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Journal of Cellular Biochemistry 108:660–667 (2009)

Journal of Cellular Biochemistry

The Biological Activity of Ubiquitinated BoNT/B Light Chain In Vitro and in Human SHSY-5Y Neuronal Cells

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ABSTRACT

BoNT/B light chain is a zinc-dependent endopeptidase. After entering its target, the neuronal cell, BoNT/B is responsible for synaptobrevin-2 (VAMP-2) cleavage. This results in reduced neurotransmitter (acetylcholine) release from synaptic vesicles, yielding muscular paralysis. Since the toxin persists in neuronal cells for an extended period, regeneration of VAMP-2 is prevented. We evaluated therapeutic targets to overcome botulinum persistence because early removal would rescue the neuronal cell. The ubiquitination/proteasome cellular pathway is responsible for removing "old" or undesirable proteins. Therefore, we assessed ubiquitination of BoNT/B light chain in vitro, and characterized the effects of ubiquitination modulating drugs, PMA (phorbol 12-myristate 13-acetate) and expoxomicin, on ubiquitination of BoNT/B light chain in neuronal cells. Both drugs altered BoNT/B light chain ubiquitination. Ubiquitination in vitro and in cells decreased the biological activity of BoNT/B light chain. These results further elucidate BoNT protein degradation pathways in intoxicated neuronal cells and mechanisms to enhance toxin removal. J. Cell. Biochem. 108: 660–667, 2009.

KEY WORDS: UBIQUITINATION; UBIQUITIN; BoNT; BOTULINUM NEUROTOXIN; SYNAPTOBREVIN-2; VAMP-2

Ubiquitin is a small protein (8.5-kDa, 76 amino acids) expressed universally in all eukaryotic cells [Schlesinger and Goldstein, 1975; Schlesinger et al., 1975]. Ubiquitin can be added to a single lysine residue or multiple lysine residues on target proteins through a process referred to as ubiquitination; an isopeptide bond of ubiquitin carboxyl group attaches to the target lysine residue [Ciechanover et al., 1978, 1980; Wilkinson et al., 1980]. Since ubiquitin itself possesses seven lysine residues, additional ubiquitin molecules can be added, yielding a polyubiquitin chain [Chau et al., 1989]. Typically, ubiquitin molecules in a polyubiquitin chain are linked via lysine 48- or lysine 63 [Chau et al., 1989; Hofmann and Pickart, 1999; Thrower et al., 2000; Petroski et al., 2007], but a variety of linkages including all possible lysine residues also form [Kim et al., 2007; Xu and Peng, 2008]. Many cellular processes including cell cycle and division, apoptosis, immune response and inflammation, neural and muscular degeneration, transcriptional regulation, modulation of cell surface receptors, and protein degradation involve protein ubiquitination [reviewed by Hershko and Ciechanover, 1998]. Proteasome-dependent degradation is a major pathway of eukaryotic intracellular protein degradation [Ciechanover, 1998; Tanaka and Chiba, 1998; Kornitzer and Ciechanover, 2000]. In this pathway, the "selective" old proteins that are no longer needed in cells are tagged with a lysine 48-linked polyubiquitin (at least four ubiquitins) in order for them to be recognized by the 26S proteasome complex [Thrower et al., 2000]. Once proteins are targeted, they are destined for degradation in the proteasome complex within which the condemned proteins are degraded into small peptides. However, ubiquitin molecules are released from the proteins prior to destruction and recycled [Ciechanover, 1998; Ciechanover et al., 2000]. In contrast, if a ubiquitinated protein is de-ubiquitinated, it avoids degradation [Wilkinson, 2000].

The family of botulinum neurotoxins (BoNTs) produced by *Clostridium* bacteria are one of the most lethal bacterial toxins

Abbreviations used: BoNT, botulinum neurotoxin; FRET, fluorescence resonance energy transfer; HT, holotoxin; HC, heavy chain; LC, light chain; NA, noradrenaline; ELISA, enzyme-linked immunosorbent assay; PBS, Phosphate-buffered saline; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor; SNAP-25, 25 kDa synaptosomal-associated protein; TMB, tetramethyl benzidine; VAMP-2, vesicle-associated membrane protein-2/synaptobrevin-2; PMA, phorbol 12-myristate 13-acetate; Ub, ubiquitin; UbA, ubiquitin aldehyde.

Grant sponsor: Defense Threat Reduction Agency (DTRA); Grant number: #3.10017_06_WR_B.

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Received 23 February 2009; Accepted 8 July 2009 • DOI 10.1002/jcb.22300 • © 2009 Wiley-Liss, Inc.
Published online 13 August 2009 in Wiley InterScience (www.interscience.wiley.com).

