



Original Article

Botulinum toxin blocks mast cells and prevents rosacea like inflammation

Jae Eun Choi^{a,1}, Tyler Werbel^{b,1}, Zhenping Wang^a, Chia Chi Wu^a, Tony L. Yaksh^c, Anna Di Nardo^{a,*}^a Department of Dermatology, University of California, San Diego, CA, USA^b School of Medicine, University of California, San Diego, CA, USA^c Department of Anesthesiology and Pharmacology, University of California, San Diego, CA, USA

ARTICLE INFO

Article history:

Received 1 November 2018

Received in revised form 11 December 2018

Accepted 25 December 2018

Keywords:

Botox

Botulinum toxin

Mast cell

Mechanism of action

Rosacea

ABSTRACT

Background: Rosacea is a chronic inflammatory skin condition whose etiology has been linked to mast cells and the antimicrobial peptide cathelicidin LL-37. Individuals with refractory disease have demonstrated clinical benefit with periodic injections of onabotulinum toxin, but the mechanism of action is unknown. **Objectives:** To investigate the molecular mechanism by which botulinum toxin improves rosacea lesions. **Methods:** Primary human and murine mast cells were pretreated with onabotulinum toxin A or B or control. Mast cell degranulation was evaluated by β -hexosaminidase activity. Expression of botulinum toxin receptor Sv2 was measured by qPCR. The presence of SNAP-25 and VAMP2 was established by immunofluorescence. *In vivo* rosacea model was established by intradermally injecting LL-37 with or without onabotulinum toxin A pretreatment. Mast cell degranulation was assessed *in vivo* by histologic counts. Rosacea biomarkers were analyzed by qPCR of mouse skin sections.

Results: Onabotulinum toxin A and B inhibited compound 48/80-induced degranulation of both human and murine mast cells. Expression of Sv2 was established in mouse mast cells. Onabotulinum toxin A and B increased cleaved SNAP-25 and decreased VAMP2 staining in mast cells respectively. In mice, injection of onabotulinum toxin A significantly reduced LL-37-induced skin erythema, mast cell degranulation, and mRNA expression of rosacea biomarkers.

Conclusions: These findings suggest that onabotulinum toxin reduces rosacea-associated skin inflammation by directly inhibiting mast cell degranulation. Periodic applications of onabotulinum toxin may be an effective therapy for refractory rosacea and deserves further study.

© 2019 Japanese Society for Investigative Dermatology. Published by Elsevier B.V. All rights reserved.

1. Introduction

Rosacea is a chronic inflammatory skin disorder which is characterized by facial flushing, telangiectasia and inflammatory papules and pustules on the central area of the face [1]. Although the pathogenesis of rosacea is complicated and not yet fully understood, abnormal neurovascular signaling, dysregulation of innate immune system, and imbalance of commensal skin microbiota are considered

to be involved [2]. Various factors trigger flushing in rosacea such as alcohol, heat, exercise, or spicy foods which supports the role of neurogenic inflammation in the development of the disease [2,3]. It has been hypothesized that the activation of transient receptor potential vanilloid (TRPV) and transient receptor potential ankyrin (TRPA) on peripheral sensory neurons, by rosacea triggers, stimulates the release of vasoactive neuropeptides that cause rosacea flares [2,4].

In addition to vascular hyper-reactivity, the dysregulation of the innate immune system through abnormal levels of cathelicidins also appears to have a central role in the pathogenesis of rosacea [5]. Cathelicidins are antimicrobial peptides in human skin that protect against infection from a variety of microbes [6]. In addition to their direct antimicrobial activity, they also promote and regulate the host immune response by several mechanisms, including angiogenesis [7], expression of extracellular matrix proteins [8], and leukocyte chemotaxis [9].

Human cathelicidin is initially secreted as a biologically inactive pro-protein called cationic antimicrobial protein 18 (CAP18) [10].

Abbreviations: ACh, acetylcholine; BoNT, botulinum toxin; CAP, cationic antimicrobial protein; CMA, chymase; KLK, kallikrein-related peptidase; MMP, matrix metalloproteinases; SNAP, Synaptosomal-associated protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TRPA, transient receptor potential ankyrin TRPV transient receptor potential vanilloid; VAMP, vesicle-associated membrane protein.

* Corresponding author.

E-mail address: adinardo@ucsd.edu (A. Di Nardo).¹ Authors equally contributed to the paper.

Serine proteases, such as kallikrein-related peptidase 5 (KLK5), cleave CAP18 to form active peptides in the epidermis [11]. Abnormally increased KLK5 activity causes increased expression of cathelicidin LL-37, a molecule which has been shown to induce rosacea-like skin inflammation in a mouse model [5]. Interestingly, small doses of doxycycline, a proven therapy for rosacea, inhibit matrix metalloproteinases (MMPs), which subsequently blocks the activity of KLKs, preventing the formation of active cathelicidin peptides in the epidermis [12]. Thus, cathelicidin LL-37 appears to have a central role in the pathogenesis of rosacea.

Recently, mast cells (MCs) have emerged as key enablers of cathelicidin LL-37-induced skin inflammation [13]. Specifically, LL-37 induces MC chemotaxis, degranulation, and release of proinflammatory cytokines [14,15]. Muto et al demonstrated that MC-deficient mice did not develop rosacea-like features after LL-37 injection. Furthermore, stabilization of MCs with cromolyn sodium reduced skin inflammation and rosacea biomarkers in both humans and mice [13]. These results not only emphasized the importance of MCs in cathelicidin-induced inflammation but also provided potential therapeutic targets for rosacea.

A variety of systemic and topical treatment options are used for rosacea, such as oral tetracyclines and topical ivermectin, metronidazole, azelaic acid and brimonidine [16,17]. However, it can be challenging to treat severe flushing and persistent erythema in refractory cases of rosacea. Rosacea has been associated with serious social stigma and therefore is considered a significant psychological problem [18].

