Urodynamic and Immunohistochemical Evaluation of Intravesical Botulinum Toxin A Delivery Using Liposomes

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Abbreviations and Acronyms

AA = acetic acid

BoNT-A = botulinum toxin A

CGRP = calcitonin gene-related peptide

CMG = cystometrogram

IC/PBS = interstitial cystitis/painful bladder syndrome

ICI = intercontraction interval

LP = liposome

PBS = phosphate buffered saline

SNAP = synaptosomal associated membrane protein

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Purpose: Botulinum toxin A (Allergan, Irvine, California) is a high molecular weight neurotoxin used to treat hypersensitive bladder by direct injection to pass the urothelial barrier. We investigated the feasibility of intravesical botulinum toxin A delivery using liposomes (Lipella Pharmaceuticals, Pittsburgh, Pennsylvania), which are phospholipid bilayered vesicles, and evaluated the urodynamic and immunohistochemical effect on acetic acid induced bladder hyperactivity in rats.

Materials and Methods: Liposomes (1 ml), botulinum toxin A (20 U/1 ml saline) or botulinum toxin A encapsulated in liposomes (lipotoxin, that is 20 U botulinum toxin A plus 1 ml liposomes) was administered in the bladder and retained for 1 hour on day 1 after baseline cystometrogram. Continuous cystometrogram was performed on day 1 by filling the bladder with saline and on day 8 by filling the bladder with saline, followed by 0.3% acetic acid. The bladder was then harvested. Cystometrogram parameters, histology, SNAP25 and calcitonin gene-related peptide expression were measured by Western blotting or immunostaining.

Results: The intercontraction interval was decreased 57.2% and 56.0% after intravesical acetic acid instillation in liposome and botulinum toxin A pretreated rats, respectively. However, rats that received lipotoxin showed a significantly decreased intercontraction interval response (21.1% decrease) to acetic acid instillation but without compromised voiding function. Also, lipotoxin pretreated rats had a better decrease in the inflammatory reaction and SNAP-25 expression, and increase in calcitonin gene-related peptide immunoreactivity than those in liposome or botulinum toxin A pretreated rats.

Conclusions: Intravesical lipotoxin administration cleaved SNAP-25, inhibited calcitonin gene-related peptide release from afferent nerve terminals and blocked the acetic acid induced hyperactive bladder. These results support liposomes as an efficient vehicle for delivering botulinum toxin A without injection.

Key Words: urinary bladder, urinary incontinence, botulinum toxin type A, liposomes, drug delivery systems

Botulinum toxin A, which acts by cleaving the cytosolic translocation protein SNAP-25 and inhibiting the process of exocytosis, decreases the release of various neurotransmitters.[1–3] The toxin has been used effectively for different muscular disorder conditions.[1] Cystoscopic guided injec-

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tions of BoNT-A have a therapeutic effect on overactive bladder and IC/PBS.[3,4] However, BoNT-A injection therapy has some limitations, including drug leakage outside the bladder, hematuria, pain at injection sites and uneven distribution.[5,6] Therefore, there is a need for a simpler and lower risk method to deliver BoNT-A without injection.

LPs are spherical vesicles composed of concentric phospholipid bilayers separated by aqueous compartments.[7] LPs have the characteristics of adhering to and creating a molecular film on cellular surfaces.[7,8] A previous study in a different animal model showed that intravesical LP instillation enhanced the barrier properties of dysfunctional urothelium and partially reversed high micturition frequency in a rat model of hyperactive bladder induced by breaching the uroepithelium with protamine sulfate and then irritating the bladder with KCl.[9] In addition, LPs serve as vehicles for drug delivery and gene therapy.[7] Tyagi et al reported that LPs are a superior vehicle for intravesical administration of capsaicin with less vehicle induced inflammation compared to that of 30% ethanol.[10]

BoNT-A is a neurotoxin with high molecular weight of 150 kDa. It is difficult for BoNT-A to access the submucosal nerve plexus in formal use with saline as a vehicle without direct injection to pass the urothelial barrier. Based on LP carrier potential, and the characteristics of adsorption and fusion with cells we hypothesized that certain LPs may serve as a BoNT-A delivery vehicle.