[Gill, 1982]. BoNTs are divided into seven serotypes A, B, C, D, E, F, and G based on their immunoreactivity [DasGupta, 1990]. Each member of the toxin family is composed of a heavy chain (HC) and a light chain (LC) held together by a disulfide bond [Simpson, 1979; Humeau et al., 2000]. The HC is responsible for binding to and translocating the holotoxin (HC + LC) into neuronal cells. The LC, a zinc-dependent endopeptidase, is responsible for cleaving toxin specific SNARE protein after it enters the neuronal cell [Bandyopadhyay et al., 1987; Montecucco and Schiavo, 1994; Deloye et al., 1997; Pellizzari et al., 1999]. Unlike other bacterial toxins, BoNTs can persist intracellularly for a very long time. BoNT/A and BoNT/B neuromuscular paralysis persisted for 4 and 2 months, respectively, in humans undergoing treatment for dystonias [Sloop et al., 1997; Eleopra et al., 1998]. In contrast, the toxins exhibited a much shorter period of muscular paralysis in mice than in humans, but similar to humans, BoNT/A-induced paralysis persisted longer than BoNT/B [Keller, 2006]. Although persistence of BoNTs in human neuronal cells in vitro has not been determined, in rodent neuronal cells, BoNT/A and BoNT/B persisted for about 4 and 2 weeks, respectively [Keller et al., 1999; Foran et al., 2003]. Consistently, BoNT/A persists longer than BoNT/B. However, mechanisms by which BoNTs persist in neuronal cells and avoid protein degradation by the ubiquitin/proteasome pathway or other pathways, such as the lysosomal pathway [Stoka et al., 2001], are still unknown. To elucidate the mechanism by which BoNTs are eventually removed from neuronal cells, and to investigate possible therapeutic approaches to overcoming the longevity of these neurotoxins by enhancing the removal rate, we investigated the ubiquitin pathway in the human neuronal cell line, SHSY-5Y.

We report here ubiquitination of BoNT/B LC in vitro using a ubiquitin conjugation kit and the effect of ubiquitination on the biological activity of the toxin. We also characterized the effect of PMA (phorbol 12-myristate 13-acetate), a ubiquitination enhancer [Zhang et al., 1998], on BoNT/B LC biological activity in SHSY-5Y neuronal cells. After this treatment, the biological activity of BoNT/B LC significantly decreased, as measured by cleavage of its natural substrate, VAMP-2, in SHSY-5Y cells. Inhibition of proteasome activity by expoxomicin showed an increase in BoNT/B LC ubiquitination in cells. Our data suggest that modulation of the ubiquitin pathway may provide methods to reduce BoNT intoxication.

MATERIALS AND METHODS

CELL CULTURE

The human neuroblastoma cell line, SHSY-5Y, was obtained from the American Type Culture Collection (ATCC), 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 a 12-well plate while 2×10^6 cells were seeded in a 10 cm plate. Cell monolayers were ~70% confluent when they were treated with BoNT/B toxin.

UBIQUITINATION OF BoNT/B LC IN VITRO

Recombinant BoNT/B LC (List Biological Laboratories, Campbell, CA) was ubiquitinated using a ubiquitin conjugation kit based on the manufacturer's instructions (BIOMOL, Plymouth Meeting, PA). Each reaction in a volume of 50 μl contained 1 mg/ml HeLa S100 lysate, 100 μg/ml ubiquitin (Ub), 2 μM ubiquitin-aldehyde (UbA), 0.5 μM BoNT/B LC protein, and 5 mM cofactors/enzymes (ATP-ERS) in the supplied ubiquitination buffer. For the negative control, distilled water was used instead of ATP-ERS. Briefly, to start the reaction, all assay components were added except BoNT/B LC to eppendorf tubes, and the tubes mixed gently. Following pre-incubation at 37°C for 10 min, the BoNT/B LC was added, the tubes mixed again, and then incubated at 37°C for 2–4 h.

BoNT/B LC FRET VAMPTide ASSAY

The endopeptidase activity of BoNT/B LC was measured using a FRET VAMPTide™ assay as described by List Biological Laboratories (Campbell, CA) in a 96-well plate (Costar, Corning Life Science, Corning, NY) with minor modifications [Shi et al., 2008]. Briefly, 6 nM BoNT/B LC or ubiquitinated BoNT/B LC 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 dithiothreitol (DTT), and 0.25 mM $ZnCl_2$. The plates were sealed with plastic and incubated at 37°C for 15 min, after which 1.5 μl of 200 μM VAMPTide™ was added to start the reaction. The plates were mixed for 30 s by the plate reader, and then immediately read using a Tecan Safire Microplate Reader and kinetic program (Tecan Systems, San Jose, CA). The wells were read for 60 cycles each per 30 s at an excitation wavelength of 300 nm and emission wavelength of 415 nm. The rate of the proteolytic activity of BoNT LC endopeptidase was determined from the slope of the linear portion of the kinetic curve with GraphPad Prism Ver. 5.0 software (GraphPad Software, San Diego, CA).

