Blockade of glutamate release by botulinum neurotoxin type A in humans: A dermal microdialysis study

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BACKGROUND: The analgesic action of botulinum neurotoxin type A (BoNTA) has been linked to the blockade of peripheral release of neuropeptides and neurotransmitters in animal models; however, there is no direct evidence of this in humans.

OBJECTIVES: To investigate the effect of BoNTA on glutamate release in humans, using an experimental model of pain and sensitization provoked by capsaicin plus mild heat.

METHODS: Twelve healthy volunteers (six men, six women) were pretreated with BoNTA (10 U) on the volar forearm and with a saline control on the contralateral side. Dermal microdialysis was applied one week later to collect interstitial samples before and after the application of a capsaicin patch (8%) plus mild heat (40°C/60 min) to provoke glutamate release, pain and vasodilation. Samples were collected every hour for 3 h using linear microdialysis probes (10 mm, 100 kD). Dialysate was analyzed for glutamate concentration. Pain intensity and skin vasomotor reactions (temperature and blood flow changes) were also recorded.

RESULTS: BoNTA significantly reduced glutamate release compared with saline (P<0.05). The provoked pain intensity was lower in the BoNTA-pretreated arm (P<0.01). The reduction in pain scores was not correlated with glutamate level. Cutaneous blood flow (P<0.05), but not cutaneous temperature (P≥0.05), was significantly reduced by BoNTA. There was a correlation between glutamate level and skin blood flow (r=0.58/P<0.05) but not skin temperature (P≥0.05). No differences according to sex were observed in any response.

CONCLUSIONS: The present study provided the first direct evidence supporting the inhibitory effect of BoNTA on glutamate release in human skin, which is potentially responsible for some of the analgesic action of BoNTA.

Key Words: Botulinum neurotoxin type A; Capsaicin; Glutamate; Human experimental pain model; Microdialysis; Vasodilation

Botulinum neurotoxin type A (BoNTA) is produced by the Gram-positive anaerobic bacterium Clostridium botulinum (1) and has long been used in the treatment of disorders characterized by increased muscle tone. It is well known that, when injected intramuscularly, the toxin inhibits the exocytotic release of acetylcholine at the neuromuscular junction, leading to muscle relaxation (2). Interestingly, pain relief occurs before muscular relaxation or in areas with no muscle tension (3), indicating that different mechanisms are responsible. In addition, an analgesic effect has also been observed in patients with pain treated using BoNTA (4,5) and in experimental pain studies involving animals and humans (6-8). BoNTA has been found to be efficient in painful conditions including neuropathic pain (9-11), low back pain (12), myofascial pain (13,14), fibromyalgia (15), trigeminal neuralgia (16), postherpetic neuralgia (17), headaches (18) and migraine (19,20). Although BoNTA is effective in several pain conditions, as mentioned above, migraine is the only approved condition, while the remaining conditions continue to be treated off-label. Most of the proposed mechanisms underlying analgesic effects of the toxin are based on in vivo animal models (8,21-23) and in vitro culture systems (24-29), which have shown that BoNTA is able to suppress the local release of substances involved in pain and vasodilation including glutamate (23), substance P (SP) (21,25) and calcitonin gene-related peptide (CGRP) (29). It has been suggested that this phenomenon may contribute to the pain relief observed in animal and clinical studies.

CONCLUSIONS: The present study has provided the first direct evidence supporting the inhibitory effect of BoNTA on glutamate release in human skin, which is potentially responsible for some of the analgesic action of BoNTA.
inhibition of peripheral sensitization and pain and, possibly, prevent central sensitization (27,30).

Glutamate is a well-known excitatory neurotransmitter with an important mediatory and modulatory role in nociception and sensitization (31,32). It has been previously shown that activation of peripheral glutamate receptors contributes to nociception and peripheral sensitization (33-35). Moreover, an increased glutamate level is known to be involved in a number of painful conditions such as migraine and fibromyalgia (36,37). Blockade of glutamate release by BoNTA has only been investigated in animal models, and there is no evidence regarding whether similar phenomenon would occur in humans following pretreatment with BoNTA. Therefore, the present study investigated this in humans using dermal microdialysis.

