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Neuroscience Letters 381 (2005) 207–210
Neuroscience Letters
www.elsevier.com/locate/neulet

Plenary

Gene expression of myogenic regulatory factors following intramuscular injection of botulinum A toxin in juvenile rats

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Received 5 August 2004; received in revised form 27 September 2004; accepted 15 November 2004

Keywords: Botulinum toxin; Gene expression; Myogenic regulatory factors; Rats; Muscle regeneration

Myogenesis is regulated by myogenic regulatory factors (MRFs) of the MyoD family of basic helix-loop-helix proteins, such as MyoD, Myf5, MRF4, and myogenin. The MRFs encode for transcription factors capable of converting cultured non-muscle cells to a muscle phenotype [15]. Binding of MRFs to DNA initiates the transcription and regulates the expression of certain muscle-specific genes, such as myosin heavy and light chains, α-actin, or creatine kinase [8]. MyoD and Myf5 are early-acting factors, mainly involved in myoblast formation and in satellite cell proliferation during regeneration, while myogenin and MRF-4 are late-acting factors, expressed during muscle differentiation [4].

Myogenesis is characterized by the sequential expression of specific gene families, including those that mediate synaptic transmission at the neuromuscular junction, such as nicotinic acetylcholine receptor (nAChR). The nAChR is a pentameric complex of four homologous subunits with a molar stoichiometry of α_2, β, δ, and γ or ε [12]. Expression of the nAChR subunits and the distribution of the receptors among muscular fibers is regulated developmentally with nAChR mRNA levels at their highest levels during myogenic differentiation [2]. Control of nAChR gene transcription is crucial to the development and maintenance of synapses in muscle.

Members of the MyoD gene family are likely to play a central role in the regulation of nAChR subunit expression [3]. All the genes coding for nAChR subunits contain one or more E-boxes in their regulatory regions, to which the MRFs indifferently recognize and bind [7].

A widely used treatment modality for the management of spasticity in cerebral palsy patients is intramuscular injection of botulinum A toxin (BoNT-A), a neurotoxin produced by *Clostridium botulinum*, a facultative anaerobe [6]. BoNT-A injection causes flaccid muscular paralysis by preventing the release of acetylcholine from cholinergic nerve endings at the neuromuscular junction [5].

Currently, no information is available regarding the possible changes in expression of myogenic regulatory factor gene family proteins in mammalian skeletal muscles following intramuscular BoNT-A injection. The specific aim of this study was to use real-time RT-PCR to investigate the gene transcription changes for MRF4, MyoD, Myf5, and myogenin in rat gastrocnemius muscles, from three days to one year after intramuscular injection of BoNT-A.

Thirty-six Sprague–Dawley rats (one month of age) supplied by Harlan (Indianapolis, Indiana) were used. Animals were housed in the animal resources facility in a room with controlled temperature (20–22 °C) on a 12 h light/12 h dark cycle. Rat chow and water were provided ad libitum. All experimental procedures were approved by the Wake Forest University Animal Care and Use Committee according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. After anesthesia was induced, animals were taken to the operative suite. Using aseptic surgical technique, a small incision was made along the posterior aspect of the left hind limb to expose the gastrocnemius. Vials of lyophyllized botulinum toxin A (BOTOX®, Allergan, Irvine, CA, USA) were reconstituted with the instillation of 2 ml of

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0304-3940/$ – see front matter
doi:10.1016/j.neulet.2004.11.098

normal saline solution in a 100-unit vial (50 units/ml). The toxin was injected into the gastrocnemius muscle at a dosage of 6 units/kg body weight in injection volume of 10–30 μL with a Hamilton syringe (Fischer Scientific, Pittsburgh, PA, USA). Equal volumes of toxin were injected in both the medial and lateral heads of the muscle under direct visualization. After injection, the soft tissues and skin were closed with 4.0 vicryl suture. An equivalent volume of saline was injected into the right gastrocnemius muscle to serve as a contralateral control. Muscle samples were harvested from both hind limbs at 3, 7, 15, 30, 60, 90, 180, and 360 days after neurotoxin/vehicle injection. In addition, the gastrocnemius muscles from 3 one-month old rats that received no injections were harvested to serve as a normal, uninjected control group.