[^{3}H]-NA RELEASE ASSAY

The release of [^{3}H]-NA (noradrenaline) was determined as previously described by Murphy et al. [1991] with minor modifications. Briefly, SHSY-5Y cultures were grown in 12-well plates to 70% confluency in RPMI 1640 media, as described in Cell Culture Section. After incubation with BoNT/B HT (holotoxin, 10 μg/ml) for 48 h, cell cultures were then incubated for 1–5 weeks in media without toxin. Untreated control cultures were also prepared without incubation in holotoxin. After the indicated times, the cells were washed 2× with 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 (dextrose), 0.2 mM ascorbic acid, 0.2 mM Pargyline). Cells were radio-labeled by incubation with HBS containing 50 nM [^{3}H]-NA (10.9 Ci/mmol, PerkinElmer, Inc., Waltham, MA) for 1 h. Next, the isotope-supplemented HBS was removed, and the cells were washed four times with HBS. The cells were then evoked to release [^{3}H]-NA by stimulation with 100 mM KCl for 5 min at 37°C. Following the stimulation, the cells were washed 2× with HBS. Unreleased [^{3}H]-NA was extracted from the cells with 0.1% Triton X-100 (1 ml per well). Cell lysate (0.5 ml) was added to 3 ml scintillation cocktail (Ultima Gold, PerkinElmer, Inc.), and the radioactivity was measured with a 1450 LSC & Luminescence counter (PerkinElmer, Inc.). The amount of [^{3}H]-NA released by stimulation with KCl was determined as the difference of [^{3}H]-NA remaining in cells after KCl exposure expressed as a percentage of unstimulated control cells.

UBIQUITINATED BoNT/B LC ELISA ASSAY

BoNT/B LC ubiquitination was measured by capture ELISA assay. SHSY-5Y cell cultures were prepared in 10 cm plates, and treated with BoNT/B HT (10 μg/ml) in RPMI 1640 culture medium for 48 h. The BoNT/B HT-treated cells were further treated with either 1 μM PMA only, or 1 μM PMA and 1 μM expoxomicin, for an additional 4 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 phosphatase and protease inhibitors (1 mM orthovanandate, 10 mM NaF, 10 μg/ml leupeptin, 25 μg/ml aprotinin and 50 μg/ml phenylmethanesulphonylfluoride/PMSF). BoNT-untreated control cultures were prepared and processed similarly. The ELISA was performed in 96-well microtiter plates coated with 1–2 μg/ml rabbit anti-BoNT/B LC antibody (BBTech, Dartmouth, MA) at 4°C overnight. Lysates from cell cultures or in vitro ubiquitinated BoNT/B LC 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 1:1,000 diluted mouse anti-ubiquitinated protein antibody (BIOMOL, Plymouth Meeting, PA) was added in a volume of 0.1 ml. Following 1 h incubation at room temperature, a 1:1,000 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 tetramethyl benzidine (TMB) reagent color reaction (KPL, Gaithersburg, MD) for 10 min. The plate was read in a Spectramax Plus plate reader (Molecular Devices, Sunnyvale, CA). 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.

VAMP-2 ELISA ASSAY

Cleavage of VAMP-2 by BoNT/B LC endopeptidase was quantified as previously described [Shi et al., 2008]. Briefly, 96-well microtiter plates were coated with 1–2 μg/ml goat anti-human VAMP-2 antibody (Proteintech, Chicago, IL) at 4°C overnight. Cell lysates isolated from cell cultures treated with BoNT/B HT only or BoNT/B HT and PMA together were added to antibody-coated wells and incubated at room temperature for 2 h. After several washes with 0.5% Tween-20 in PBS, a 1:1,000 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 1:1,000 diluted anti-mouse antibody conjugated with horseradish peroxidase (Cell Signaling) was added in a volume of 0.1 ml, and incubated at room temperature for an additional 1 h. Immunoreactivity was measured in a Spectramax Plus plate reader as described above. The ELISA plate results were normalized to protein content.

WESTERN BLOTTING ASSAY

Approximately 20 μg of cell lysate proteins were separated by 10% SDS–PAGE and transferred to PVDF Immobilon-P transfer membrane (Millipore, Billerica, MA). The blots were developed with specific primary antibodies that included rabbit anti-human VAMP-2 (Proteintech), rabbit anti-BoNT/B light chain (BBTech, Dartmouth, MA), rabbit anti-β-actin (Cell Signaling), and rabbit anti-ubiquitin (BioMol, Plymouth Meeting, PA) antibodies. The secondary antibody used in these blots was HRP-conjugated anti-rabbit antibody (Cell Signaling). The ECL (enhanced chemiluminescent) detection reagents (Amersham, Louisville, CO) were used to visualize bands with a FUJIFILM LAS-3000 (FUJI Medical Systems, Stamford, CT). The signal intensity of bands was analyzed by densitometry using ImageQuant version 5.2 software (Molecular Dynamics, CA).