There are a couple of case reports that successfully treated refractory erythema and flushing with β -adrenergic blockers, naloxone (opiate antagonist), ondansetron (serotonin antagonist), or endoscopic thoracic sympathectomy [19–23]. Recently, intradermal botulinum toxin (BoNT) has also been investigated as a novel treatment of refractory facial erythema and flushing [24–26]. BoNT A is a potent neurotoxin produced by the bacterium *Clostridium botulinum*, which inhibits the release of acetylcholine (ACh) from the presynaptic vesicle [27]. BoNT not only inhibits the release of ACh but also modulates several other neuropeptides, including substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) [28,29]. Since ACh and VIP are key mediators of vasodilation and flushing [30,31], inhibition of their release is a reasonable mechanism of action for BoNT in rosacea. However, the mechanism by which BoNT acts on the immune system in rosacea remains unclear.

Our group has recently demonstrated that human and mouse MCs express SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins, including synaptosomal-associated protein-25 (SNAP-25) and vesicle-associated membrane protein (VAMP) [32]. SNAREs are the main components of a protein complex involved in the docking and fusion of vesicles with the presynaptic membrane. On sensory nerve endings, BoNT A and B block the exocytosis of vesicles containing neuropeptides by cleaving SNAP and VAMP respectively [33,34]. The presence of SNAREs on MCs suggests that BoNT's mechanism of action in the treatment of rosacea may extend beyond the regulation of neuronal activity. We hypothesized that BoNT could improve erythema and flushing in rosacea by direct inhibition of MC degranulation, a key step in the development of the disease [13]. In the present study, we further investigate this novel mechanism.

2. Materials and methods

2.1. Mouse experiments

C57BL/6 mice were bred at our facility. Institutional Animal Care and Use Committee (IACUC) of UC San Diego approved all

animal experiments. Mice were shaved 24 h before intradermal injection of 0.5 units of botulinum toxin A (BoNT A 25 μ g in 100 μ l PBS, ALLERGAN), 12 h later, intradermally injected with 50 μ l of 320 μ M cathelicidin LL-37 (AnaSpec) or phosphate-buffered saline (PBS) (n = 3) twice a day for 2 days. Images of clinical lesions were processed with Image J software, and the area of cutaneous erythema was calculated. Skin biopsies were taken after 72 h of observation, and tissues were immersed immediately into RNA stabilization reagent (RNAlater; QIAGEN) and stored at -80°C .

2.2. Mast cells

Primary murine MCs (mMCs) were generated by extracting bone marrow cells from the femurs of 5- to 8-week-old mice and culturing cells in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% inactivated fetal bovine serum (FBS) (Thermo Fisher), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 4 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 100 IU/ml penicillin and 100 μ g/ml streptomycin. Recombinant murine interleukin-3 (IL-3) (1 ng/ml, R&D Systems) and recombinant murine stem cell factor (20 ng/ml, R&D Systems) were also included. After 4 weeks, mMCs were consistently generated as confirmed by the expression of CD117 (c-Kit) and Fc ϵ RI; cell maturation was confirmed by metachromatic staining with toluidine blue. The purity of mMCs was greater than 98% [35].

Primary human MCs (hMCs) were derived from human cord blood CD34⁺CD45⁺ cells (Astarte Biologics) according to Kirshenbaum and Metcalfe [36]. Briefly, CD34⁺CD45⁺ cells were cultured in serum-free culture media (Stemline II, Sigma) containing recombinant human stem cell factor (100 ng/ml, R&D Systems), recombinant human IL-6 (100 ng/ml, R&D Systems), and recombinant human IL-3 (20 ng/ml, R&D Systems, first week only). After 10 weeks, hMCs were consistently generated as confirmed by the expression of CD117 and Fc ϵ RI. Cell maturation was confirmed by metachromatic staining with toluidine blue. The purity of hMCs was greater than 98%.

2.3. Real-time quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN), and 0.5 μ g of total RNA was used for cDNA synthesis using the iSCRIPT cDNA Synthesis Kit (Bio-Rad). Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed in an ABI 7300 Real-Time PCR system (Applied Biosystems). We used the comparative $\Delta\Delta\text{CT}$ method to determine the quantification of gene expression, normalized the target gene expression in the test samples to the endogenous reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level and reported them as the fold difference relative to GAPDH gene expression in untreated baseline control [37]. TaqMan gene expression assays (Applied Biosystems) were used to analyze the expressions of mouse *Mmp9*, mouse *Trpv2*, mouse *Klk5*, and mouse chymase (*Cma1*). SYBR Green gene expression assays (Bio-Rad) were used to analyze the expressions of mouse synaptic vesicle glycoprotein 2a (*Sv2a*), mouse *Sv2b*, and mouse *Sv2c*. The following primer sequences were used: mouse *Sv2a* forward, 5'-TCGTCCTTCGTCACGGGTTA-3', and reverse, 5'-ATGAATCGTTTTAATGTGGGTCAC-3'; mouse *Sv2b* forward, 5'-CACTGTCTACAGGATACCGC-3', and reverse, 5'-CCCACTATGACAATCTGCTAG-3'; mouse *Sv2c* forward, 5'-TGA-GATGCTTCAACTACCCAGTCAGG-3', and reverse, 5'-CCTTTTGACATTCCTAGTTAGCAG-3' [38]; mouse *Gapdh* forward, 5'-CTTAG-CACCCCTGGCCAAG-3', and reverse, 5'-TGGTCATGAGTCCTTC-CACG-3'. We performed all the assays in triplicate and repeated the experiments at least three times.