MATERIALS AND METHODS

LP, BoNT-A and Lipotoxin Preparation

LPs (10 mg) were dispersed in physiological saline (1 ml) with the dispersion in liposomal form. BoNT-A was dissolved in physiological saline (1 ml, 20 U/ml in saline). LPs encapsulating BoNT-A, termed lipotoxin, were prepared by a modified dehydration-rehydration vesicle method that loads 20 U BoNT-A into 10 mg LP dispersion (1 ml).[11] Briefly, lipotoxin was prepared before application by hydrating freeze-dried LPs (50 mg) in saline (3 cc) and BoNT-A (100 U) in saline (2 cc) at room temperature.

Cystometrogram

All experimental procedures were performed in female Sprague-Dawley rats weighing 220 to 280 gm, and were reviewed and approved by the institutional animal care and use committee before the study began. Animals were anesthetized by subcutaneous injection of urethane (1.2 gm/kg). PE-50 tubing (Clay-Adams, Parsippany, New Jersey) was inserted into the bladder through the urethra and connected via a 3-way stopcock to a pressure transducer and to a syringe pump to record intravesical pressure and infuse solutions into the bladder. On day 1 control CMG was performed by filling the bladder with saline (0.08 ml per minute) to elicit repetitive voiding. The amplitude (peak pressure minus baseline pressure during each contraction period), pressure threshold (pressure immediately before the reflex contraction), pressure baseline (pressure at the start of CMG with an empty bladder or immediately after reflex contraction) and ICI (average time between contractions) of reflex bladder contractions were recorded. Measurements in each animal represented the average of 3 to 5 bladder contractions.

On day 8 after baseline measurement during saline infusion we infused 0.3% AA into the bladder at 0.08 ml per minute to acutely promote bladder hyperactivity. We measured 3 to 5 bladder contractions after 30 minutes of infusion.

Drug Instillation and Transcardiac Perfusion

On day 1 after baseline CMG PE-50 tubing was tied in place by a ligature around the urethral orifice using halothane anesthesia. The bladder was emptied of urine and filled with LPs, BoNT-A or lipotoxin for 1 hour through the catheter. On day 8 after CMG 6 animals per group were deeply anesthetized and sacrificed via transcardiac perfusion with Krebs buffer, followed by 4% paraformaldehyde fixative. Animals were dissected to harvest the bladder.

Histology

For histology bladder tissues were fixed in 4% paraformaldehyde in PBS for 4 hours and then in 30% sucrose in PBS overnight. Samples for histology were embedded in paraffin, cut in 10 μm pieces and stained with hematoxylin and eosin. The AA induced inflammatory reaction was graded on a scale of 0—no evidence of inflammatory cell infiltrates or interstitial edema, 1—mild (few inflammatory cell infiltrates and little interstitial edema), 2—moderate (moderate inflammatory cell infiltrates and interstitial edema) and 3—severe (large diffuse inflammatory cell infiltrates and severe interstitial edema).[12,13]

Immunofluorescence Microscopy for CGRP and SNAP-25

Alternative samples were frozen and mounted in Tissue-Tek® O.C.T.™ mounting medium. Longitudinal sections (10 μm) were cut on a cryostat and mounted on Superfrost® slides. Sections were fixed by immersion in acetone for 15 minutes and washed in PBS. Endogenous peroxidase activity was blocked by incubating slides in 0.3% H_2O_2 solution in PBS for 10 minutes. After further washing in PBS sections were incubated in Image-iT^{TW} fix signal enhancer for 1 hour. For CGRP immunostaining slides were then stained with goat anti-CGRP polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California) (1:50 dilution) at 4C for 48 hours. The next day sections were washed in PBS and incubated with donkey anti-goat IgG fluorescein isothiocyanate (Santa Cruz Biotechnology) (1:2,000 dilution) for 1 hour. Sections were washed and mounted in SlowFade® Gold antifade reagent. For SNAP-25 immunostaining slides were then stained with mouse anti-SNAP-25 monoclonal antibody (AbD Technology, Durham, North Carolina (1:2,000 dilution) at 4C overnight. The next day sections were washed in PBS and incubated with goat anti-mouse IgG Alexa Fluor® 488 for 1 hour. To detect muscle fibers sections were counterstained with tetramethylrhodamine isothiocyanate labeled phalloidin (Sigma®) (1:2,000 dilution), washed and mounted in SlowFade Gold antifade reagent.