The microdialysis technique has long being used to examine metabolic changes or dynamic patterns of substance release (eg, glutamate [38,39]) in different tissues under physiological or pathological conditions (40-42). Previously, it has been shown that capsaicin stimulation provokes release of glutamate from primary afferent fibres in rats (43). Thus, we designed an experimental model of pain evoked by capsaicin plus mild heat in healthy volunteers to provoke glutamate release and to further investigate potential inhibitory action of BoNTA on glutamate release in humans.

We hypothesized that intradermal injection of BoNTA alters the release of glutamate in human skin. The aims of the present study were to: investigate glutamate release following application of a topical capsaicin patch (8%) plus mild heat in healthy volunteers to provoke glutamate release and to further investigate potential inhibitory action of BoNTA on glutamate release in humans.

The effect of BoNTA on glutamate release in humans

Subjects
The group of healthy volunteers consisted of six men (mean ± SEM age 25.0±1.5 years) and six women (mean age 25.7±0.72 years), who were recruited among students through advertisements at Aalborg University, Aalborg, Denmark. Participants were informed about the experimental procedures, safety issues, goals and perspectives of the research and provided written informed consent before the start of the experiments.

Study design
The study was designed as a double-blinded, randomized, placebo-controlled trial, and was approved by the local ethics committee (Region of North Jutland, Denmark; N-20110075) and the Danish data protection agency. Good Clinical Practice guidelines and the Declaration of Helsinki were followed during the course of the study. Participants attended three sessions in total. The first session consisted of a screening session, at which medical history was reviewed and the subject was excluded if any previous or present skin, systemic or neuromuscular diseases were present including migraine or chronic tension-type headache, diabetes, peripheral neuropathies, fibromyalgia, myasthenia gravis, Lambert-Eaton syndrome, amyotrophic lateral sclerosis or any other significant disease that may interfere with neuromuscular function. In addition, they could not participate if they had any severe allergy or history of current or past alcohol and/or drug abuse. Female volunteers were not pregnant or experiencing irregularities in their menstrual cycles. They could not be taking any medication during the sessions, nor could they be participating in another experiment. Weight and height were measured, and only participants with body mass index between 19 kg/m² and 35 kg/m² were included.

In the screening session, a challenge using the capsaicin pain model was also performed to evoke pain and flaire. Only subjects who rated pain intensity >5 (on a visual analogue scale ranging from 0 to 10) and developed a visible flare in response to this challenge were included in the present study. Individuals who passed the screening session were scheduled for the experimental sessions, at which they first received BoNTA and saline (control) injection in each forearm in a randomized, blinded fashion; the physician who injected BoNTA and saline did not know the content of the syringes and the investigator did not receive the code until the end of the trial. The subjects also did not know which arm was pretreated with the toxin or the vehicle, which was also randomized. All safety precautions were taken during the injections. After a one-week interval, which has been shown to be the peak of the toxin’s analgesic effect in the authors’ previous experimental pain models in humans (7,44,45), subjects were challenged using a topical capsaicin patch (8%) plus mild heat while a microdialysis membrane was placed on the pretreated area. They were asked to refrain from ingesting chili peppers 48 h before and caffeine-containing products 24 h before the experimental session. During the study sessions, each volunteer rested in a supine position. An overview of the study design is presented in Figure 1. Sensory and vasomotor assessments performed for each experimental session are summarized in Figure 2. The same investigator (LBS) performed all of the tests at the pain laboratory, a quiet environment with a controlled temperature of 22°C to 23°C located in the Center for Sensory-Motor Interaction, Aalborg University, Denmark.

BoNTA and saline injections
Each vial of BoNTA (BOTOX®, Allergan Incorporated, USA) was reconstituted using preservative-free 0.9% sodium chloride. Each participant received a single injection of BOTOX (10 U/0.2 mL) using a disposable needle (Neuroline Inojet, 27 gauge, Ambu A/S, Denmark) intradermally in the volar part of the forearm, 5 cm distant from the cubital fossa, as shown in Figure 3. An equal volume of sterile physiological saline (0.2 mL, 0.9%) was injected in the contralateral forearm as a control. The injection side was randomized and blinded. The
**Capsaicin + mild heat-evoked pain model**

Before the application of the capsaicin patch, the area was presensitized with heat stimulation at 45°C for 5 min. Capsaicin patch (8% with mild heat (40°C) delivered using the PATHWAY ATS advanced thermal stimulator (Medoc Ltd, Israel) through the thermode (30 mm × 30 mm), avoiding the microdialysis membrane.

**Microdialysis samples**

Samples from all subjects were collected and included in the analysis. Samples were stored at −80°C for later analysis.

