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Antinociceptive Effect of Botulinum Toxin Type A in Rat Model of Carrageenan and Capsaicin Induced Pain

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Aim To test antinociceptive properties of botulinum toxin type A (BTX-A) in rats with carrageenan- and capsaicin-induced pain and inflammation.

Methods Pain was provoked with carrageenan (1%) or capsaicin (0.1%) injection into the plantar surface of the rat paw-pad. The effect of BTX-A 5 U/kg on carrageenan- and capsaicin-induced mechanical and thermal hypersensitivity, as well as the size of carrageenan-induced paw edema were tested 24 hours and 6 days following the toxin injection into the rat paw-pad. In the dose-response experiment, the effect of different doses of BTX-A (2, 3, 3.5, 5, and 7 U/kg) on carrageenan-induced mechanical hypersensitivity was investigated on day 5 after BTX-A application.

Results Pretreatment with 5 U/kg BTX-A significantly reduced or completely abolished the enhanced sensitivity to mechanical and thermal stimuli provoked by peripheral carrageenan or capsaicin injections. This reduction was significant when BTX-A was applied 6 days before the induction of pain and inflammation, but the toxin was ineffective when applied 24 hours before the challenge. In the dose-response experiment, the lowest effective dose was 3.5 U/kg, but apparently the effect was not dose-dependent. In contrast to the antinociceptive effect, 5 U/kg BTX-A had no effect on the carrageenan-induced paw edema.

Conclusion The study demonstrated the efficacy of peripherally applied BTX-A pretreatment on the pain component of inflammatory process in experimental animals.

Botulinum toxin type A (BTX-A) is one of the seven serotypes of the neurotoxin synthesized by the anaerobic bacterium *Clostridium botulinum*. BTX-A is synthesized as a large protein that must be cleaved into di-chain structures consisting of a 100 kD heavy chain and 50 kD light chain to be active (1). The classic mechanism of the botulinum neurotoxin action is the inhibition of acetylcholine release from the peripheral nerve terminal (2). The first step in this process is the binding of the toxin heavy chain to the presynaptic acceptors on axon terminals. Following binding, the toxin is internalized into nerve cells via acceptor-mediated endocytosis and translocated into the neuronal cytosol. The ultimate result of this translocation is the reduction of the disulfide bond and the release of the light chain into the intracellular space where it acts as a zinc-dependent endopeptidase that cleaves one of the proteins required for vesicle docking and fusion with plasma membrane named synaptosomal associated protein of 25 kD (SNAP-25). Accordingly, BTX-A prevents acetylcholine release into the synaptic cleft (2,3).

The administration of nanogram quantities of BTX-A manufactured for medical purpose has been used as a treatment for various neuromuscular disorders such as cervical dystonia, juve-

nile cerebral palsy, focal spasticity, etc (4). However, several reports indicate that BTX-A might be effective in various pain syndromes (5). According to open-label and a few double-blind studies, BTX-A treatment was found to be effective in chronic tension-type headache (6), cervical dystonia (7), chronic low back pain (8), pain of musculoskeletal origin (9), migraine (10,11), and neuropathic pain (12). In clinical practice, pain alleviation was usually observed to occur before and last beyond the muscular relaxation effect, and to be more pronounced than muscular improvement. Furthermore, based only on individual case reports, it has been suggested that BTX-A might have antinociceptive properties in patients, independent of its known action on muscular spasm (10-14). If botulinum toxin does have an antinociceptive effect, the doses required for pain treatment, the duration of the antinociceptive effect, and the range of indications are unknown (15,16). To our knowledge, there are only 3 literature reports on the studies investigating the antinociceptive effect of BTX-A in experimental animals.

In the laboratory of the manufacturer (Allergan), Cui et al (17) were the first to show in the rat formalin model that peripheral subcutaneous BTX-A pretreatment inhibited the formalin-related spontaneous nociceptive behaviors such as shaking and licking of the injected paw in a dose-dependent fashion during the tonic inflammatory second phase of the test. They observed the effect to start within the first 24 hours and last for 12 days, which was the latest time point tested. The authors also found the formalin-induced peripheral glutamate release in the rat foot pad to be significantly reduced by BTX-A (7 U/kg) compared with the vehicle when measured 5 days after the toxin injection.