Slides were examined with a fluorescence microscope to record the image.

Western Blot for SNAP-25 Expression

Six animals per group were deeply anesthetized and sacrificed without transcardiac perfusion. The bladder was removed for Western blot analysis of SNAP-25 expression according to the standard protocol (GE Healthcare, Little Chalfont, United Kingdom) and our previous study.[13,14] Samples were homogenized in T-PER protein extraction solution (Thermo Scientific, Rockford, Illinois) before sonication and purification. Total protein was measured with the Bradford protein assay method (Bio-Rad®). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the Laemmli buffer system. Briefly, an aliquot of extracts equivalent to 30 μg protein was loaded onto 8% polyacrylamide gel, electrophoresed at a constant voltage of 100 V for 1 hour and transferred to Hybond™-P polyvinylidene difluoride membrane. The membrane was blocked with blocking agent and immunoblotted overnight at 4C with mouse anti-SNAP-25 monoclonal antibody (1:500 dilution) and mouse anti-β-actin monoclonal antibody (Rockland Immunochemicals, Gilbertsville, Pennsylvania) (1:2,000 dilution). After washing the membrane was incubated with secondary antibody using 5% defatted milk powder in tris buffered saline for 2 hours at room temperature using horseradish peroxidase linked anti-rabbit or anti-mouse IgG. Western blots were visualized by an enhanced chemiluminescence detection system (GE Healthcare). The amount of β-actin was also detected as the internal control. Quantitative analysis was done using LabWorks™ image acquisition and analysis software.

Statistical Analysis

Quantitative data are expressed as the mean ± SEM. Statistical analysis was performed using 1-way ANOVA with the Bonferroni post test or the Kruskal-Wallis test with Dunn's post test when applicable with $p < 0.05$ considered significant.

RESULTS

LP, BoNT-A and Lipotoxin Pretreatment

CMG response. CMG parameters in the LP control, BoNT-A and lipotoxin groups during intravesical saline instillation on day 1 were not significantly different from those on day 8 (table 1 and fig. 1). Results indicated that bladder function on day 8 under normal condition was not affected by LP, BoNT-A and lipotoxin pretreatment. Furthermore, bladder contraction amplitude did not significantly change between days 1 and 8 from baseline on CMG. Baseline pressure after each contraction was stable and did not progressively increase.

There was no infection, weight loss, distress signs or evidence of constant dribbling indicative of overflow incontinence in the lipotoxin treated group. Therefore, we assumed that lipotoxin intravesical delivery did not affect bladder emptying. In future studies we will directly evaluate post-void residual urine volume. On day 8 the irritative effect of AA was evident 20 to 30 minutes after the start of infusion. In the LP and BoNT-A pretreated groups the detrusor overactivity parameter ICI was significantly decreased by 57.2% and 56.0%, respectively, after AA intravesical instillation. However, rats that received prior lipotoxin treatment showed a significantly decreased response to AA instillation (21.1% decreased ICI). These results indicate that lipotoxin pretreatment suppressed AA induced bladder overactivity, which was not seen at the identical pretreatment dose of BoNT-A.