RESULTS

BoNT/B MAINTAINED ITS ABILITY TO CLEAVE VAMP-2 IN HUMAN NEURONAL CELLS FOR WEEKS

As described in the Introduction, BoNT/B HT produced neuromuscular paralysis for approximately 2 months in humans [Sloop et al., 1997] and its biological activity persisted for at least 2 weeks in rat cerebellar neuronal cells [Foran et al., 2003], but the duration of its persistence in the human neuronal cell line SHSY-5Y has not been reported. To determine BoNT/B persistence in human SHSY-5Y cells, we treated the cells with 10 μg/ml BoNT/B HT and measured [^{3}H]-NA release from the cells weekly for up to 5 weeks. Since toxin LC in cells causes cleavage of VAMP-2, neurotransmitter release, which depends on intact VAMP-2, is also inhibited. As shown in Figure 1, BoNT/B inhibited NA release by about 95% for 2 weeks, which decreased to 50% inhibition by 3 weeks. Full restoration of NA release in the SHSY-5Y cells was observed after 5 weeks of incubation. Throughout this 5-week period, control cells not treated with BoNT/B exhibited normal neurotransmitter release. Thus, our data for BoNT/B intoxication in SHSY-5Y cells exhibits slightly

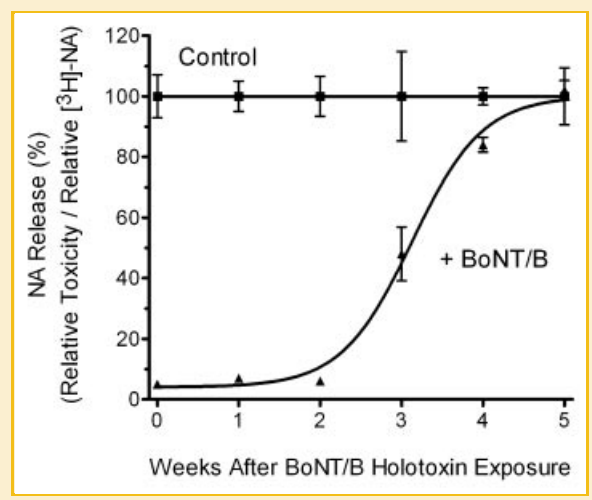

Fig. 1. Persistence of BoNT/B toxicity in human neuronal cells, as measured by FRET assay. SHSY-5Y cells in 12-well plates were treated with 10 μg/ml BoNT/B HT for 48 h and then incubated for up to 5 weeks in medium without toxin. The inhibition of [^{3}H]-noradrenaline (NA) release by BoNT/B was determined at each time point. NA release requires intact VAMP-2, which is the substrate of the toxin. Experiments were performed in triplicate wells (mean ± SD of three experiments).

longer persistence (3 weeks) than that observed in rat cerebellar neuronal cells [2 weeks; Foran et al., 2003].

UBIQUITINATION OF BoNT/B LIGHT CHAIN IN VITRO AND IN VIVO

In eukaryotic cells, ubiquitin-tagged proteins are degraded through the proteasome-dependent pathway [Ciechanover, 1998], but BoNT/B LC may avoid ubiquitination and subsequent degradation, resulting in its long duration of action in neuronal cells. To determine if BoNT/B LC could be ubiquitinated, we treated the LC with all the necessary ubiquitination components (present in the BIOMOL ubiquitin conjugation kit) and then measured ubiquitination using a capture ELISA assay. As shown in Figure 2, the toxin LC was significantly ubiquitinated compared to the control without ubiquitination cofactors/enzymes (ATP-ERS). In the presence of UbA, a deubiquitination inhibitor, there was no significant change in ubiquitination compared to that observed in the absence of UbA. Western blotting with anti-ubiquitin and anti-BoNT/B LC antibodies independently demonstrated that BoNT/B LC was ubiquitinated (Fig. 3). Ubiquitinated protein bands including polyubiquitins and ubiquitinated BoNT/B LC were observed in the treated LC with all the necessary ubiquitination components regardless of the presence or absence of UbA (Fig. 3A). Note that there was no protein band corresponding to ubiquitinated BoNT/B LC observed in control LC (Fig. 3A). When the blot was stripped and re-probed with anti-BoNT/B LC antibody (Fig. 3B), the control LC yielded a strong band reflecting the ~53 kDa LC. In contrast, the LC treated with the conjugated kit yielded higher molecular weight bands corresponding to multiple ubiquitinations added to the toxin where each ubiquitin moiety is ~9 kDa (Fig. 3B). Thus, BoNT/B LC ubiquitination measured by ELISA (Fig. 2) correlated well to that measured by Western blotting (Fig. 3), showing conclusively that BoNT/B LC is ubiquitinated in vitro.