The instillation of 25 U/ml BTX-A into the rat bladder prevented the protamine sulfate and acetic acid induced decrease in the intercontraction interval of the reflex bladder contractions (as an indicator of hyperalgesia) when measured after 7 days of the toxin treatment (18). The authors interpreted it as an antinociceptive effect. In this experiment, BTX-A was effective after 7 days but not after 3 days of the treatment. The authors found evidence for reduced bladder inflammation. Immunofluorescence studies of the calcitonin gene-related polypeptide (CGRP) showed the CGRP immunoreactivity to occur in the "mucosal layer of the bladder" only 7 days after BTX-A instillation. The cellular source of the immunoreactivity was not specified. The authors interpreted the findings as a possible indicator of CGRP release but also as an "increased CGRP synthesis or decreased CGRP breakdown" (18).

In our study in a rat model of surgical neuropathic pain induced by partial sciatic nerve transection (19), BTX-A reduced thermal and mechanical hyperalgesia from day 5 to day 15 (or more) but not within 24 hours after the toxin peripheral application.

Other studies indirectly related to the postulated antinociceptive effect of BTX-A were performed on cultured neurons *in vitro*. For example, one study investigated the effect of BTX-A on the substance P secretion from cultured embryonic rat dorsal root ganglia neurons (20), whereas another one dealt with the effect of the toxin on the CGRP release from cultured trigeminal ganglia neurons (21). Both studies found that BTX-A inhibited the release of substance P and CGRP from cultured neurons *in vitro*. However, the relevance of these observations for the antinociceptive activity of BTX-A *in vivo* is not clear.

Since antinociceptive drugs produce short-lasting analgesia, the potential long-term effect (observed in animals after more than 15 days) (19) of BTX-A might be of great importance and therefore there is an obvious need of both controlled clinical experiments and more preclinical studies on different animal models to characterize the antinociceptive effect, ie to identify the conditions in which it could be expected, as well as the effective doses and duration of the effect.

Peripheral injections of small volumes of different irritants such as formalin, carrageenan, and capsaicin in the rat paw-pad are well-characterized and commonly employed models for the investigation of anti-inflammatory and antinociceptive effects of different substances. The injection 5% formalin solution, employed in the experiment by Cui et al (17), elicits a biphasic response characterized by spontaneous pain behaviors such as flinching, shaking, biting, and licking of the injected paw, where the first phase is a consequence of direct stimulation of nociceptors, whereas the second phase is thought to reflect an inflammatory process (22).

Peripheral application of carrageenan or capsaicin produces hypersensitivity to thermal and mechanical stimuli. In addition, 2-6 hours after the injection, carrageenan produces inflammatory edema which is more intensive than edema induced by formalin.

Carrageenan, a sulfated polysaccharide, promotes inflammation by activating proinflammatory cells. It is assumed that it causes hyperalgesia by promoting the local release of mediators such as substance P, glutamate, prostaglandins, histamine, and serotonin. These noxious chemicals sensitize primary afferents resulting in primary hyperalgesia (23). It has been shown that carrageenan-induced inflammation and pain activate early genes such as c-fos, which can then activate other genes leading to changes in the synthesis of various neuropeptides in the dorsal horn of the spinal cord (24). The injection of carrageenan in the rat paw induces COX-2 mRNA in the lumbar spinal cord and releases glutamate, aspartate, substance P, nitric oxide, and prostaglandin E_2 in the dorsal horn. Carrageenan can also cause an acute increase in immunoreactive CGRP and substance P in the spinal cord that lasts for at least 1 week (23). Some recent findings from the study of fos-labeled cells indicate that unilateral hind paw carrageenan injection produces bilateral activation of the descending modulation system from the locus coeruleus/nucleus subcoeruleus (25). Therefore, it seems that different peripheral, as well as central mechanisms contribute to carrageenan-evoked hyperalgesia.

