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The effect of local injection of botulinum toxin A on the immunoreactivity of nNOS in the rat submandibular gland: An immunohistochemical study

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Summary

Purpose: In our study, we intend to investigate the influence of local injections of botulinum toxin A on the activity of neuronal nitric oxide synthase (nNOS) in submandibular glands of adult rats. Since interest has been focused on the role of nitric oxide (NO) as a possible neuromodulator of secretory regulation processes in the upper aerodigestive tract, it was the aim of the present study to show that the toxin also interferes with the metabolic actions of NO on investigated rat submandibular glands. It is of great clinical interest whether the NO pathway is able to influence salivary gland secretion. Increasing of knowledge in this field maybe helpful to treat sialorrhoea, especially in juvenile otolaryngologic and neurologic patients.
Materials and methods: We performed immunohistochemical reaction of neuronal nitric oxide synthase (nNOS) in the submandibular gland of female adult Wistar rats, both in native (untreated) glands and after intraglandular injection of botulinum toxin A under general anesthesia. The immunoreactivity of nNOS was investigated on different times after injection.
Results: Other than in the untreated glands, there was a significant decrease of nNOS in the treated organs, which became stronger with extended toxin exposure time. The present study shows explicit data on the effect of botulinum toxin A injection on a higher number of examined submandibular glands and is able to analyze a time course of the effect duration.
Conclusion: In our study, it was shown that botulinum toxin A had an influence on the immunoreactivity of neuronal nitric oxide synthase (nNOS) in submandibular glands.

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Therefore, the participation of nitric oxide (NO) in the regulation of secretion from these organs seems to be evident. It might be assumed that the influence of botulinum toxin A on nNOS in the submandibular gland of the rat is able to explain the sometimes longer duration of toxin effect at the neuroglandular junction than at the motor endplate.

1. Introduction

The treatment of sialorrhoea, or extensive hypersalivation and drooling is a therapeutic problem so far unsolved in children with severe otolaryngological and neurological diseases. There is a constant risk for life-threatening pulmonary infections, since swallowing is frequently distorted in those children. In these cases, the diminishing of salivary secretion by pharmacological treatment could be a great help. The families of the affected children are subjected to extreme stress, and social isolation. To date, permanent hypersalivation has largely been treated by extended surgical procedures such as gland resection, transposition of the excretory ducts, and tympanic neurectomy [1—3]. These invasive measures are stressful for the patient and not suited for passing hypersalivation states because of their permanent consequences. A systemic anticholinergic therapy is often contraindicated because its side effects interfere with circulation.

A promising therapeutic approach is topical intraglandular application of botulinum toxin A [4,5]. The duration effect lasts about 3 months [6] and the local application of the toxin even in children was without severe side-effects [5].

Recently, interest has been focused on the role of nitric oxide (NO) as a possible neuromodulator of secretory regulation processes in the upper aerodigestive tract. As an indicator of NO activity, neuronal NO synthase (nNOS) is an important marker at the nervous endplates of the salivary glands [7,8]. Inhibition of this enzyme by L-NAME and L-NMMA results in reduced saliva flow from the submandibular gland [9]. This is an indication that besides acetylcholine other transmitters, too, may be involved in regulating salivary gland function. It was the aim of the present study to show that the toxin also interferes with the metabolic actions of NO on investigated rat submandibular glands. Maybe we could detect that NO like the well-known acetylcholine and the second messengers cGMP and Ca^{2+} could be another messenger in the pathway of salivary gland secretion. Especially, investigations on the time course of duration of toxin effect were carried out in this study. Extended knowledge about the mode of botulinum toxin action may be helpful in its clinical application for reducing salivary flow in hypersalivation states [10,11].

2. Materials and methods

Adult female Wistar rats (200—360 g) were used in the experiments. The animals were fed a standard diet and had free access to food and water. To exclude hormonal influences, sexually mature rats were used. The day—night cycles were 12 h each and, in view of a possible circadian rhythm, all experimental manipulations were conducted between 2:00 and 4:00 p.m.

The animals were divided into two groups. The rats of group 1 (controls) were killed in carbon dioxide hyperkapnia by cervical dislocation. Both submandibular glands were excised and immediately placed in buffered 3.7% paraformaldehyde solution. The animals of group 2 (toxin-treated) were anesthetized by intraperitoneal injection of ketamine (50 mg/kg body weight) and xylazine (4 mg/kg body weight). The submandibular gland on each side was exposed by a cervical incision and 2.5 U botulinum toxin A (Botox®, Allergan, Irvine, CA, USA), reconstituted in 0.1 mL of physiological saline, was injected into the right gland; 0.1 mL saline was injected into the left gland (sham operation). Five, 7, 14, and 28 days later, the rats were sacrificed as described. Again, the submandibular glands were excised and immediately placed in buffered 3.7% paraformaldehyde. Altogether, eight glands from control animals and 32 glands from treated animals were investigated.

After dehydration and paraffin embedding, 8—10-mm serial sections were cut. For the demonstration of nNOS immunoreactivity, a rabbit polyclonal antiserum raised against human nNOS was used (Upstate Biotechnology, Lake Placid, USA) that specifically binds to the enzyme [12,13]. In a second step, a secondary peroxidase-labeled antibody was added (porcine anti-rabbit immunoglobulin, Dako, Hamburg, Germany). Binding of this antibody was shown by reaction with the peroxidase substrate diaminobenzidine (DAB), dissolved in Tris—HCl buffer, resulting in the formation of a brown product. Hemalum was used for counterstaining. To exclude non-specific staining, controls were made by omitting either the primary antiserum or the secondary antibody. Three randomly choiced sections of each gland were subject to further evaluation. The evaluation rater did not know about the treatment conditions and was blind to the fact whether he investigated a treated or a non-treated section. For the evaluation of staining