**Measurement of glutamate**

Samples (10 µL) were analyzed for their glutamate content and investigation of pattern change over the time-course of the trial. An ISCUSflex Microdialysis Analyzer (M Dialysis AB, Sweden) was used, which applies enzymatic reagents and colorimetric measurements to monitor a number of different substances including glutamate. The results are presented in µM, as a comparison between the BoNTA- and saline-pretreated sides.

**Capsaicin + mild heat-evoked pain**

Pain scores were recorded right after the membrane insertion, following the heat sensitization period and every 15 min from the capsaicin patch application. Subjects were asked to rate their pain intensity on a visual analogue scale, on which 0 = ‘no pain’ and 10 = ‘the most pain imaginable’. The peak pain intensity scores were manually recorded and used for statistical analysis.

**Skin blood flow**

Skin blood flow was measured using a laser speckle contrast imager (FLPI, Moor Instruments, United Kingdom) to monitor vasomotor changes in the treated area. This device uses a full-field laser technique and provides real-time and high-resolution images of blood flow. The technique is noninvasive and is based on a random speckle pattern that is generated when tissue is illuminated by the laser light to capture the movement of the blood cells. With the lenses placed 40 cm perpendicularly above the tissue, it was possible to obtain an image of the entire forearm at baseline, and every hour after the membrane insertion, up to 3 h. To better follow the magnitude of the blood flow changes, an area of approximately 9 cm² was later chosen as a region of interest (ROI) for the analysis, which comprised the patch surface along with the surrounding area. The software was set to capture pictures using high-resolution/low speed with 1 s per frame in a free-run mode. An 8 ms exposure time was selected. The images were stored on the computer’s hard disk for offline analysis. The average blood flow within the ROI was calculated using the designated Moor software (mFLPIV4, Moor Instruments), expressed in arbitrary units and used for statistical analysis. Measurements were performed in a semidark room to eliminate artifacts from ambient room lighting.

**Skin temperature**

Skin temperature was assessed using a noninvasive infrared thermographic camera (FLIR Systems Inc, Sweden) to record the surface tissue temperature changes. The temperature resolution of the device is 0.09°C. The distance between the lenses and the tissue surface was set to 50 cm to capture the entire forearm image in a single frame. Measurements were performed before the membrane insertion (baseline) and every hour for 3 h after the insertion. While the baseline measurement was performed with the intact and uncovered skin, the subsequent measurements were taken after probe insertion or placement of the patch on the camouflaged area. To obtain the profile and magnitude of temperature changes, a square ROI (approximately 9 cm²) was defined that comprised the patch surface and the surrounding area. Thermographic images were stored on the computer’s hard disk for offline analysis. The average temperature within the ROI was calculated by ThermaCAM researcher Pro 2.8 (FLIR Systems Inc) and used for statistical analysis. To eliminate artifact from ambient room lighting, measurements were performed in a semidark room.

**Statistical analysis**

The number of participants was estimated using the formula $n = ((12\times SD^2/\beta^2) \times k)$, where $k$ was 7.9 constant value, $\alpha$ was set to 0.05 (corresponding to a significance level of $P<0.05$) and $\beta$ was set to 0.20, corresponding to a statistical power of 0.8 (48). $E$ and $SD$ are the minimal clinically relevant difference between the two situations (BoNTA...
versus saline) and the SD of the mean difference between the two, respectively. For a better estimation, data from a previous study (42) were used, in which E (for CGRP) was found to be 2.04 (as expected to yield a difference of 30%) and SD was found to be 1.7 in a microdialysis study involving human skin. Applying these, the number of participants was calculated to be n=10.97. Therefore, 12 subjects were recruited to have sufficient number to detect a possible suppressive effect of BoNTA in the current study.

Data are presented as mean and SEM in the text and figures, unless otherwise specified. Data were analyzed using ANOVA with three factors, defined as: treatment (BoNTA and saline), time (baseline and different time points) and sex (male and female). The Bonferroni test was used for post hoc analysis. All statistical tests were performed using SPSS version 20 (IBM Corporation, USA); P<0.05 was considered to be statistically significant.

RESULTS
All volunteers completed the study and no safety issues were reported. The main findings were: that BoNTA showed a significant inhibitory effect on glutamate release in the skin; and that BoNTA reduced capsaicin + mild heat-evoked pain intensity and skin blood flow. None of the observed responses were sex dependent.