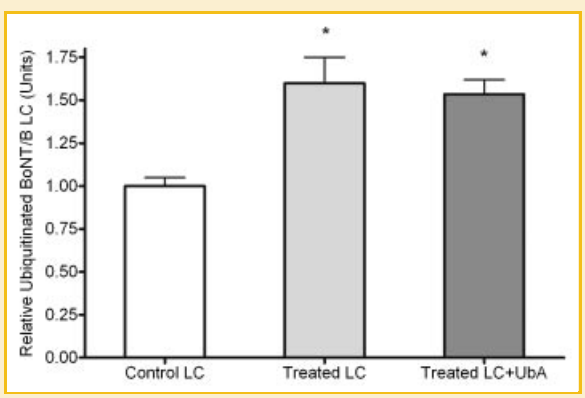

Fig. 2. Determination of ubiquitinated BoNT/B LC generated in vitro. BoNT/B LC was ubiquitinated using a ubiquitin conjugation kit. Control LC: contains the reaction mixture without ATP-ERS (the cofactors and conjugation enzymes); Treated LC: contains complete reaction mixture without UbA (deubiquitination inhibitor); Treated LC + UbA: contains the full component of reagents and UbA. A capture ELISA was used to measure ubiquitinated BoNT/B LC. The level of ubiquitinated BoNT/B LC in control (Control LC) was set to 1 unit. Data shown were obtained in triplicate wells (mean ± SD of three experiments). $^{*}P < 0.05$ versus Control LC (Student's *t*-test, two-tailed).

To demonstrate that BoNT/B LC was ubiquitinated in neuronal cells, human SHSY-5Y neuronal cells were treated with 10 μg/ml BoNT/B HT for 48 h, followed by 1 μM PMA, a ubiquitination enhancer [Zhang et al., 1998; Srivastava et al., 2002], for an additional 4 h. Next, isolated cell lysates from the treated and non-treated SHSY-5Y cells were measured for toxin LC ubiquitination. As shown in Figure 4, PMA treatment significantly increased ubiquitination of the BoNT/B LC by approximately 2.5-fold compared to the control without PMA treatment. Thus, PMA promoted BoNT/B LC ubiquitination in SHSY-5Y neuronal cells, and BoNT/B LC could be ubiquitinated in vitro and in SHSY-5Y cells.

UBIQUITINATION DECREASED BoNT/B LIGHT CHAIN BIOLOGICAL ACTIVITY

Next, we determined if ubiquitination affects the biological activity of BoNT/B LC. The activity of BoNT/B LC ubiquitinated in vitro (verified by ELISA, see Fig. 2) was quantified using the FRET assay. As shown in Figure 5, ubiquitination decreased the biological activity of the toxin LC by approximately 34% compared to the non-ubiquitinated toxin LC. Thus, ubiquitinated BoNT/B LC exhibits moderately decreased biological activity and substrate cleavage in vitro.

To demonstrate that ubiquitinated BoNT/B LC exhibits decreased activity in SHSY-5Y neuronal cells, cleavage of VAMP-2 by the LC in PMA-treated cells was determined. The amount of VAMP-2 was measured by the capture ELISA assay. In the cells treated with BoNT/B and PMA, the VAMP-2 level increased approximately 22% compared to the cells treated with only BoNT/B, as shown in Figure 6. In other words, PMA increased ubiquitination and moderately decreased BoNT/B LC activity (see Figs. 4 and 6). To confirm the increase in VAMP-2 in cells treated with PMA, Western blotting was used with anti-human VAMP-2 antibody to measure VAMP-2 levels. Consistent with the ELISA assay analysis, VAMP-2 levels in cells treated with PMA increased compared to those that were untreated (Fig. 7). Densitometric values (normalized to β-actin) clearly demonstrated that the biological activity of BoNT/B LC (VAMP-2 cleavage) also decreased after ubiquitination in the neuronal cells. Since it is likely that ubiquitin-tagged toxin LC molecules were degraded through the proteasome-dependent pathway, in part, the increased level of VAMP-2 in cells after PMA treatment may be due to degradation of the toxin LC.

EXPOXOMICIN TREATMENT INCREASED THE LEVEL OF UBIQUITINATED BoNT/B LIGHT CHAIN IN HUMAN NEURONAL CELLS

If ubiquitinated BoNT/B LC is degraded through a proteasome-dependent pathway, then its ubiquitinated form would be expected to increase if the activity of the proteasome were inhibited. To test this possibility, SHSY-5Y cells were treated with 10 μg/ml BoNT/B HT for 48 h, followed by 1 μM of the proteasome inhibitor expoxomicin [Meng et al., 1999] and proteasome enhancer PMA for an additional 4 h. Next, the level of ubiquitinated toxin LC in cell lysates was determined. Expoxomicin treatment increased the level of ubiquitinated BoNT/B LC by approximately 30% compared to PMA treatment alone (Fig. 8). While these results suggest that the