Table 1. *Effect of saline and 0.3% AA on CMG parameters on days 1 and 8 in 6 treated rats per group*

	Mean ± SE Intravesical Instillation		
	Saline Day 1	Saline Day 8	AA Day 8
ICI (mins):			
LPs	11.9 ± 1.1*	15.2 ± 1.7†	6.5 ± 1.1
BoNT-A	13.5 ± 2.3*	13.4 ± 1.3‡	5.9 ± 1.4
Lipotoxin†	14.3 ± 1.1	16.1 ± 1.5	12.7 ± 1.8
Contraction amplitude (cm H_2O):§			
LPs	33.0 ± 3.2	30.1 ± 3.1	36.0 ± 2.7
BoNT-A	29.5 ± 1.8	29.5 ± 3.3	33.5 ± 4.1
Lipotoxin	29.3 ± 1.0	30.5 ± 4.1	33.6 ± 3.3
Baseline pressure (cm H_2O):§			
LPs	3.3 ± 0.7	4.6 ± 1.0	4.5 ± 0.4
BoNT-A	3.7 ± 0.4	4.1 ± 0.4	4.5 ± 0.6
Lipotoxin	2.9 ± 0.1	3.7 ± 0.5	3.5 ± 0.3
Pressure threshold (cm H_2O):§			
LPs	7.0 ± 0.3	6.6 ± 0.5	6.2 ± 0.7
BoNT-A	6.5 ± 0.6	6.9 ± 1.2	5.3 ± 0.8
Lipotoxin	5.7 ± 0.9	5.9 ± 0.9	4.8 ± 0.5

* Vs saline day 8 and AA Bonferroni multiple comparison test $p > 0.05$ and < 0.05, respectively.

† Vs AA Bonferroni multiple comparison test $p < 0.01$.

‡ Vs AA Bonferroni multiple comparison test $p < 0.05$.

§ Saline days 1 vs 8 and saline day 8 vs AA Bonferroni multiple comparison test $p > 0.05$.

Histological response. AA instillation induced a moderate inflammatory reaction in the LP and BoNT-A pretreated groups on histopathological evaluation of tissue sections stained with hematoxylin and eosin (table 2 and fig. 2). However, the AA induced inflammatory reaction was significantly decreased in the lipotoxin vs the LP pretreated group (48.9% decrease, $p < 0.05$), although it was not statistically significantly different in the lipotoxin vs BoNT-A pretreated group (33% decrease, $p = 0.0681$). These results indicate that lipotoxin pretreatment inhibited AA induced bladder inflammation. Previous studies showed that untreated control bladder has intact urothelial CGRP and abundant SNAP-25.[10,14]

CGRP immunostaining. CGRP immunostaining was confirmed at the bladder mucosal layer in the lipotoxin pretreated group but not in the LP or BoNT-A pretreated group (fig. 3). These results suggest that