Glutamate concentration
Pretreatment with BoNTA significantly reduced capsaicin + mild heat-evoked glutamate release compared with saline (F=5.028; P<0.05), as shown in Figure 4. Furthermore, post hoc analysis revealed a significant interaction between treatment and time in the levels of glutamate release in the final samples collected following the capsaicin + mild heat-evoked pain stimulation (F=7.974; P<0.05).

Pain characteristics
Capsaicin + mild heat-evoked pain intensity was lower in the BoNTA-pretreated arm (F=9.894; P<0.01). Moreover, post hoc analysis showed a significant interaction between treatment and time, revealing higher pain ratings (F=8.670; P<0.05) during the sensitization period, before the membrane insertion and 60 min after the capsaicin + heat stimulation, with the BoNTA-pretreated side showing higher pain ratings (F=9.894; P<0.01). Moreover, post hoc analysis revealed a significant interaction between treatment and time; the response was greatest 3 h after the membrane insertion (F=7.113; P<0.05), as shown in Figure 6.

Skin temperature
No significant difference was observed in the skin temperature measurements between BoNTA- and saline-pretreated areas (F=0.008; P=0.95).

Correlation
When comparing all outcomes, a correlation was found between the glutamate levels and blood flow (r=0.58; P<0.05). This correlation showed that blood flow was higher with higher levels of glutamate. All other outcomes did not reveal any correlation.

DISCUSSION
The present study examined glutamate release following cutaneous human experimental pain provoked by capsaicin + mild heat before and after localized BoNTA treatment. BoNTA decreased pain intensity, suppressed the capsaicin-evoked elevated skin blood flow and lowered the release of glutamate. These responses were sex-independent. This is the first evidence in humans to show that BoNTA attenuates glutamate release in the skin and is consistent with previous findings in animals. The blockade of glutamate release may contribute to the peripheral analgesic effects of BoNTA.

The effect of BoNTA on glutamate release
Glutamate is one of the most important excitatory neurotransmitters involved in pain transmission, both in the central nervous system and in the periphery. Evidence shows that an increased level of glutamate is found in inflammatory disease and pain conditions, such as myalgia (49), temporal mandibular disorder (39) and chronic tendinitis (41), all measured using the microdialysis technique, which enables the collection of fluid samples from the interstitial space of targeted tissues through a permeable membrane. Protein-unbound molecules move freely from one side of the membrane to the other, based on the concentration gradient of these substances (38,50).

In the present study, we used a capsaicin patch (8%) plus mild heat as a pain model, which is known to activate polymodal mechanoheat receptors located on nociceptive primary C-fibres (51,52). This activation leads to sensitization and local release of neurotransmitters, such as glutamate, CGRP and SP, from the peripheral nerve endings (51,52). Here, we demonstrated that provoked glutamate release by this pain model was attenuated by BoNTA. In a previous study by our group, BoNTA decreased glutamate concentration in the temporalis...
were made in each arm (BoNTA and control). The graph shows mean and the lower sequence shows the matching control.

Figure 6) A Typical blood flow image at baseline, 1 h and 2 h after probe insertion, and after sensitization and capsaicin (cap) + 40°C heat stimulation, shown with a corresponding image of the region of interest. The upper sequence shows the botulinum neurotoxin type A (BoNTA)-pretreated side and the lower sequence shows the matching control. B Blood flow was measured before probe insertion (baseline) and every hour for 3 h. Assessments were made in each arm (BoNTA and control). The graph shows mean and SEM; *P<0.05. BoNTA versus control. **P<0.001. 2 h timepoint versus capsaicin + 40°C heat. A correlation was found between the level of glutamate release and blood flow (r=0.58; P<0.05). au Arbitrary units

muscle of rats more rapidly and to a greater extent compared with saline (53). This effect has also been shown in an animal model of formalin-induced pain, in which rats were pretreated with BoNTA and challenged with formalin in the paw. Application of BoNTA five days before formalin-induced pain led to a significant decrease in glutamate release (23). In preclinical settings, it has been shown that substance release is blocked by BoNTA through cleavage of synaptosomal-associated protein 25 (54,55). Based on our observation and some previous animal data, it is likely that BoNTA may also influence the release of other substances, such as CGRP and SP, from the primary afferent fibres. Thus, microdialysis may prove useful for further discoveries and for recording dynamic patterns of substance release (eg, CGRP, SP) following BoNTA treatment in human tissue in vivo. In rats, it has been shown that some endogenous analgesic compounds are elevated following BoNTA administration (56); whether enkephalin, endorphin and/or dynorphin levels are modulated by BoNTA in humans, and whether these changes are detectable by microdialysis, remains unknown.