Capsaicin, the pungent component of the chili pepper, excites sensory neurons directly by acting on vanilloid receptors type 1 (VR-1), which are selectively expressed on C-fibers (26). VR-1 receptors are present on primary afferent nerve fibers that contain neuropeptides such as substance P and CGRP, which are released from nerve endings following receptor excitement ie capsaicin application (26). *In vivo* exposure to capsaicin decreases the threshold of nociceptive afferents to thermal or mechanical stimuli. It seems that hyperalgesia to heat may involve excitation and sensitization of C-fiber nociceptors, whereas mechanical hyperalgesia appears to be mediated primarily by central sensitization whereby responses of dorsal horn neurons evoked by low-threshold mechanoreceptors become facilitated (27).

In the present study, carrageenan and capsaicin models were used to investigate the possible influence of BTX-A on thermal and mechanical hyperalgesia induced by these substances, and in case of carrageenan on local edema formation. Dose-response and time-course of the toxin action were investigated. The most important observation was that BTX-A reduced capsaicin- and carrageenan-induced pain but had no effect on carrageenan induced edema.

Methods

Animals

A total of 120 male Wistar rats (Zagreb University School of Medicine, Zagreb, Croatia) weighing 250-300 g, were used in all experiments. The experiments were carried out according to the Croatian Act on animal welfare (Narodne novine 19/1999). The Principles of Laboratory Animal Care (NIH Publication No. 86-23, 1985) were followed. The experiments were approved by the Ethical Committee of Zagreb University School of Medicine (permit No. 07-76/2005-43).

Drugs

The following drugs were used: botulinum toxin type A (BOTOX®, Allergan, Inc., Irvine, CA, USA); carrageenan (α-carrageenan, Sigma, St. Louis, MO, USA); and capsaicin (Sigma, St. Louis, MO, USA).

Each vial of BOTOX® contains 100 U (~4.8 ng) of purified *Clostridium botulinum* toxin type A. BTX-A was reconstituted in 0.9% saline solution. The doses of BTX-A used in dose-response experiment were 2, 3, 3.5, 5 and 7 U/kg. In all other experiments, only one dose of the toxin was employed (5 U/kg). BTX-A was injected into the plantar surface of the rat hind paw in a volume of 20 μl with a 27-gauge syringe.

Experimental Procedures

Carrageenan-induced edema and hyperalgesia. 1% Carrageenan (dissolved in saline) was injected into the plantar surface of the hid paws of rats, which were awake, in a volume of 100 μl with a 27-gauge tuberculin syringe. Intraplantar injection of carrageenan in freely moving rats produced an acute restricted inflammation associated with thermal and mechanical hyperalgesia. Carrageenan was injected 3 hours before nociceptive testing (28). The sensitivity to

mechanical stimuli was tested after the measurement of thermal sensitivity.

Paw edema measurement. The volume of the paw was determined with a plethysmometer as described by Winter et al (29). Volumes of the treated and contralateral-untreated paws were determined 3 hours after carrageenan application. Three measurements of each paw were performed at 10-min intervals.

Capsaicin-induced hyperalgesia. 0.1% Capsaicin (dissolved in saline containing 10% Tween and 10% ethanol) was administered into the plantar region of the hind paws of rats, which were awake, in a volume of 50 µl with a 27-gauge tuberculin syringe. Withdrawal responses to thermal and mechanical stimuli were measured 15 minutes after capsaicin application (30). The measurement of thermal sensitivity was followed by the measurement of sensitivity to mechanical stimuli.

Instead of carrageenan or capsaicin solution, control groups of animals in all experiments received saline in the same volumes.

On preparation for intraplantar injection, rats were gently restrained with one hind paw exposed. During injection, the needle penetrated the skin in the center of the plantar surface. Successful intraplantar injection was noted by the appearance of a bleb within the injection site on the hind paw.

Measurement of Pain Reactivity

Unilateral hot-plate test. Thermal sensitivity was tested using a slight modification of the unilateral hot-plate test originally described for mice (31). The temperature of the hot-plate surface was 52 ± 0.5 °C and the cut off time was 20 seconds in order to prevent paw tissue damage. Rats were gently restrained and the plantar side of the tested paw was placed on the hot-plate surface. The latency of paw withdrawal from the heated surface was recorded 3 times at 10-minute intervals.

Paw pressure test. The sensitivity to mechanical stimuli was measured by the paw-pressure test as described by Randall and Selitto (32). Mechanical nociceptive thresholds expressed in grams were measured 3 times at 10-minute intervals by applying increased pressure to the hind paw until the paw-withdrawal or overt struggling was elicited.

