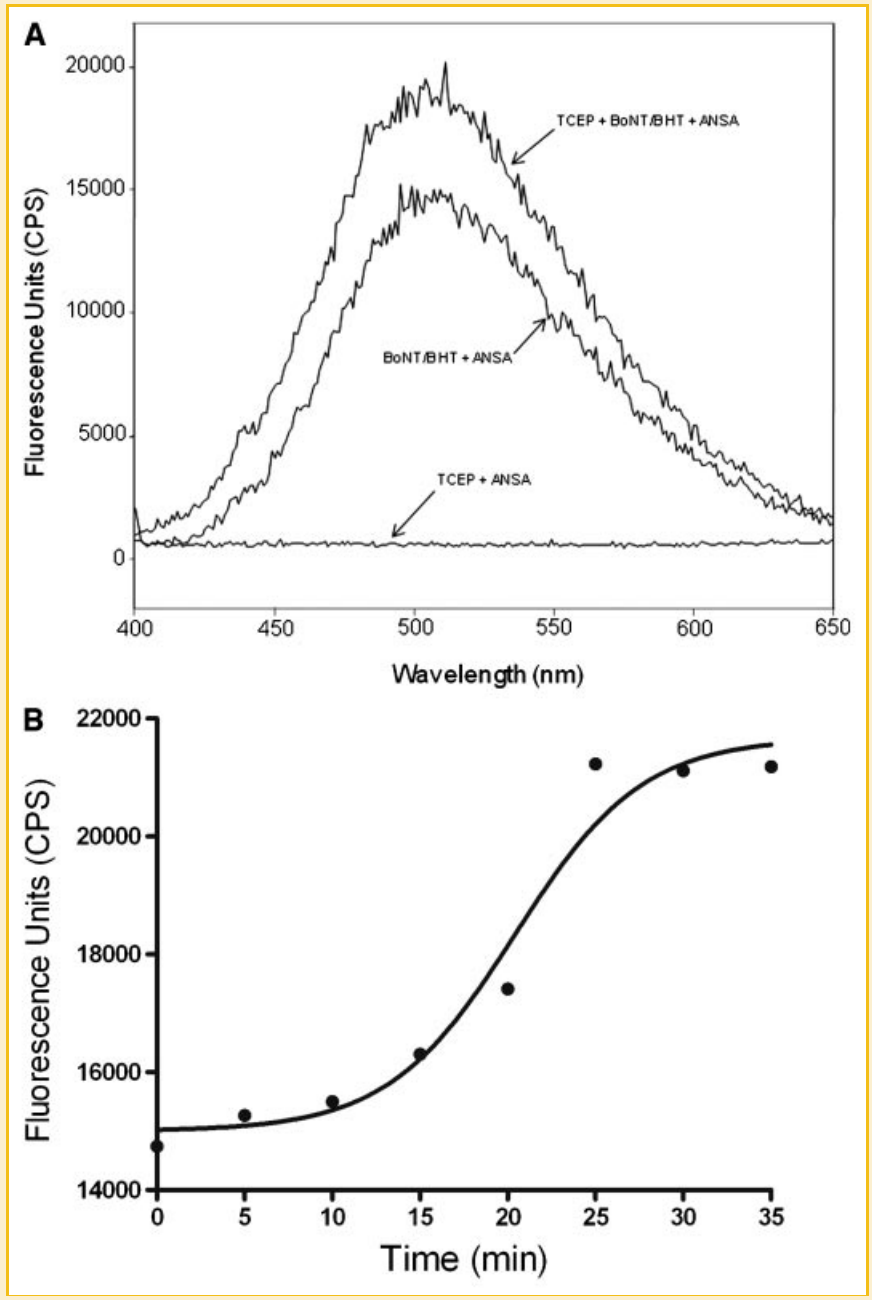

Fig. 8. Effect of TCEP on conformation of BoNT/B HT measured by fluorometric analysis. A: BoNT/B HT conformational change 30 min after TCEP treatment. ANSA (8-anilino-1-naphthalenesulfonic acid) fluorescence intensities were estimated by averaging three readings at room temperature. The excitation wavelength was 380 nm, and the emission spectra collected from 400 to 650 nm. The toxin and TCEP concentrations were 0.8 μM and 1 mM, respectively. B: Time course of BoNT/B HT conformational change after TCEP treatment.

[reviewed by Smith and Rusnak, 2007]. Post-exposure treatment with antibody also shows efficacy either because toxin is still being produced from gut *Clostridia* and/or toxin has not yet entered the neuronal cell [Arnon et al., 2006]. Therefore, it is likely that post exposure treatment will consist of a combination of multiple therapies. TCEP, or other non-toxic sulfhydryl reducers, offer the potential for additional means to abrogate, in a general manner, BoNT toxicity prior to neuronal cell penetration.

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