The effect of BoNTA on evoked pain

The capsaicin patch (8%) plus mild heat applied in the present study was found to be a reliable pain model to induce pain and vasomotor responses. Capsaicin-sensitive neurons release several neuropeptides, such as SP, neurokinin A, somatostatin, and CGRP, some of which are known to be involved in pain induction and transmission (57). It is also known that glutamate receptors, which are present on the primary afferent neurons, can be activated by glutamate and provoke pain in both animals and humans (35,58).

The inhibitory effect of BoNTA on glutamate release has not only been shown in formalin-induced pain in the hind paws of rats (23), but also in rat trigeminal nerve cells (29), rabbit iris sphincter and dilator muscles (21), and embryonic rat dorsal root ganglia neurons (25). Moreover, several animal and human studies have demonstrated an analgesic effect of BoNTA (6-8,45,53), while others have not (59-63). Differences among studies could be due to various reasons. For example, the inconsistency of the botulinum toxin types used may be one factor. Differences in doses (in units), sites of application (muscle or skin), methodology of BoNTA administration, assessment techniques and outcome parameters may also lead to these contradictions. In addition, the experimental pain models used in each of these studies differ to a large extent. Vollmer et al (59) used capsicin-induced pain and Krämer et al (63) used electrical-induced pain, while Sycha et al (61) used an experimental ultraviolet B pain model.

In the present study, pretreatment with BoNTA significantly reduced pain and demonstrated an antinociceptive effect of BoNTA on the peripheral nociceptors sensitized by capsaicin and mild heat. These results confirm the suitability of the chosen model. Because we measured glutamate levels in parallel with pain responses, we were interested to determine whether these two parameters are correlated; however, the results did not indicate a correlation. Therefore, it is assumed that glutamate release inhibition is only partially responsible for the analgesic action of BoNTA and other factors/substances are potentially involved.

The effect of BoNTA on vasomotor responses

Capsaicin provokes neurogenic inflammation. This flare can be attributed to neuropeptides (eg, CGRP and, to some extent, SP) released from peripheral nerve endings (64). These substances are released from primary afferents (65). It has been shown that SP is colocalized in primary afferent terminals with CGRP and glutamate (66). CGRP is a potent vasodilator affecting precapillary arterioles, and SP enhances vascular permeability by acting on capillaries and postcapillary venules. These substances are involved in the physiological control of blood flow (52,67-69). It is believed that the 40°C heat stimulation would enhance these reactions (neuropeptide release and vasodilation) through the same receptor involved in the capsaicin reaction (transient receptor potential cation channel subfamily V member 1 [TRPV1]), in addition to the TRPV3 receptor (which is insensitive to capsaicin). The latter is known to be present on the primary afferent fibres in the skin and is sensitive to heat stimulation >39°C (70). Consistent neurogenic inflammation was found in the present study, and BoNTA reduced the provoked blood flow, as previously observed (7,45). On the contrary, no temperature change by BoNTA was detected, which may be a result of the interference in the irradiated heat while the patch was placed on the skin for the final measurement. The plastic-based patch may affect the exact temperature or correct recording due to its different composition compared with the skin. Moreover, a linear association has not always been drawn between temperature and blood flow measurements (71). Considering all of these limitations, the blood flow reduction by BoNTA adds further support to the concept that skin blood flow is a reliable parameter and could be used as an element to monitor some aspects of analgesic efficacy in specific candidate compounds. A correlation was found between the reduction of glutamate release and blood flow changes following BoNTA pretreatment, supporting the contribution of glutamate in cutaneous vasodilation in human skin and the inhibitory role of BoNTA. To determine the role of CGRP and SP and potential inhibitory effect of BoNTA, further investigation is required to measure the levels of these substances along with glutamate levels using the method described in the current study.

CONCLUSIONS

The present study demonstrated that BoNTA attenuated the release of glutamate in human skin when provoked by capsaicin + mild heat stimulation. BoNTA inhibited the provoked pain and reduced the blood flow. An association was found between BoNTA reduction in glutamate release and provoked blood flow response.
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