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.

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

Le blocage de la libération du glutamate par la neurotoxine botulique de type A chez les humains : une étude de microdialyse cutanée

HISTORIQUE: L’action analgésique de la neurotoxine botulique de type A (NBdTA) est liée au blocage de la libération périphérique des neuropeptides et des neurotransmetteurs. Cependant, il n’y en a pas de preuves directes chez les humains.

OBJECTIFS: Examiné l’effet de la NBdTA sur la libération du glutamate chez les humains, au moyen d’un modèle expérimental de douleur et de sensibilisation provoquées par la capsaïcine et une chaleur légère.

MÉTHODOLOGIE: Douze volontaires en bonne santé (six hommes, six femmes) ont reçu un traitement préalable à la NBdTA (10 U) sur l’aspect antérieur de l’avant-bras et une injection de soluté physiologique du côté controlatéral. La microdialyse cutanée a été appliquée une semaine plus tard pour recueillir des échantillons interstitiels avant et après l’application d’un timbre de capsaïcine (8 %) associé à une chaleur légère (40 °C pendant 60 minutes) pour provoquer une libération de glutamate, de la douleur et une vasodilatation. Les échantillons ont été recueillis toutes les heures pendant trois heures au moyen de sondes pour microdialyse linéaire (10 mm, 100 kD). Le dialysat a été analysé pour déterminer la concentration de glutamate. L’intensité de la douleur et les réactions vasomotrices cutanées (températures et modifications du débit sanguin) ont également été enregistrées.

RÉSULTATS: La NBdTA réduit la libération de glutamate beaucoup plus que le soluté physiologique (P<0.05). L’intensité de la douleur provoquée était plus faible dans le bras prétraité par NBdTA (P<0.01). La réduction des indices de douleur n’était pas corrélée avec le taux de glutamate. La NBdTA réduisait considérablement le débit sanguin cutané (P<0.05), mais pas la température cutanée (P≥0.05). Il y avait une corrélation entre le taux de glutamate et le débit sanguin cutané (r=0,58/P<0.05), mais pas la température cutanée (P≥0.05). Aucune des réponses ne comportait de différences en fonction du sexe.

CONCLUSIONS: La présente étude a fourni les premières preuves directes de l’effet inhibiteur de la NBdTA sur la libération de glutamate sur la peau humaine, potentiellement responsable de l’action analgésique de la NBdTA.

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inhibition of peripheral sensitization and pain and, possibly, prevent central sensitization (27,30).

Glutamate is a well-known excitatory neurotransmitter with an important mediator 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.

METHODS

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 study. Weight and height were measured, and only participants containing products 24 h before the experimental session. Participants were requested 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 (Neurline Injekt, 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...


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 ISCUStFlex 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 before 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 part 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 cannulated area. To obtain the profile and magnitude of temperature changes, an area of 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

\[ n = \left( \frac{Z_{1-\alpha/2} \times SD}{E} \right)^2 \times k, \]

where \( Z_{1-\alpha/2} = 1.96 \) (corresponding to a significance level of \( P = 0.05 \) and \( \beta = 0.2 \) corresponding to a statistical power of 0.8 (48). E and SD are the minimal clinically relevant difference between the two situations (BoNTA vs. saline-pretreated areas).
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 microdi-
alysis 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 cap-
saicin + 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, reveal-
ing higher pain ratings (F=8.670; P<0.05) during the sensitiza-
tion period, before the membrane insertion and 60 min after the cap-
saicin + heat stimulation, with the BoNTA-pretreated side showing
lower pain ratings compared with saline. Detailed information is presented
in Figure 5.

Skin blood flow

The elevated skin blood flow evoked by capsaicin + mild heat was
significantly attenuated by BoNTA (F=5.822; P<0.05) compared with
saline. Post hoc analysis showed a significant interaction between
treatment 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 measure-
ments between BoNTA- and saline-pretreated areas (F=0.008;
P>0.05).

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 cuta-
neous 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 noted 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 col-
lection 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 con-
centration 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 mechanosheat
receptors located on nociceptive primary C-fibres (51,52). This activ-
ation 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 temporals

The effect of BoNTA on glutamate release in humans
were made in each arm (BoNTA and control). The graph shows mean and
measured before probe insertion (baseline) and every hour for 3 h. Assessments
and the lower sequence shows the matching control.
sequence shows the botulinum neurotoxin type A (BoNTA)-pretreated side
insertion, and after sensitization and capsaicin (cap) + 40°C heat stimula-
Figure 6)
Our observation and some
release (23). In preclinical settings, it has been shown that substance
release and provoked blood flow response. 

but also in rat trigeminal nerve cells (29), rabbit iris sphincter and
but 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 tech-

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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