The experiments were partially blinded, ie the animals with different treatments were mixed in each particular cage and measurements were made without prior recollection of the type of treatment. However, the treatment and measurement were performed by the same investigators.

Statistical Analysis

Results were presented as mean ± standard deviation. Statistical analysis was performed by the analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test for between-group differences. *P*-value less than 0.05 was considered significant. On result presentation, *P* values from *post hoc* test were given.

Results

Injection of carrageenan into the rat paw pad resulted in increased sensitivity to both thermal and mechanical stimuli. Thermal latencies as well as paw withdrawal thresholds in these animals were significantly decreased ($P = 0.006$ and $P = 0.001$, respectively) in comparison with the control group of rats (Fig. 1). When injected 6 days before carrageenan, BTX-A 5 U/kg significantly reduced ($P = 0.016$) thermal hypersensitivity (Fig. 1A) and completely abolished ($P = 0.001$) mechanical hypersensitivity in the carrageenan-treated animals (Fig. 1B). In the dose-response experiment, the lowest effective dose that reduced mechanical hypersensitivity evoked with carrageenan on the day 5 of BTX-A application was 3.5 U/kg ($P = 0.001$). There was no significant difference in the intensity of analgesic effect obtained by either 3.5, 5 ($P = 0.001$) or 7 U/kg ($P < 0.001$) of BTX-A (Fig. 2).

BTX-A 5U/kg did not affect the size of carrageenan-induced paw edema at any of the test time-points (Fig. 3).

Injection of capsaicin produced immediate guarding behavior that consisted of the animal lifting the injected paw and not applying pressure on it. This "nocifensive" behavior lasted for approximately 3 minutes, after which the animals appeared to use the paw normally for locomotion. BTX-A pretreatment did not affect this behavior (data not shown). Capsaicin also provoked exaggerated responses to both thermal and mechanical stimuli. BTX-A pretreatment almost completely returned the pain threshold to the control values.

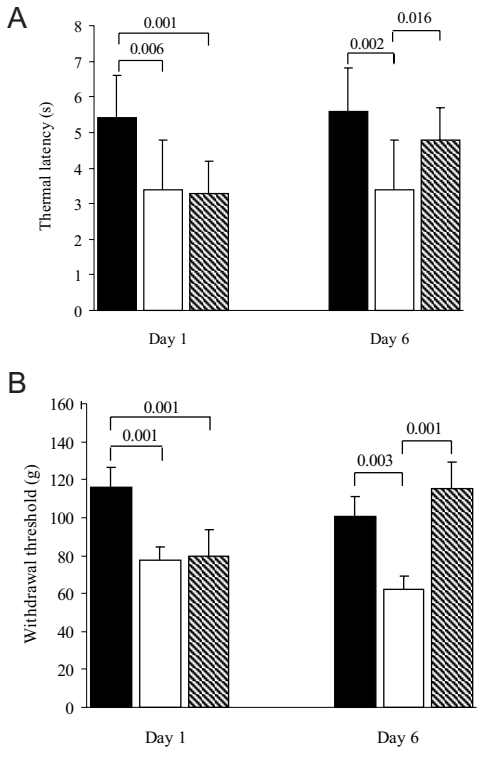

Figure 1. Influence of BTX-A (5 U/kg) on thermal (**A**) and mechanical (**B**) hyperalgesia in carrageenan model of inflammation in rats. Closed bars – control; open bars – carrageenan; grey bars – BTX-A+carrageenan. Control group n=7; carrageenan- and BTX-A+carrageenan-treated groups (n=6 each). *P* values are shown above bars. Newman-Keuls *post hoc* test was used for between-group differences.

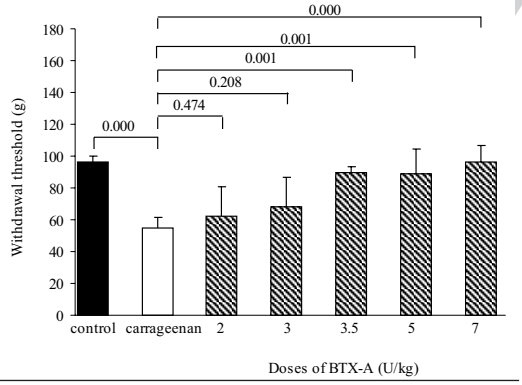

Figure 2. Influence of different doses of BTX-A on carrageenan-induced mechanical hyperalgesia measured on day 5 after peripheral toxin application in rats. Groups treated with 2 and 5 U/kg of BTX-A (n=5 each, all other groups n=6 each). *P* values are shown above bars. Newman-Keuls *post hoc* test was used for between-group differences.