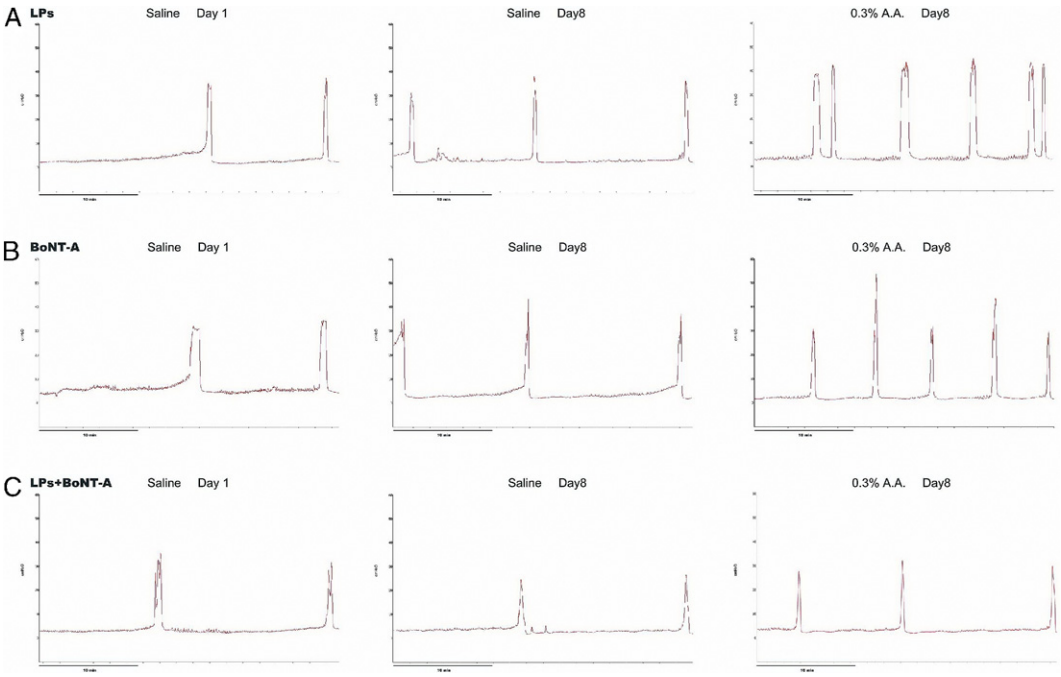

Figure 1. Representative in vivo continuous CMG traces in urethane anesthetized, LP (*A*), BoNT-A (*B*) and lipotoxin (*LPs + BoNT-A*) (*C*) pretreated rats show that parameters during intravesical saline instillation were not significantly different on days 1 and 8. After AA infusion LP and BoNT-A pretreated rats had significantly decreased ICI but ICI was significantly decreased at identical BoNT-A dose in lipotoxin pretreated rat.

lipotoxin pretreatment inhibited AA induced CGRP release. AA depleted CGRP in the other 2 groups and the presence of CGRP, as shown by green staining, supported the inhibition of AA induced CGRP release by lipotoxin instilled 8 days previously. Absent green staining in the urothelium of the LP and BoNT groups showed that BoNT alone or LPs did not prevent the CGRP depletion induced by AA.

SNAP-25 expression. SNAP-25 positive neuronal fibers were detected in LP and BoNT-A pretreated animal bladder samples (fig. 4). However, SNAP-25 positive neuronal fibers were rare in lipotoxin pretreated animals. Western blotting showed that the mean SNAP-25 protein level was decreased 66.4% and 58.1% compared to that in the LP and BoNT-A pretreated groups, respectively (table 2 and fig. 4, *D*). These results indicate that lipotoxin pretreatment decreased SNAP-25 expression.

Table 2. *Day 8 LP, BoNT-A and lipotoxin bladder inflammation, and SNAP-25 expression scores in 6 rats per group*

	Mean ± SE Inflammation		Mean ± SE SNAP-25
	Edema	Inflammatory Cell	
LPs*	2.5 ± 0.2	2.2 ± 0.3	1.0 ± 0.00
BoNT-A	1.7 ± 0.2	1.5 ± 0.2	0.79 ± 0.08
Lipotoxin	1.2 ± 0.2	1.2 ± 0.2	0.33 ± 0.11
p Value (Bonferroni multiple comparison test):			
Vs LPs	<0.01	<0.05	<0.01
Vs BoNT-A	>0.05	>0.05	<0.05

* Vs BoNT-A Dunn multiple comparison test p >0.05.

DISCUSSION

Major findings in the current study are that intravesical lipotoxin pretreatment suppressed AA induced bladder hyperactivity and inflammatory reaction, which were not observed in LP pretreated groups and were less significant in BoNT-A pretreated groups in this animal model. Furthermore, SNAP-25 expression was significantly decreased and CGRP was significantly increased in the lipotoxin pretreated group compared to those in the LP and BoNT-A pretreated groups in this model. Importantly bladder contraction amplitude did not show a significant change between days 1 and 8 baseline CMGs, and urinary retention or overflow incontinence was not seen in the lipotoxin treated group. Therefore, intravesical lipotoxin instillation may be a simpler, effective method to deliver BoNT-A without injection.