2.4. Mast cell degranulation assay

5×10^4 hMCs or 1×10^5 mMCs were pretreated with BoNT A or B (0.5–5 pM) for 24 h. After being washed 2 times with PBS, MC degranulation was assessed by measuring the activity of β -hexosaminidase in the supernatants [39–41] of 1×10^5 mMCs in 200 μ l Tyrode's buffer (0.1% BSA, 0.1% glucose, 2 mmol/l $MgCl_2$, 137.5 mmol/l NaCl, 12 mmol/l $NaHCO_3$, 2.6 mmol/l KCl, pH 7.4) or 5×10^4 hMCs in 100 μ l saline phosphate buffer (0.9% NaCl, 10 mM NaH_2PO_4 , 45 mM glucose) incubated for 30 min at 37 °C with Compound 48/80 (10 μ g/ml, Sigma) which was used to promote MC degranulation in an IgE-independent manner [42]. For each sample assayed, MC supernatant aliquots (20 μ l) were mixed with substrate solution (100 μ l), which consisted of 1 mM 4-methylumbelliferyl-2-acetamide-2-deoxy- β -D-glucopyranoside (Calbiochem) in 0.1 M sodium citrate buffer (pH 4.5), and were incubated for 2 h at 37 °C in the dark. The reaction was then stopped by the addition of 12 μ l of 0.2 M glycine (pH 10.7). The reaction mixtures were excited at 365 nm and measured at 460 nm in a fluorescence plate reader (Gemini EM microplate spectrofluorometer, Molecular Devices). To determine the total cellular content of this enzyme, an equivalent number of cells were lysed with 1% triton-X-100 (Sigma). Release of β -hexosaminidase was calculated as the percentage of the total enzyme content.

2.5. Histologic analysis of mast cell degranulation

To assess the degree of dermal mast cell degranulation *in vivo*, a quantitative analysis was performed. Animal skin samples were collected, fixed with buffered formalin, and embedded in paraffin. After deparaffinizing and rehydrating the sections, sections were stained with toluidine blue (RICCA Chemical) to identify MCs [43]. After staining, randomly selected sections were examined under light microscopy. For each section, the total, intact, and degranulating mast cells in the dermis were counted per 20 fields of view using a magnification of $\times 200$. A mast cell was considered to be degranulating if four or more extruded granules were visible adjacent to the cell or if the cell outline appeared disrupted [44]. In contrast, intact mast cells demonstrate a deep blue (orthochromatic) staining. All photos were captured at original magnifications of $\times 400$.

2.6. Fluorescence images

Mast cells were treated with 0.1 pM BoNT A or B for 24 h, then attached to a glass slide by using Shandon Cytospin 2 cytocentrifuge (Thermo Fisher). The cells were stained with 1 μ g/ml anti-BoNT A-cleaved-SNAP-25 antibody and anti-VAMP2 antibody

(Synaptic Systems) according to the manufacturer's instructions. Slides were mounted in ProLong anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes). We imaged the cells using the Bx51 research microscope (Olympus) and X-Cite 120 fluorescence illumination systems (EXFO Photonic Solutions). All isotype controls showed negative staining (data not shown). The fluorescence intensity of each cell was measured using ImageJ software (NIH) and the corrected total cell fluorescence (CTCF) was calculated.

2.7. Statistical analyses

All data are presented as the mean \pm SD. At least three independent experiments were performed to assess the reproducibility of the different experiments. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software). For 48/80-induced mast cell degranulation, histologic mast cell degranulation, and gene expression experiments, results were compared using one-way ANOVA with post hoc Tukey's tests. For comparisons of CTCF and area of erythema on skin, results were analyzed with Student's *t*-test. For all statistical tests, *P*-values < 0.05 were considered statistically significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

3. Results

3.1. Mast cell degranulation is blocked by onabotulinum toxin *in vitro*

To confirm the interaction between BoNT and MCs, primary hMCs and mMCs were treated *in vitro* with compound 48/80, and β -hexosaminidase release was measured as a marker for MC degranulation. In comparison to controls, pretreatment of both hMCs and mMCs with BoNT A or B for 24 h significantly decreased MC degranulation. Interestingly, BoNT A had a dose-dependent effect on MC function (Fig. 1).

3.2. Mast cells express SNAP-25 and VAMP2

To verify the presence of SNARE proteins on MCs, immunostaining for SNAP-25 was performed on mMCs with or without BoNT A pretreatment. The SNAP-25 antibody used only recognizes the cleaved product of SNAP-25 (cSNAP-25) and not the full-length molecule. In comparison to control, pretreatment of the mMCs with BoNT showed a quantitative increase in cSNAP-25 staining (Fig. 2a–c). The VAMP2 antibody used in the present study detects the intact molecule. Therefore, the reduction of VAMP2 protein expression was used as a measure of VAMP2 cleavage by BoNT B. Following pretreatment with BoNT B, mMCs demonstrated a decrease in VAMP2 staining. Therefore, VAMP2 cleavage was

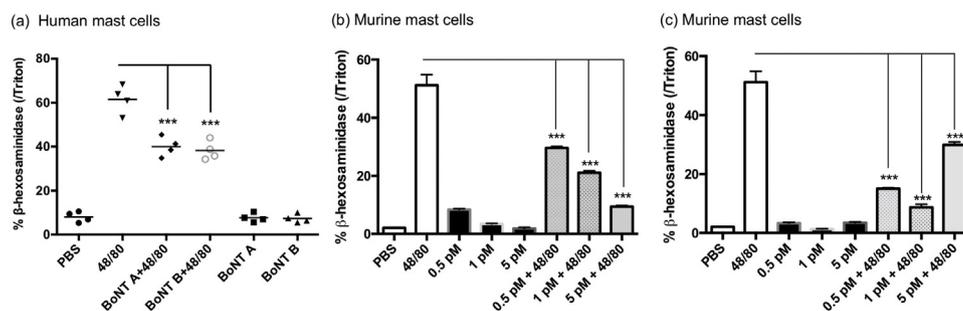


Fig. 1. Mast cell degranulation is blocked by onabotulinum toxin *in vitro*. (a) Human mast cell (MC) degranulation was assessed by measuring release of β -hexosaminidase. The cells were pretreated with 5 pM botulinum toxin (BoNT) A or BoNT B for 24 h, then treated with compound 48/80 (10 μ g/ml) for 30 min; (b–c) Murine MC degranulation was assessed by measuring release of β -hexosaminidase. The cells were pretreated with 0.5–5 pM BoNT A (b) or BoNT B (c) for 24 h, then treated with compound 48/80 (10 μ g/ml) for 30 min. ****P* < 0.001 (*n* = 3).

significantly greater in the BoNT B treated cells in comparison to the control group (Fig. 2d–f).