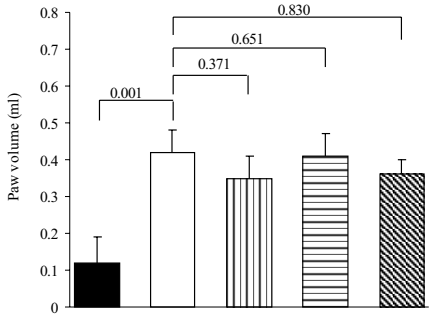

Figure 3. Influence of BTX-A 5 U/kg on paw edema after carrageenan challenge measured on day 1, 5, and 10 following toxin application in rats. Closed bar – control; open bar – carrageenan; bar with vertical lines – BTX-A day 1+carrageenan; bar with horizontal lines – BTX-A day 5+carrageenan; bar with slanting lines – BTX-A day 10+carrageenan. Control, carrageenan-treated and BTX-A day 10+carrageenan-treated groups (n=8 each, other two groups n=5 each). *P* values are shown above bars. Newman-Keuls *post hoc* test was used for between-group differences.

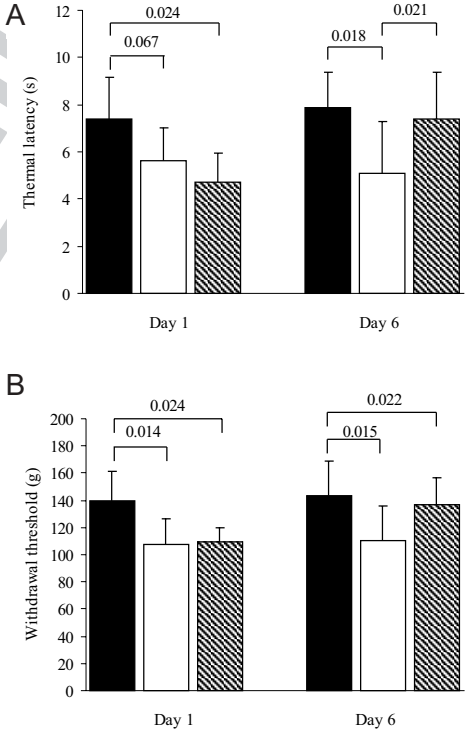

Figure 4. Influence of BTX-A on thermal (**A**) and mechanical (**B**) hyperalgesia in capsaicin model of inflammatory pain in rats. Closed bars – control; open bars – capsaicin; grey bars – BTX-A+capsaicin. Capsaicin-treated group n=5, control group n=6, and BTX-A+capsaicin-treated group (n=7). *P* values are shown above bars. Newman-Keuls *post hoc* test was used for between-group differences.

However, BTX-A was found to be effective only when nociceptive measurements were carried out 6 days after the toxin injection (Fig. 4), whereas no differences in response to either carrageenan or capsaicin were observed at 24 hours of BTX-A application.

BTX-A had no direct antinociceptive action. When applied peripherally 5 days before nociceptive measurement, 5 U/kg BTX-A modified neither thermal (8.18 ± 0.92 seconds for BTX-A vs 7.85 ± 1.4 seconds for saline) nor mechanical pain thresholds (152.7 ± 13.0 g for BTX-A vs 147.8 ± 14.4 g for saline) in the saline-treated control animals.

None of the applied doses of BTX-A produced any muscle weakening effect, ie the movement and muscular tone during pain measurement appeared normal.

Discussion

The present study demonstrated the effectiveness of peripherally applied BTX-A (5 U/kg) in reducing hyperalgesia induced by capsaicin or carrageenan injection in the rat paw pad. Although many details remain unknown, thermal and mechanical hyperalgesia produced by these two substances seems to have different mechanisms of action. Capsaicin-induced pain seems to be primarily peripheral in origin (26,27), connected with neuropeptides release, whereas the action of carrageenan might involve central and peripheral mechanisms (23-25).