The PCR primers for MRF4, MyoD, Myf5, myogenin, and GAPDH used in the present study were the same as those reported in the literature [1,13]. Muscle samples were pulverized in liquid nitrogen, and mRNA was extracted using Trizol reagent. Residual genomic DNA was removed with DNAse I (0.4 μg/μg RNA) at 37 °C for 20 min. The enzyme then was inactivated with an inactivation agent (Ambion, Austin, TX, USA). The quality and quantity of the isolated RNA samples were assayed prior to their downstream applications. The prepared total RNA was reverse-transcribed by MMLV reverse transcriptase and random primers (Retroscript RT kit; Ambion, Inc). The resulting first strand cDNA was used as the template for the subsequent real-time PCR analysis. Real-time PCR assays were performed on a Bio-Rad iQ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) in a final volume of 25 μl containing 1× SYBR Green PCR Master Mix (Sigma) and primers at a final concentration of 200 nM. One microliter of each appropriately diluted sample (standard curve points and experimental samples) was added to 24 μl of the PCR master mixture. A typical quantification cycling protocol was: 95 °C for 10 min, followed by 35–40 cycles at 95 °C for 15 s, and 60 °C for 45 s. At the end of each run, a dissociation protocol was performed to ensure that only the specific PCR product was present. Experiments were performed with duplicates for each sample and with triplicates for each standard curve point. Each PCR run included the standard curve, established by serially diluted plasmid containing the target sequence and a no-template negative control. At the end of each real-time RT-PCR run, the presence of PCR products (amplicons) and the size of the corresponding fragments were subjected to analysis using DNA 1000 LabChip (Agilent). All mRNA levels measured by real-time PCR were normalized to GAPDH levels in each sample, respectively.

All values were expressed as the mean ± standard error of the mean. Comparisons between samples were performed using ANOVA (time series) and Student's unpaired *t*-test for gene expression studies. A probability value of less than 0.05 was considered significant.

Study results show that mRNA coding for MRF4 increased significantly at 3 days and peaked at 7 days, returning to both the contralateral and uninjected control levels 15 days after BoNT-A injection (Fig. 1, $^*p < 0.05$). Myogenin mRNA levels also increased significantly at 3 days and peaked at 7 days; however, a second peak occurred 60 days after toxin injection. Myogenin levels returned to control values 90 days after toxin injection (Fig. 2, $^*p < 0.05$). There was no significant

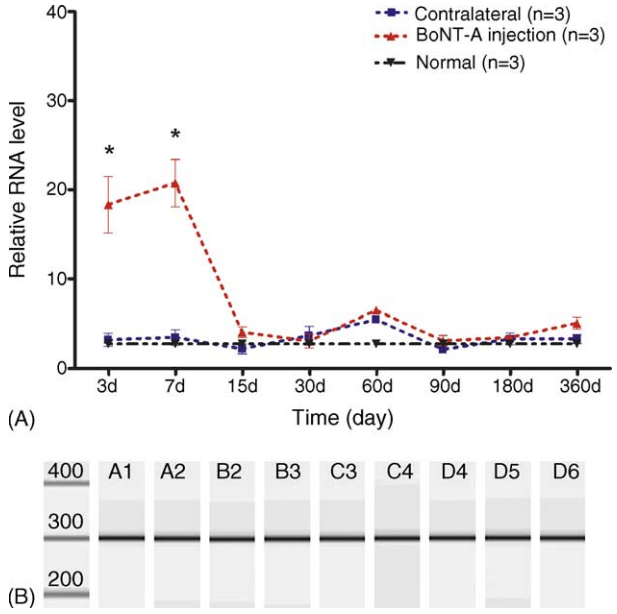

Fig. 1. (A) Time course of MRF4 expression following BoNT-A injection in juvenile rats ($n = 3$) at 6 units/kg of body weight ($^*p < 0.05$). (B) DNA capillary electrophoresis of PCR products on a DNA 1000 LabChip (Agilent). From left to right: lane 1, markers; lanes 2–10, samples from selected wells.

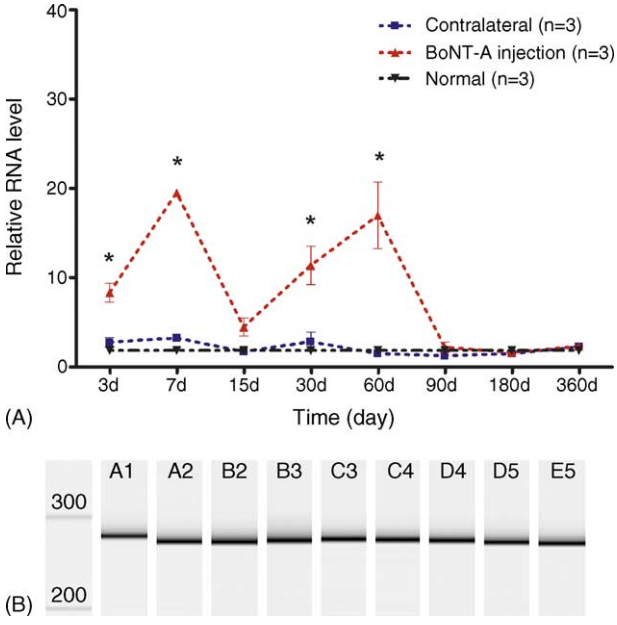

Fig. 2. (A) Time course of myogenin expression following BoNT-A injection in juvenile rats ($n = 3$) at 6 units/kg of body weight ($^*p < 0.05$). (B) DNA capillary electrophoresis of PCR products on a DNA 1000 LabChip (Agilent). From left to right: lane 1, markers; lanes 2–10, samples from selected wells.