Intravesical administration of drug solutions provides high local drug concentrations in the bladder and a low risk of systemic side effects.[7,15] Intravesical pharmacotherapy has been used to treat refractory overactive bladder and IC/PBS.[4,15,16] An impor-

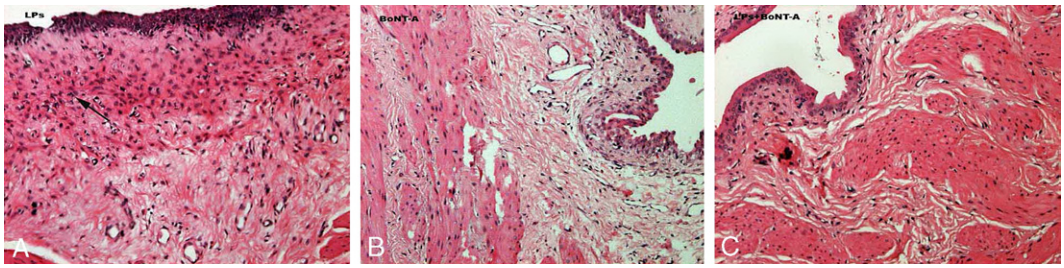

Figure 2. Bladder section photomicrographs reveal AA induced inflammatory cell accumulation (arrow) and edematous change in bladder urothelium in LP (*A*) and BoNT-A (*B*) preparations. AA effects were decreased by lipotoxin (*C*). Reduced from ×100.

tant obstacle in the success of intravesical drug delivery arises from bladder epithelial low permeability. The watertight barrier is generally located at the umbrella cells, which are the superficial layer of bladder epithelium augmented by glycosaminoglycans and uroplakins.[7,15]

Bladder epithelium and sensory axons in the bladder have an important role in afferent transduction mechanisms that modulate micturition, particularly in conditions of increased sensory nerve transmission after bladder inflammation, bladder hypersensitivity and detrusor overactivity.[17] Sensory mediators, including CGRP, substance P, adenosine triphosphate and nerve growth factor, are released in response to noxious stimuli and BoNT-A decreases the release of these neurotransmitters.[2,3,18,19] BoNT has efficacy on detrusor overactivity as well as on IC/PBS.[1,4,18] The large molecular weight of BoNT-A of 150 kDa makes it difficult to diffuse passively across the intact bladder epithelium. Previous experiments in rats showed that exposing the bladder to protamine sulfate could slough off the bladder epithelium and increase epithelial permeability.[2,20] Thereafter BoNT-A instillation could inhibit CGRP release from afferent nerve terminals and produce an analgesic effect without compromising the voiding phase if it passed the urothelial barrier.[2] The current study demonstrates similar results without a need for protamine sulfate in the LP encapsulating BoNT-A pretreated group but not in the LP or BoNT-A pretreated group. Thus, LPs improved BoNT-A bladder uptake without breaking down the bladder permeability barrier. Fraser et al previously reported a physiological effect of intravesical LP alone in a hyperactive bladder model involving protamine and KCl.[9] In an impaired permeability model they noted that LP acted as a barrier to block KCl from reaching the sensory afferent nerves. AA was used by Chuang et al to study the effect of BoNT-A but in that study protamine was also used to allow BoNT-A to pass the urothelial barrier.[2] In the current study to our knowledge we report for the first time that the large BoNT-A was able to have a beneficial physiological effect when delivered using liposomes. This method using LPs encapsulating BoNT-A overcomes the need for physical or chemical trauma to break the urothelial barrier before delivering BoNT-A into the bladder.

Furthermore, we report that lipotoxin decreased SNAP-25 expression and the AA induced inflammatory reaction in the bladder. Although the true mechanism of lipotoxin on bladder epithelium is not clear, the ability of LPs to transport drugs and macromolecules across epithelial membranes was used to deliver BoNT-A.[21] In contrast to lipotoxin, BoNT-A instillation in saline solution cannot pass the urothelial barrier and it has no significant difference on neurotransmission and bladder activity compared with LP instillation.