Intraplantar injection of capsaicin provokes immediate nocifensive behavior, which consists of guarding and licking of the injected paw, and it was short-lasting (for about 3 minutes) without pronounced edema formation. Mechanical hyperalgesia observed after capsaicin injection is more robust than thermal hypersensitivity, lasting for up to 4 hours compared with approximately 45 minutes and occurring over most of the plantar surface of the paw, ie extending beyond the area of injection (30). On the other hand, carrageenan-induced marked inflammatory edema and thermal, as well as mechanical hyperalgesia are most pronounced at 2-6 hours after carrageenan challenge (28).

In the present experiments, BTX-A, when injected 6 days before carrageenan or capsaicin in the rat paw pad, reduced thermal as well as mechanical hyperalgesia. Thermal and mechanical hypersensitivity was almost completely abolished in both models employed. In the dose-response experiment, the lowest effective dose that reduced mechanical hypersensitivity following carrageenan was 3.5 U/kg. Surprisingly, in the present experiments this effect was not dose-dependent. In contrast to this, Cui et al (17) reported dose-dependent inhibition of nociceptive behavior in formalin model of inflammatory pain. Peripheral injection of BTX-A in the rat paw pad reduced pain-related behavior only in the second phase of formalin test, which is thought to be connected with an inflammatory process. In the mentioned experiment (17), the doses employed ranged from 3.5 U/kg to 30 U/kg. Whereas in our study none of the doses used produced any muscle weakening effect, the higher doses (15 and 30 U/kg) employed in the experiment of Cui et al (17) caused significant muscle relaxation that, in our opinion, could affect the nociceptive measurements (time of licking and biting of the formalin-injected paw was scored).

In the present experiment, 5 U/kg of BTX-A failed to significantly affect the size of carrageenan-induced paw edema. In other words, in present experiments, BTX-A had antinociceptive but not anti-inflammatory action. Carrageenan-induced edema is a biphasic effect. The initial phase is attributed to the release of histamine and serotonin, whereas the increased vascular permeability in the second phase is maintained by the release of kinins, prostaglandins, protease, and lysosome (28,29). However, at the dose tested BTX-A had no measurable effect on carrageenan induced inflammatory edema. In contrast to this, according to Cui at al (17), BTX-A at a dose of 7 U/kg but not 3.5 U/kg reduced formalin-induced edema, which has been suggested to be the consequence of local inhibition of formalin-induced glutamate release. Accordingly, it is hypothesized that BTX-A mediates an antinociceptive activity *via* peripheral inhibition of the neurotransmitter release, which was also supported by some *in vitro* studies performed on primary culture neurons (20,21). However, based on the current knowledge, we feel that it is difficult to explain the different effects of the same doses of BTX-A on carrageenan and formalin induced edema.

In the time-course experiments, we found that BTX-A was not effective when injected 24 hours before carrageenan or capsaicin chal-

lenge. The same was observed in the rat model of experimental neuropathic pain where BTX-A in a dose of 7 U/kg decreased thermal as well as mechanical hypersensitivity on day 5 but not on day 1 of its peripheral application (19). Similarly, BTX-A was effective in reducing bladder pain on day 7 but not on day 3 (18). Thus, it is concluded that, with the possible exception of formalin induced pain, the antinociceptive effect of BTX-A is not an immediate but a slowly (over 5-7 days) developing effect.

The results obtained in the present study, along with the data of Cui et al (17), the study by Chuang et al on bladder pain (18), and our recent report on neuropathic pain (19) clearly demonstrated the antinociceptive activity of small doses of BTX-A in experimental animals. Since single application of BTX-A in rat produces an antinociceptive effect lasting for 12-15 days or longer (17,19), the potential significance for pain pharmacology is more than clear. However, (a) the several day delay in the onset of antinociceptive activity of peripherally applied BTX-A, (b) its effectiveness in different models of acute and chronic experimental pain, as well as (c) its ineffectiveness in reducing carrageenan induced paw edema indicate that the mechanism of the antinociceptive action of BTX-A might be much more complex than the suggested inhibition of transmitter release in the periphery (17,18).

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