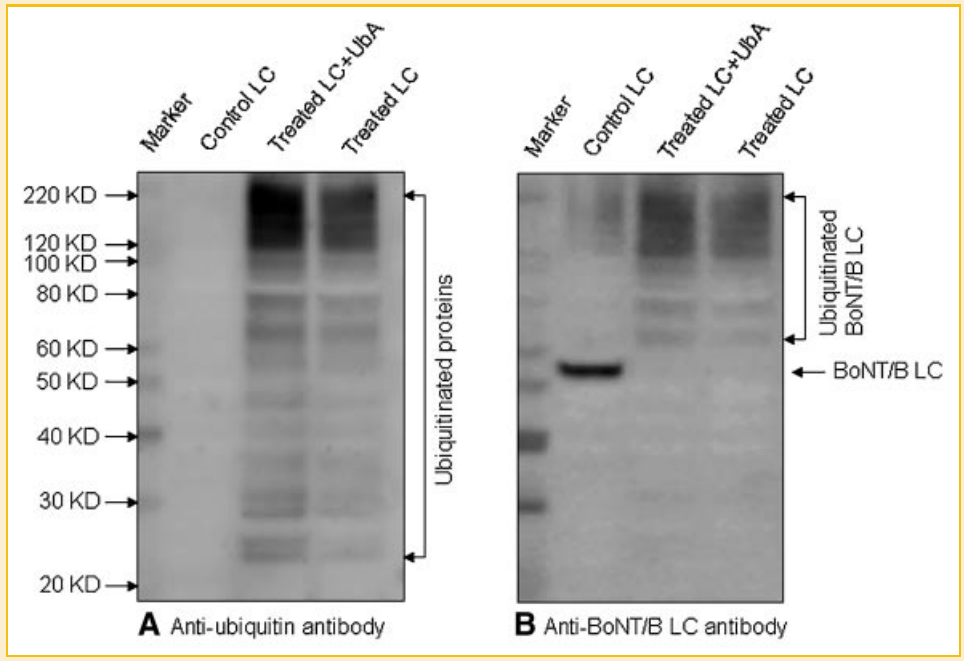

Fig. 3. Western blot of ubiquitinated BoNT/B LC generated in vitro. BoNT/B LC was ubiquitinated using the BIOMOL ubiquitin conjugation kit. Control LC: contains the reaction mixture without ATP-ERS (cofactors and conjugation enzymes); Treated LC: contains complete reaction mixture without UbA (deubiquitination inhibitor); Treated LC + UbA: contains the full component of reagents and UbA. Treated LC and control LC proteins (0.5 μg) were separated on 10% SDS–PAGE and transferred to a PVDF Immobilon-P membrane. A: The blot was developed with rabbit anti-ubiquitin and detected with HRP conjugated anti-rabbit antibodies. B: The blot was detected with anti-BoNT/B LC and HRP conjugated anti-rabbit antibodies after it was stripped with buffer.

BoNT/B LC is processed for removal via the proteasome-dependent degradation pathway after ubiquitination in neuronal cells, there are as yet unknown mechanism(s) for the prolonged persistence of the BoNT/B in neuronal cells (Fig. 1).

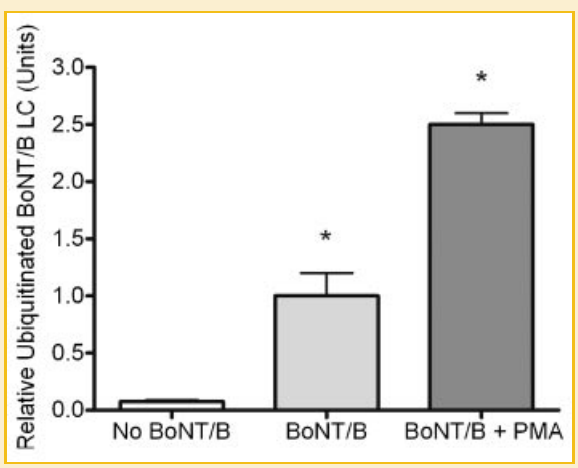

Fig. 4. PMA treatment increased ubiquitination of BoNT/B LC in human neuronal cells. SHSY-5Y cells pre-treated with 10 μg/ml BoNT/B for 48 h were then treated with 1 μM PMA for an additional 4 h. A capture ELISA was used to measure ubiquitinated BoNT/B LC in whole cell lysates following treatment. The level of ubiquitinated BoNT/B LC in the cell lysate from PMA untreated cells was set to 1 unit. Data shown were obtained in triplicate wells (mean ± SD of three experiments). $^{*}P < 0.05$ versus Control, no BoNT/B (Student's *t*-test, two-tailed).

DISCUSSION

The [^{3}H]-noradrenaline release assay has been widely used to measure the proteolytic activity of BoNTs in neuronal cells [Ashton and Dolly, 1991; Purkiss et al., 2001; Stigliani et al., 2003]. In human neuronal SHSY-5Y cells, we found that the full proteolytic activity of BoNT/B HT, as measured by [^{3}H]-NA release, persisted for approximately 2 weeks, and the toxin continued to block 50% of NA release at 3-week post-exposure (Fig. 1). In our previous report, cleavage of VAMP-2, the substrate for BoNT/B LC, correlated well with inhibition of neuronal transmitter release [Shi et al., 2008]. Foran et al. [2003] also demonstrated good correlation between target cleavage and inhibition of neurotransmitter release. Our data are in agreement with other published work showing that BoNT/B has shorter persistence than BoNT/A in neuronal cells. BoNT/B maintained its half-life of activity to cleave VAMP-2 in rat cerebellar granular neurons for about 2 weeks [Foran et al., 2003], while BoNT/A persisted in its ability to cleave SNAP-25 for much longer (approximately 11 weeks) in mouse cultured spinal cord cells [Keller et al., 1999] and about 4 weeks in rat cerebellar granular neurons [Foran et al., 2003].