3.3. Mast cells express BoNT receptor SV2

It has been shown that BoNT enters neurons by binding to synaptic vesicle proteins, such as synaptic vesicle glycoprotein 2 (SV2), and subsequently taking advantage of the physiological recycling of synaptic vesicles [45]. To investigate the presence of BoNT receptors on primary mMCs, we measured the mRNA expression of the three isoforms of Sv2 by RT-qPCR. Although all isoforms were present, Sv2b demonstrated significantly higher expression (Fig. 2g). We subsequently examined how BoNT treatment affects the expression of Sv2 isoforms and found no appreciable differences (data not shown).

3.4. Onabotulinum toxin reduces LL-37-induced skin inflammation

To investigate the clinical effects of BoNT on rosacea inflammation, we used a well-established mouse model of rosacea-like inflammation [5]. We injected cathelicidin LL-37 intradermally into C57BL/6 mice twice a day for two consecutive days and compared the resulting inflammation with mice that were pretreated with BoNT A. Our clinical endpoint observation at 72 h demonstrated visibly and quantitatively reduced inflammation in the skin of the pretreatment group (Fig. 3).

3.5. Dermal mast cell degranulation is blocked by onabotulinum toxin in vivo

Skin biopsies from each mouse were subsequently taken to assess the degree of mast cell degranulation *in vivo*. In comparison to mice only treated with cathelicidin LL-37, the BoNT A pretreatment group showed a significantly decreased percentage of dermal mast cells that were degranulating (Fig. 4). Furthermore,

the degree of mast cell degranulation in the pretreatment group approached that of the control group.

3.6. Onabotulinum toxin reduces expression of rosacea biomarkers

Chymase, KLK5, matrix metalloproteinase 9, and transient receptor potential cation channel proteins are involved in the mast cell immune response and have been previously shown to be reliable biomarkers for rosacea [4,5,12,13]. To quantitatively assess the clinical response to BoNT A, we measured the expression of *Cma1*, *Klk5*, *Mmp9*, and *Trpv2* in skin tissue taken from each mouse. In comparison to mice only treated with cathelicidin LL-37, the BoNT A pretreatment group showed a significantly decreased expression of these rosacea biomarkers (Fig. 5).

4. Discussion

The treatment of rosacea typically involves a combination of behavioral modification and therapeutic intervention. However, satisfactory improvement of symptoms is uncommon, necessitating the investigation of new therapies. BoNT is a neuromuscular blocking agent that was initially approved by the Food and Drug Administration (FDA) in 1989 for blepharospasm, hemifacial spasm, and strabismus [46]. Since then, there have been many additional indications, including axillary hyperhidrosis, cervical dystonia, chronic migraine, limb spasticity, and overactive bladder, in addition to several cosmetic uses [46,47]. Recently, several individuals with papulopustular rosacea have demonstrated clinical improvement with intradermal injections of BoNT A, but the mechanism of action has not been investigated [24–26].

To our best knowledge, this is the first study to investigate the direct action of BoNT on MCs in rosacea. Interestingly, one prior study described BoNT activity on MC infiltration in wound healing. The number of infiltrated MCs was markedly fewer in the skin pretreated with BoNT A compared to the control group [48].

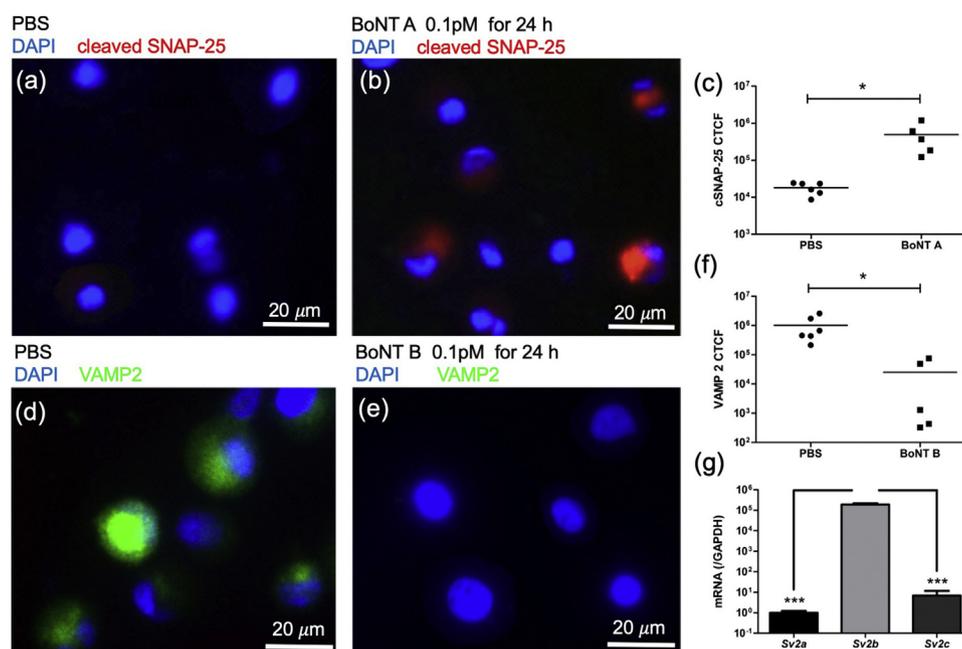


Fig. 2. Mast cells express SNAP-25, VAMP2, and SV2. Immunostaining for cleaved synaptosomal-associated protein-25 (SNAP-25) (a–c) or vesicle-associated membrane protein 2 (VAMP2) (d–f) was performed on mouse MCs. (a) Cells treated with PBS; (b) Cells treated with 0.1 pM BoNT A for 24 h; (d) Cells treated with PBS; (e) Cells treated with 0.1 pM BoNT B for 24 h; (c, f) Corrected total cell fluorescence (CTCF) intensity for cells treated with PBS or BoNT A or B. * $P < 0.05$ ($n = 5–6$). Expression of the three isoforms of synaptic vesicle glycoprotein 2 (Sv2) in mouse MCs were measured by RT-qPCR; (g) Although all isoforms were detected, Sv2b was expressed at significantly higher levels. *** $P < 0.001$ ($n = 3$).