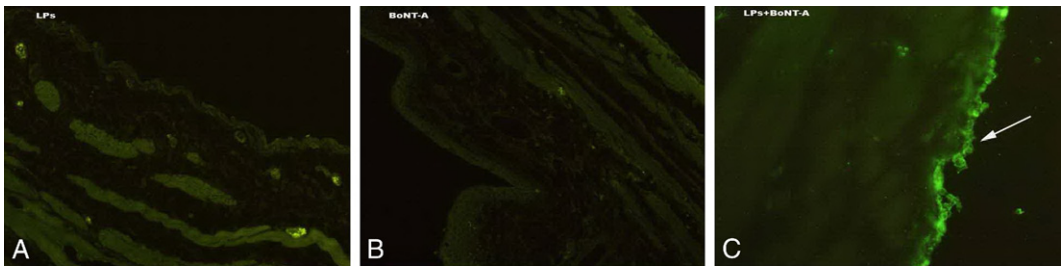

Figure 3. In representative experimental rat bladder section CGRP staining in bladder mucosal layer was not noted in LP (*A*) or BoNT-A (*B*) pretreated group but was seen in lipotoxin pretreated group (*C*). Reduced from ×100.

Figure 4. Representative experimental rat bladder sections show neuronal fibers (arrows) stained positive for SNAP-25 in LP (*A*) and BoNT-A (*B*) pretreated animal bladder samples but SNAP-25 positive neuronal fibers were rare in lipotoxin pretreated animals (*C*). Reduced from ×100. Western blot for SNAP-25 in bladder reveals that SNAP-25 protein was significantly decreased in lipotoxin pretreated group vs LP and BoNT-A pretreated groups (*D*).

Our results show that BoNT-A can be combined with LPs to be administered as a liquid instillation without cystoscopic injection. The LP lipid bilayer is similar to the cell membrane structure and phospholipids composing LPs may provide an affinity for lipotoxin. The affinity of LPs for urothelium allow them to deliver BoNT-A without significant irritation. In addition, studies show that synaptobrevin, SNAP-23 and syntaxin are colocalized with uroplakin III at the apical plasma membrane of umbrella cells in urothelium.[22] Expression of these proteins on umbrella cells may assist in BoNT-A adherence and bladder uptake from lipotoxin. LPs encapsulating BoNT-A may be endocytosed into bladder and BoNT-A may leach out from the LPs adsorbed onto urothelium. BoNT-A taken up by the bladder can then cleave SNAP-25 and halt CGRP release from sensory afferents. CGRP release inhibition by BoNT-A may be important to suppress AA induced bladder hyperactivity and inflammation.

To our knowledge this is the first report of the promise of liquid instillation of BoNT-A. Although we provide strong physiological evidence for efficacy, the mechanism of action remains to be determined, that is whether lipotoxin activity is restricted to the urothelium and blocks urothelial release of acetylcholine and other sensory neurotransmitters or lipotoxin is further trafficked to the suburothelial space and has activity at this area. Liposomes enhance the barrier properties of wounded urothelium and in this study it aided BoNT-A uptake into the urothelium. The mechanism of action of lipotoxin uptake and trafficking in the urothelium remains to be explored. In a future study it would be interesting to determine whether fluorescently labeled lipotoxin could penetrate the bladder epithelium and exist in the subepithelial area. In addition, CGRP is only one of the neurotransmitters involved in neurogenic inflammation. Further study with the measurement of other neurotransmitters could be more informative.

CONCLUSIONS

Intravesical lipotoxin administration cleaved SNAP-25, inhibited CGRP release from afferent nerve terminals and blocked AA induced bladder hyperactivity and inflammation. These results support the concept of LPs as an efficient vehicle for delivering BoNT-A. Intravesical lipotoxin instillation might be a simpler effective treatment for overactive or hypersensitive bladder without injection.

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