We have demonstrated, for the first time, that ubiquitination significantly decreased the biological activity of BoNT/B LC (Figs. 5–7) and that the ubiquitinated BoNT/B is in part likely degraded by the proteasome-dependent pathway (Fig. 8). The proteasome is a major pathway for removal of unneeded proteins in the eukaryotic cells [Ciechanover et al., 2000]. In the studies reported here, ubiquitination played a dual role that (a) decreased the activity of BoNT/B LC

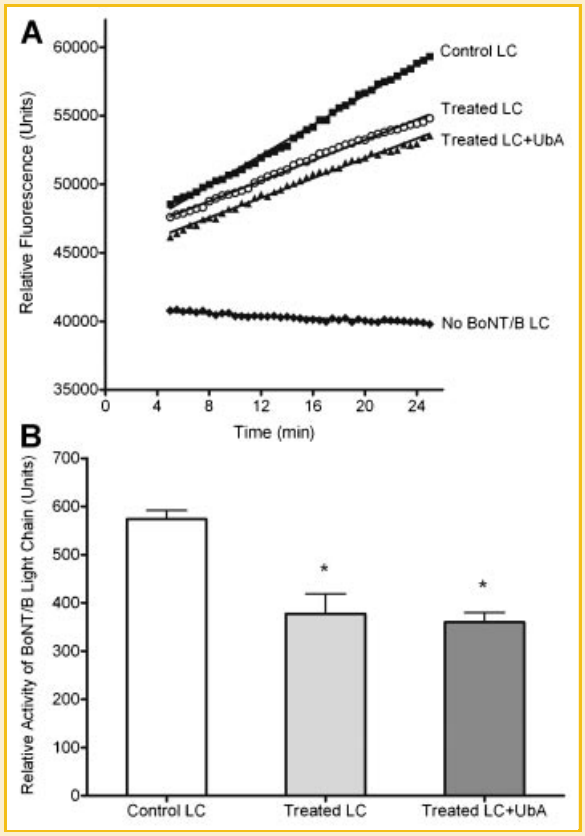

Fig. 5. Comparison in the activities of ubiquitinated and non-ubiquitinated BoNT/B LC. The BoNT/B LC was ubiquitinated with the BIOMOL conjugation kit and verified by ELISA assay. The proteolytic activities of both ubiquitinated and non-ubiquitinated BoNT/B LC were measured by using a modified BoNT/B LC FRET assay. A: Relative fluorescence data showing the activities of ubiquitinated and non-ubiquitinated BoNT/B LC over time. B: Representation of the activities of the treated light chains. Data shown were obtained in triplicate wells (mean ± SD of three experiments). $^{*}P < 0.05$ versus Control LC (Student's *t*-test, two-tailed).

(Figs. 5–7) and (b) increased its removal through the proteasome dependent pathway (Fig. 8). This dual role of ubiquitination was also reported in a transcription factor containing the VP16 TAD (transcriptional activation domains), whose function was regulated by ubiquitination as a dual signal for activation and activator destruction [Salghetti et al., 2001]. In addition to removal of unwanted proteins, ubiquitination has been reported to modulate gene expression. For instance, ubiquitination did not target the viral transcriptional activator Tax for destruction, but instead it decreased its transcriptional ability to activate gene expression [Peloponese et al., 2004]. In contrast, the transcriptional function of the viral transactivator Tat was stimulated by ubiquitination [Brès et al., 2003].

PMA promoted ubiquitination of BoNT/B LC in the human SHSY-5Y neuronal cells (Fig. 4), leading to a significant decrease in the toxin LC biological activity and cleavage of VAMP-2 (Figs. 6 and 7). The reduction in VAMP-2 cleavage is either due to the loss of the BoNT/B LC molecules because of degradation after ubiquitination or due to ubiquitination modulation of toxin activity, as we observed