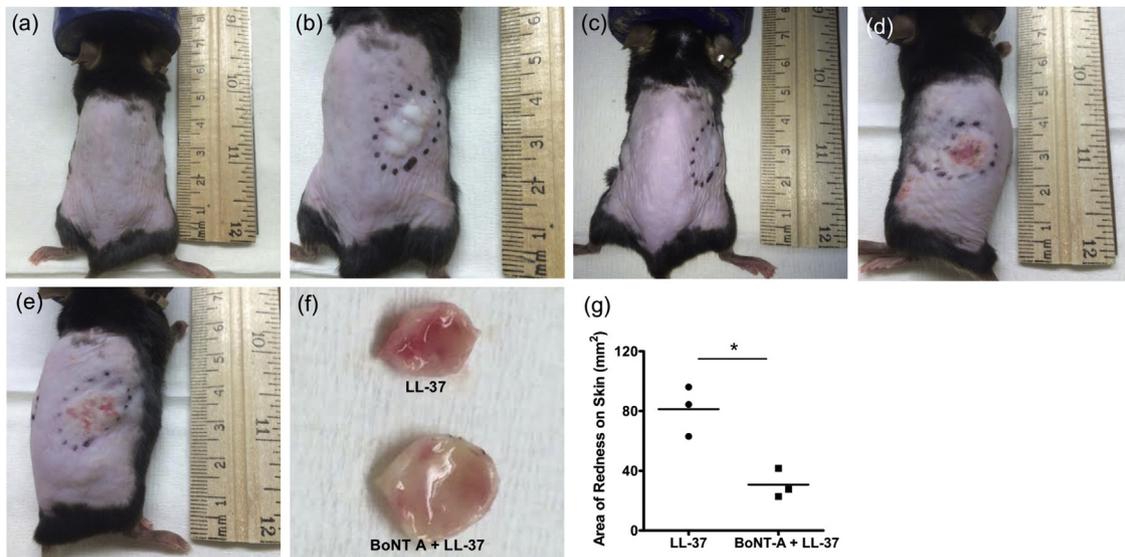


Fig. 3. Onabotulinum toxin reduces LL-37-induced skin inflammation in mice. Cathelicidin LL-37 50 μ l of 320 μ M was injected intradermally into C57BL/6 mice twice a day for two consecutive days with or without 0.5 unit of BoNT A pretreatment. (a) Control (injected with PBS); (b) Immediately after LL-37 intradermal injection; (c) 12 h after BoNT A pre-treatment and just before LL-37 injection; (d–e) Three days after LL-37 injection without (d) or with (e) BoNT A pretreatment; (f) Collected skin tissues from LL-37 treated mice without and with BoNT A pretreatment; (g) Image J software quantification of area of erythematous skin on LL-37 treated mice without and with BoNT A pretreatment. * $P < 0.05$ ($n = 3$).

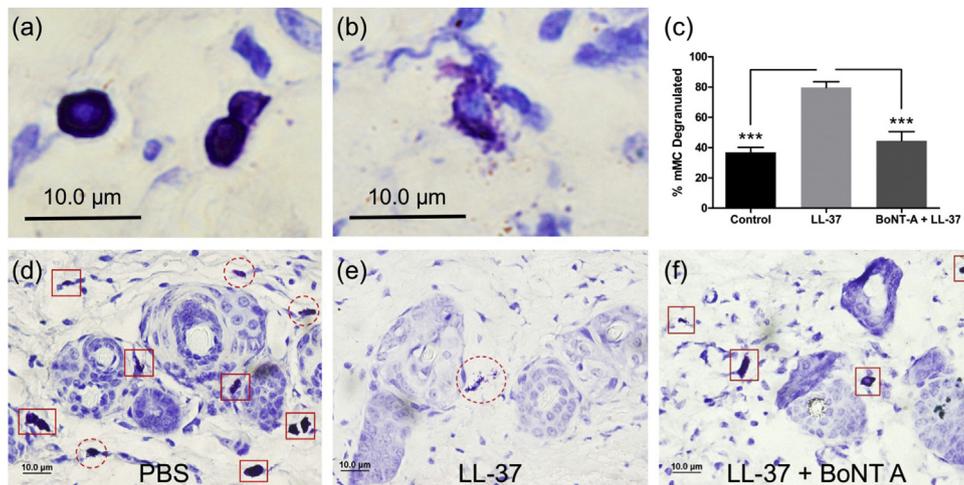


Fig. 4. Mast cell degranulation is blocked by onabotulinum toxin *in vivo*. Intact (a) and degranulating (b) dermal MCs were counted in murine skin to assess the effect of BoNT A *in vivo*. Representative toluidine blue staining of skin tissue taken from mice treated with PBS (d) or cathelicidin LL-37 without (e) or with (f) BoNT A pretreatment; Intact and degranulating mast cells are indicated by red square and red dashed red circle outlines respectively; (c) Quantification of the percentage of dermal mast cells that are degranulating. *** $P < 0.001$ ($n = 20$ fields of view).

Similarly, in a NC/Nga mouse model of atopic dermatitis, the skin pretreated with intradermal BoNT injection had less MC infiltration compared to control [49]. We confirmed this observation in the LL-37-induced rosacea mouse model (data not shown).

