学 位 論 文 の 要 旨

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学　位　論　文　名	Blocking Insulin-Like Growth Factor 1 Receptor Signaling Pathway Inhibits Neuromuscular Junction Regeneration After Botulinum Toxin-A Treatment
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論 文 内 容 の 要 旨

INTRODUCTION

Botulinum toxin-A (BTX) administration into muscle is an established treatment for conditions with excessive muscle contraction. BTX has effects at the neuromuscular junction (NMJ) which is a specialized synapse between motor nerve endings and their muscle fibers. However, botulinum therapy has short-term effectiveness, and high-dose injection of BTX could induce neutralizing antibodies against BTX. Therefore, prolonging its effects could be beneficial in a clinical situation. Insulin-like growth factor-1 receptor (IGF1R) and its ligands, insulin-like growth factor (IGF) -I and II, regulate the physiological and pathological processes of the nervous system. It has been suggested that IGF1R is involved in the process after BTX administration, but the specific regeneration mechanism remains unclear. Therefore, this study aimed to determine how inhibition of the IGF1R signaling pathway affects BTX-induced muscle paralysis.

MATERIALS AND METHODS

Male B6.Cg-Tg(Thy1-YFP)16jrs/j mice (Thy1-YFP mice) (The Jackson Laboratory, Bar Harbor, ME, USA) and C57BL/6J mice (SLC Co., Shizuoka, Japan) aged 6–8 weeks were used in this study. Thy1-YFP mice were used for analysis of the NMJ and C57BL/6J mice were used for muscular and satellite cells (SCs) analysis. All experiments with animals in this study were approved by the Animal Care and Use Committee of Shimane University and Shimane University Safety Committee for Recombinant DNA Experiments (IZ31-57, 58, IZ2-124, IZ4-11and protocol No.595).

BTX and anti-IGF1R antibodies were administered to the gastrocnemius (GC) muscle of mice. The control-IgG (cont IgG) antibody combination group was used for comparison. Muscle

paralysis was evaluated using a footprint test (FPT) and the tibial functional index (TFI). After the FPT procedure, the GC muscle was collected for immunochemical staining to evaluate the NMJ, muscle fiber area, and SCs. We quantified the protein and gene expression levels involved in the process of NMJ regeneration using immunoblotting and quantitative reverse transcription-polymerase chain reaction (qPCR). Statistical analyses were performed with unpaired t-tests, one-way or two-way analysis of variance (ANOVA) method followed by post-hoc Tukey's multiple tests using PRISM 9 software (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Western blot analyses were performed to examine the protein levels of IGF-I, IGF-II, IGF1R, and phosphorylated-IGF1R in the GC muscle 2 weeks following BTX treatment. All the protein levels compared to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) increased after BTX administration.

BTX treatment in the GC muscle induced nearly complete paralysis at 2 or 4 weeks, followed by a spontaneous recovery over 12 weeks. Paralysis lasted significantly longer with the addition of anti-IGF1R antibody, compared to BTX alone or BTX with cont IgG. With respect to the effects of sequential injections of anti-IGF1R or cont IgG, with or without a half dose of BTX administration, the TFI of the anti-IGF1R antibody alone group was not significantly different from that of the cont IgG alone group. In contrast, at 6 weeks after BTX injection, the TFI of the BTX+anti-IGF1R antibody group remained low, whereas that of the BTX cont IgG-treated group spontaneously recovered.

Immunohistochemical analysis of the GC muscle and NMJ revealed that IGF1R was highly expressed in the membrane of muscle fibers, $Pax7^{+}$-SCs, and NMJ. However, it was weakly expressed in the growing tip of the sprouting fiber.

The treatment of BTX caused morphological changes in the NMJ, leading to an increase in axonal sprouting fibers. Both presynaptic and postsynaptic components were significantly lower in the BTX-treated samples compared to naïve controls. The group treated with BTX+ anti-IGF1R antibody showed a lack of regeneration of both presynaptic and postsynaptic components compared with BTX+cont IgG group. However, the number of axonal inputs to the NMJ did not show significant differences between BTX+cont IgG and BTX+anti-IGF1R antibody groups. These results suggested that the NMJ, but not sprouting fibers, was regulated by the IGF1R signaling pathway.

The weight of the denervated GC muscle was significantly lower by approximately 40% than that of the contralateral control GC muscle at 2 weeks following BTX administration. At 6 weeks after BTX treatment, muscle weight in the cont IgG group was spontaneously recovered

to 64.2 ± 2.3% of the contralateral control muscle weight. In contrast, the anti-IGF1R antibody group did not show any recovery in muscle weight and maintained 44.7±1.5% of the contralateral muscle weight.

We subsequently performed a histological analysis of the cross-sectional area of the muscle fibers. The BTX-treated group had fewer muscle fibers with a larger cross-sectional area at 2 weeks. Interestingly, the distribution was spontaneously recovered after 6 weeks in the BTX+cont IgG group, while the BTX+anti-IGF1R antibody group showed a higher frequency of small muscle fibers with cross-sectional areas of 51–150 and 151–300 μm^2 and a lower frequency of muscle fibers with cross-sectional areas of >451 μm^2. These results suggested that BTX treatment induced neurogenic atrophy and that sequential treatment with anti-IGF1R antibody maintained the neurogenic atrophy induced by BTX.

The ratio of SCs in the GC muscle was not significantly different between the two groups at 2 weeks after treatment. However, the ratio of SCs was significantly higher in the BTX+anti-IGF1R antibody group than in the BTX+cont IgG group 6 weeks after treatment.

Denervation by BTX strongly activated the expression of total mTOR and Akt. In addition, phosphorylated mTOR (Ser2448) and S6 kinase were more significantly activated than total mTOR and S6 kinase. However, the light chain 3BII/light chain 3BI (LC3BII/ LC3BI) ratio was not changed. The relative expression of phosphorylated mTOR and S6 kinase was significantly affected by anti-IGF1R antibody administration. Also, the genes related to neural recovery, except Trim63/ MuRF1 and Chrne, were upregulated by BTX treatment, although none of them were affected by anti-IGF1R antibody treatment.

These results showed that anti-IGF1R antibody administration inhibited the recovery from BTX-induced neurogenic paralysis, and the synaptic components at the NMJ, mainly post-synaptic components, were significantly affected by the antibody. In addition, the wet weight or frequency distribution of the cross-sectional area of the muscle fibers was regulated by IGF1R, and sequential antibody administration following BTX treatment increased the number of $Pax7^+$-SCs in the GC muscle, independent of NMJ recovery. Moreover, BTX treatment upregulated the mTOR/S6 kinase signaling pathway, HDAC4, Myog, Fbxo32/MAFbx/Atrogin-1 pathway, and transcription of synaptic components, but not autophagy. Finally, IGF1R inhibition affected only mTOR/S6 kinase translational signaling in the GC muscle.

CONCLUSION

Anti-IGF1R antibody administration prolongs the effects of BTX by regulating the mTOR/S6 kinase signaling pathway and NMJ regeneration. The IGF1R signaling inhibition could be highly beneficial in clinical practice.