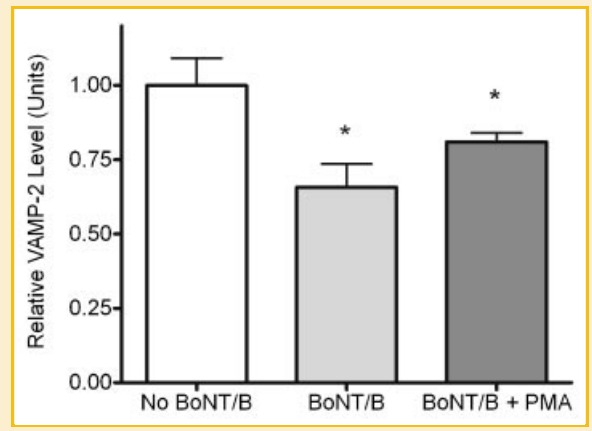

Fig. 6. Ubiquitination decreased the activity of BoNT/B LC, reflected in the level of VAMP-2 in human neuronal cells. SHSY-5Y cells pretreated with 10 μg/ml BoNT/B HT were treated with 1 μM PMA for an additional 4 h. A capture ELISA assay was used to measure VAMP-2 in whole cell lysates following treatment. The level of VAMP-2 in the cell lysate from BoNT/B untreated cells was set to 1. Data shown were obtained in triplicate wells (mean ± SD of three experiments). $^{*}P < 0.05$ versus Control, no BoNT/B (Student's *t*-test, two-tailed).

in vitro. The in vitro FRET assay showed that the proteolytic ability of BoNT/B LC to cleave its substrate, VAMP-2, was decreased (Fig. 5) after ubiquitination (Figs. 2 and 3). Therefore, based on our observation that the toxin LC in cells has reduced proteolytic ability to cleave VAMP-2 if not degraded after PMA treatment, therapeutics to overcome BoNT toxicity should also focus on increasing ubiquitination. While there have been a large number of studies to develop drugs to inhibit the toxin proteolytic activity to relieve toxic symptoms [Willis et al., 2008], the findings reported here

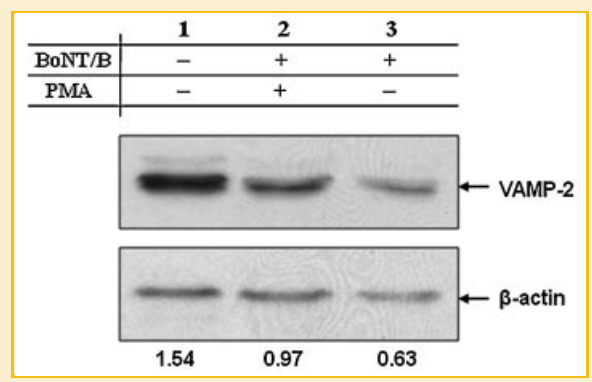

Fig. 7. Determination of VAMP-2 levels by Western blot analysis. SHSY-5Y cell lysates (20 μg) were separated on 10% SDS–PAGE and transferred to PVDF Immobilon-P membranes. The blot was first developed with rabbit anti-VAMP-2 and HRP conjugated anti-rabbit antibodies, and after being stripped with buffer, re-probed with anti-β-actin and HRP conjugated anti-rabbit antibodies. Lane 1 (Control): Cells were not treated; Lane 2 (BoNT/B + PMA): Cells pretreated with 10 μg/ml BoNT/B HT were then exposed to 1 μM PMA for an additional 4 h; Lane 3 (BoNT/B): Cells were treated with 10 μg/ml BoNT/B HT only. Densitometric values below lanes refer to the relative intensity of bands after normalization to β-actin.

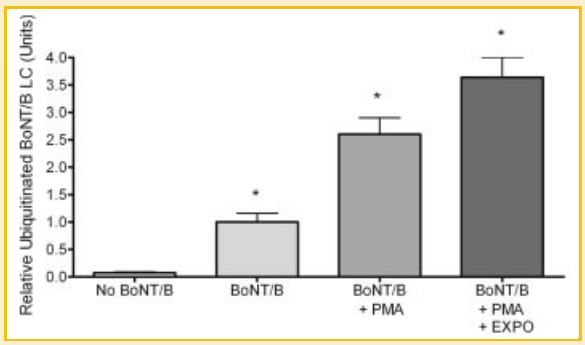

Fig. 8. Expoxomicin treatment increased the level of ubiquitinated BoNT/B LC in human neuronal cells. SHSY-5Y cells pre-treated with 10 μg/ml BoNT/B HT for 48 h followed by treatment with 1 μM expoxomicin (EXPO) plus 1 μM PMA for an additional 4 h. A capture ELISA was used to measure ubiquitinated BoNT/B LC in cell lysates. The level of ubiquitinated BoNT/B LC from expoxomicin untreated cells was set to 1. Data shown were obtained in triplicate wells (mean ± SD of three experiments). $^{*}P < 0.05$ versus Control, no BoNT/B (Student's *t*-test, two-tailed).

focused on removing the toxin, thereby rapidly reducing its toxicity by ubiquitination in the neuronal cells. Our studies may contribute to the development of therapeutics directed against the longevity of BoNTs through removal of the toxins from target neuronal cells by promoting their ubiquitination using drugs.

ACKNOWLEDGMENTS

We would like to thank Dr. Julian R. Haigh for a critical reading of the manuscript. This material has been reviewed by the Walter Reed Army Institute of Research and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. This study is supported by Defense Threat Reduction Agency (DTRA) proposal #3.10017_06_WR_B.

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