Here, we demonstrated the direct effect of BoNT on MC degranulation in the absence of its neuromodulating activity. Both BoNT A and B decreased MC degranulation in both mouse and human MCs *in vitro*. To investigate the interaction between BoNT and MCs, we established the presence of BoNT's receptor SV2 on MCs. To confirm the direct action of BoNT on MCs, VAMP2 and SNAP-25, which are cleaved by BoNT A and B respectively, were measured. Immunostaining showed decreased cleavage of SNAP-25 and VAMP2 in MCs pretreated with BoNT which supports our hypothesis that BoNT directly inhibits MC degranulation. Since MC degranulation has a critical role in promoting LL-37 rosacea

inflammation, its inhibition by BoNT should contribute to rosacea improvement.

Thus, to validate the effect of BoNT on rosacea *in vivo*, we used an LL-37-induced rosacea mouse model. Since BoNT B is only FDA-approved for cervical dystonia by intramuscular injection and since BoNT A is more widely administered by intradermal injection, only BoNT A was utilized in our *in vivo* experiments. Mice pretreated with intradermal BoNT A showed significantly less erythema compared to the control. Additionally, the pretreatment group showed an almost two-fold reduction in dermal mast cell degranulation in comparison to LL-37-injected mice.

Cathelicidin, cutaneous serine proteases, and MMPs are involved in the mast cell immune response and are known as reliable biomarkers in rosacea [5,12]. Chymase is a serine protease that is abundant in MCs and is used as marker of MC activity; KLK5

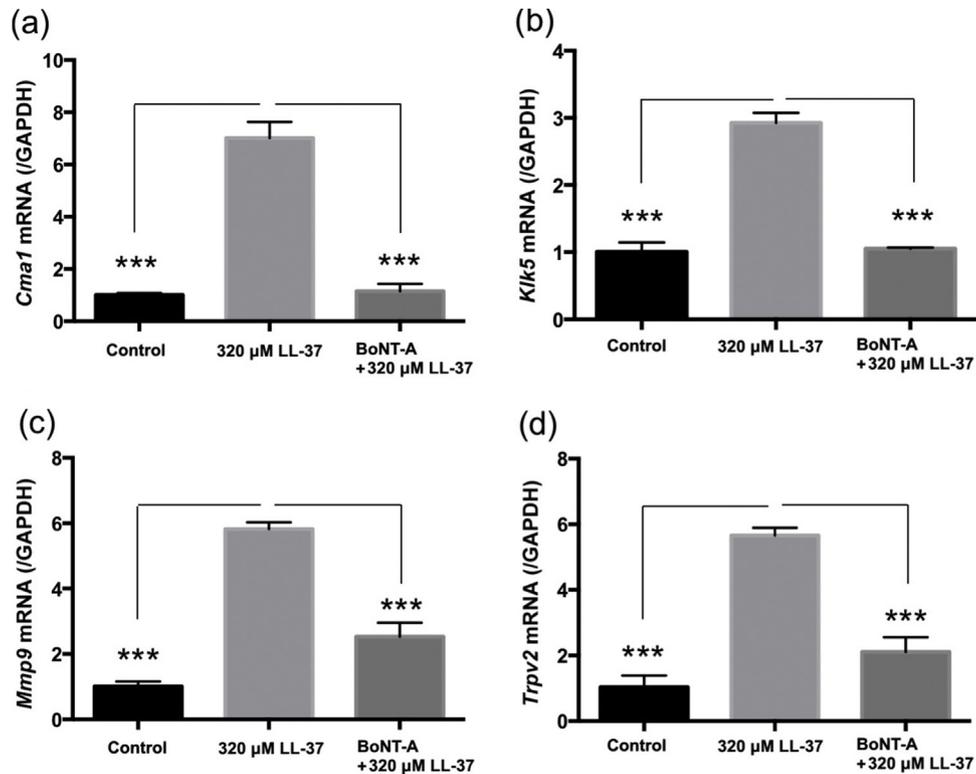


Fig. 5. Onabotulinum toxin inhibits the expression of rosacea biomarkers in LL-37-induced rosacea mouse model. LL-37 50 μ l of 320 μ M was injected intradermally into C57BL/6 mice twice a day for two consecutive days with or without 0.5 unit of BoNT A pre-treatment. Control mice were injected with PBS. *Cma1* (chymase) (a), *Kik5* (kallikrein related-peptidase 5) (b), *Mmp9* (matrix metalloproteinase 9) (c), and *Trpv2* (transient receptor potential cation channel, subfamily V, member 2) (d) mRNA expressions in the skin were measured by RT-qPCR. *** $P < 0.001$ (n = 3).

is the predominant serine protease in epidermis that is responsible for cathelicidin cleavage, leading to increased LL-37 expression in rosacea [5]; MMP9 is needed to activate KLK5 by cleaving its proenzyme form [2]. TRPV is closely related to sensory pathologies, and it has been hypothesized that the activation of TRPV and TRPA on peripheral sensory neurons by rosacea triggers stimulates the release of vasoactive neuropeptides that cause rosacea flares [4]. Additionally, TRPV2 and TRPV4 are co-localized with MCs in rosacea skin [50]. In BoNT-pretreated mice, these biomarkers were significantly suppressed compared to the control upon LL-37 administration, quantitatively supporting its clinical protective effect [12].

In summary, the results of this study suggest that the mechanism of botulinum toxin in the treatment of rosacea involves the blockage of MC degranulation by cleaving SNARE proteins within the cell. Therefore, not only does BoNT target the neurogenic inflammatory component of rosacea, but it also has direct inhibitory effects on MCs. These multiple targets of BoNT may offer therapeutic advantages over currently available remedies. Since intradermal injections are invasive and require periodic office visits, a well-formulated topical BoNT may be appropriate for further study in the treatment of rosacea.

Funding

This study was funded by grants from the National Institute of Health and the National Rosacea Society.

Disclosures

The authors have no conflict of interest to declare.

References

- [1] R.L. Gallo, R.D. Granstein, S. Kang, M. Mannis, M. Steinhoff, J. Tan, et al., Standard classification and pathophysiology of rosacea: the 2017 update by the National Rosacea society Expert Committee, *J. Am. Acad. Dermatol.* 78 (January (1)) (2018) 148–155.
- [2] A.M. Two, W. Wu, R.L. Gallo, T.R. Hata, Rosacea: part I. Introduction, categorization, histology, pathogenesis, and risk factors, *J. Am. Acad. Dermatol.* 72 (May (5)) (2015) 749–758 quiz 59–60.
- [3] J.E. Choi, A. Di Nardo, Skin neurogenic inflammation, *Semin. Immunopathol.* 40 (May (3)) (2018) 249–259.
- [4] N.L. Mascarenhas, Z. Wang, Y.L. Chang, A. Di Nardo, TRPV4 mediates mast cell activation in cathelicidin-induced Rosacea inflammation, *J. Invest. Dermatol.* 137 (April (4)) (2017) 972–975.
- [5] K. Yamasaki, A. Di Nardo, A. Bardan, M. Murakami, T. Ohtake, A. Coda, et al., Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea, *Nat. Med.* 13 (August (8)) (2007) 975–980.
- [6] V. Nizet, T. Ohtake, X. Lauth, J. Trowbridge, J. Rudisill, R.A. Dorschner, et al., Innate antimicrobial peptide protects the skin from invasive bacterial infection, *Nature* 414 (November (6862)) (2001) 454–457.
- [7] R. Koczulla, G. von Degenfeld, C. Kupatt, F. Krotz, S. Zahler, T. Gloe, et al., An angiogenic role for the human peptide antibiotic LL-37/hCAP-18, *J. Clin. Invest.* 111 (June (11)) (2003) 1665–1672.
- [8] H.J. Park, D.H. Cho, H.J. Kim, J.Y. Lee, B.K. Cho, S.I. Bang, et al., Collagen synthesis is suppressed in dermal fibroblasts by the human antimicrobial peptide LL-37, *J. Invest. Dermatol.* 129 (April (4)) (2009) 843–850.
- [9] D. Yang, Q. Chen, A.P. Schmidt, G.M. Anderson, J.M. Wang, J. Wooters, et al., LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells, *J. Exp. Med.* 192 (October (7)) (2000) 1069–1074.
- [10] M. Zaiou, V. Nizet, R.L. Gallo, Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence, *J. Invest. Dermatol.* 120 (May (5)) (2003) 810–816.
- [11] K. Yamasaki, J. Schaubert, A. Coda, H. Lin, R.A. Dorschner, N.M. Schechter, et al., Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin, *FASEB J.* 20 (October (12)) (2006) 2068–2080.
- [12] A. Di Nardo, A.D. Holmes, Y. Muto, E.Y. Huang, N. Preston, W.J. Winkelman, et al., Improved clinical outcome and biomarkers in adults with papulopustular rosacea treated with doxycycline modified-release capsules in a randomized trial, *J. Am. Acad. Dermatol.* 74 (June (6)) (2016) 1086–1092.

- [13] Y. Muto, Z. Wang, M. Vanderberghe, A. Two, R.L. Gallo, A. Di Nardo, Mast cells are key mediators of cathelicidin-initiated skin inflammation in rosacea, *J. Invest. Dermatol.* 134 (November (11)) (2014) 2728–2736.
- [14] F. Schiemann, E. Brandt, R. Gross, B. Lindner, J. Mittelstadt, C.P. Sommerhoff, et al., The cathelicidin LL-37 activates human mast cells and is degraded by mast cell tryptase: counter-regulation by CXCL4, *J. Immunol.* (Baltimore, Md : 1950) 183 (August (4)) (2009) 2223–2231.
- [15] M. Yoshioka, N. Fukuishi, Y. Kubo, H. Yamanobe, K. Ohsaki, Y. Kawasoe, et al., Human cathelicidin CAP18/LL-37 changes mast cell function toward innate immunity, *Biol. Pharm. Bull.* 31 (February (2)) (2008) 212–216.
- [16] A.D. Holmes, M. Steinhoff, Integrative concepts of rosacea pathophysiology, clinical presentation and new therapeutics, *Exp. Dermatol.* 04 (July) (2016).
- [17] A.M. Two, W. Wu, R.L. Gallo, T.R. Hata, Rosacea: part II. Topical and systemic therapies in the treatment of rosacea, *J. Am. Acad. Dermatol.* 72 (May (5)) (2015) 761–770 quiz 71–2.
- [18] T.T. Huynh, Burden of disease: the psychosocial impact of Rosacea on a patient's quality of life, *Am. Health Drug Benefits* 6 (July (6)) (2013) 348–354.
- [19] C.C. Hsu, J.Y. Lee, Pronounced facial flushing and persistent erythema of rosacea effectively treated by carvedilol, a nonselective beta-adrenergic blocker, *J. Am. Acad. Dermatol.* 67 (September (3)) (2012) 491–493.
- [20] A.M. Schram, W.D. James, Neurogenic rosacea treated with endoscopic thoracic sympathectomy, *Arch. Dermatol.* 148 (February (2)) (2012) 270–271.
- [21] U. Wollina, The response of erythematous rosacea to ondansetron, *Br. J. Dermatol.* 140 (March (3)) (1999) 561–562.
- [22] J.K. Wilkin, Effect of subdepressor clonidine on flushing reactions in rosacea. Change in malar thermal circulation index during provoked flushing reactions, *Arch. Dermatol.* 119 (March (3)) (1983) 211–214.
- [23] J.E. Bernstein, K. Soltani, Alcohol-induced rosacea flushing blocked by naloxone, *Br. J. Dermatol.* 107 (July (1)) (1982) 59–61.
- [24] K.Y. Park, M.Y. Hyun, S.Y. Jeong, B.J. Kim, M.N. Kim, C.K. Hong, Botulinum toxin for the treatment of refractory erythema and flushing of rosacea, *Dermatology* 230 (4) (2015) 299–301.
- [25] S.H. Dayan, R.N. Pritzker, J.P. Arkins, A new treatment regimen for rosacea: onabotulinumtoxinA, *J. Drugs Dermatol.* 11 (December (12)) (2012) e76–e79.
- [26] S.H. Dayan, N. Ashourian, K.A. Cho, Pilot, Double-Blind, Placebo-Controlled Study to Assess the Efficacy and Safety of IncobotulinumtoxinA Injections in the Treatment of Rosacea, *J. Drugs Dermatol.* 16 (June (6)) (2017) 549–554.
- [27] W. Huang, J.A. Foster, A.S. Rogachefsky, Pharmacology of botulinum toxin, *J. Am. Acad. Dermatol.* 43 (August (2 Pt.1)) (2000) 249–259.
- [28] J. Meng, J. Wang, G. Lawrence, J.O. Dolly, Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and inhibition by botulinum toxins reflects their anti-nociceptive potential, *J. Cell. Sci.* 120 (August (Pt. 16)) (2007) 2864–2874.
- [29] K.R. Aoki, Review of a proposed mechanism for the antinociceptive action of botulinum toxin type A, *Neurotoxicology* 26 (October (5)) (2005) 785–793.
- [30] L.A. Holowatz, C.S. Thompson, C.T. Minson, W.L. Kenney, Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin, *J. Physiol. (Lond.)* 563 (March (Pt. 3)) (2005) 965–973.
- [31] B.W. Wilkins, L.H. Chung, N.J. Tublitz, B.J. Wong, C.T. Minson, Mechanisms of vasoactive intestinal peptide-mediated vasodilation in human skin, *J. Appl. Physiol.* (Bethesda, Md : 1985) 97 (October (4)) (2004) 1291–1298.
- [32] R. Ramachandran, M.J. Marino, S. Paul, Z. Wang, N.L. Mascarenhas, S. Pellett, et al., A study and review of effects of botulinum toxins on mast cell dependent and independent pruritus, *Toxins*. 10 (March (4)) (2018).
- [33] G. Zanetti, D. Azamia Tehran, M. Pirazzini, T. Binz, C.C. Shone, S. Fillo, et al., Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism, *Biochem. Pharmacol.* 98 (December (3)) (2015) 522–530.
- [34] G. Schiavo, M. Matteoli, C. Montecucco, Neurotoxins affecting neuroexocytosis, *Physiol. Rev.* 80 (April (2)) (2000) 717–766.
- [35] Z. Wang, Y. Lai, J.J. Bernard, D.T. Macleod, A.L. Cogen, B. Moss, et al., Skin mast cells protect mice against vaccinia virus by triggering mast cell receptor S1PR2 and releasing antimicrobial peptides, *J. Immunol.* 188 (January (1)) (2012) 345–357.
- [36] A.S. Kirshenbaum, D.D. Metcalfe, Growth of human mast cells from bone marrow and peripheral blood-derived CD34+ pluripotent progenitor cells, *Methods Mol. Biol.* 315 (2006) 105–112.
- [37] V. Luu-The, N. Paquet, E. Calvo, J. Cumps, Improved real-time RT-PCR method for high-throughput measurements using second derivative calculation and double correction, *Biotechniques*. 38 (February (2)) (2005) 287–293.
- [38] C. Peng, G. Zhu, X. Liu, H. Li, Mutant huntingtin causes a selective decrease in the expression of synaptic vesicle protein 2C, *Neurosci. Bull.* 34 (October (5)) (2018) 747–758.
- [39] B. Schick, K.F. Austen, Modulation of chymase-mediated rat serosal mast cell degranulation by trypsin or diisopropyl fluorophosphate, *Immunology* 66 (March (3)) (1989) 434–438.
- [40] K. Suzuki, I.M. Verma, Phosphorylation of SNAP-23 by IkappaB kinase 2 regulates mast cell degranulation, *Cell* 134 (August (3)) (2008) 485–495.
- [41] L.B. Schwartz, K.F. Austen, S.I. Wasserman, Immunologic release of beta-hexosaminidase and beta-glucuronidase from purified rat serosal mast cells, *J. Immunol.* (Baltimore, Md : 1950) 123 (October (4)) (1979) 1445–1450.
- [42] A.M. Rothschild, Mechanisms of histamine release by compound 48-80, *Br. J. Pharmacol.* 38 (January (1)) (1970) 253–262.
- [43] E.W. Smith, W.B. Atkinson, Simple procedure for identification and rapid counting of mast cells in tissue sections, *Science* 123 (May (3204)) (1956) 941–942.
- [44] M. Dyson, D.A. Luke, Induction of mast cell degranulation in skin by ultrasound, *IEEE Trans. Ultrason. Ferroelectr. Freq. Control* 33 (2) (1986) 194–201.
- [45] M. Dong, F. Yeh, W.H. Tepp, C. Dean, E.A. Johnson, R. Janz, et al., SV2 is the protein receptor for botulinum neurotoxin A, *Science* 312 (April (5773)) (2006) 592–596.
- [46] Botox [product insert], Allergan Inc., Irvine, CA, 2017.
- [47] Botox Cosmetic [product insert], Allergan Inc., Irvine, CA, 2017.
- [48] T.H. Park, The effects of botulinum toxin A on mast cell activity: preliminary results, *Burns* 39 (June (4)) (2013) 816–817.
- [49] S.B. Han, H. Kim, S.H. Cho, J.H. Chung, H.S. Kim, Protective effect of botulinum toxin type A against atopic dermatitis-like skin lesions in NC/Nga mice, *Dermatol. Surg.* (April) (2017) 24.
- [50] M. Sulik, S. Seeliger, J. Aubert, V.D. Schwab, F. Cevikbas, M. Rivier, et al., Distribution and expression of non-neuronal transient receptor potential (TRPV) ion channels in rosacea, *J. Invest. Dermatol.* 132 (April (4)) (2012) 1253–1262.