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Original Article

Ouabain exacerbates botulinum neurotoxin-induced muscle paralysis via progression of muscle atrophy in mice

Ryu Fujikawa[1], Yoshikage Muroi[1,2], Toshihiro Unno[1,3] and Toshiaki Ishii[1,2]

[1]Department of Pathogenetic Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[2]Department of Basic Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
[3]The Laboratory of Pharmacology, Faculty of Applied Biological Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

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ABSTRACT — Botulinum neurotoxin serotype A (BoNT/A) inhibits acetylcholine release at the neuromuscular junction in isolated muscles, and ouabain can partially block its effect. However, it is not clear whether ouabain attenuates BoNT/A-induced neuromuscular paralysis *in vivo*. In this work, we investigated the effects of ouabain on BoNT/A-induced neuromuscular paralysis in mice. Ouabain was administered to mice intraperitoneally immediately after a single injection of BoNT/A into skeletal muscle. The effects of ouabain on BoNT/A-induced muscle paralysis were assessed by quantitative monitoring of muscle tension and digit abduction via the digit abduction scoring (DAS) assay. A single administration of ouabain significantly prolonged BoNT/A-induced neuromuscular paralysis. Moreover, consecutive daily injection of ouabain exacerbated BoNT/A-induced neuromuscular paralysis, and led to a significant decrease in both twitch and tetanic forces as assayed in isolated BoNT/A-injected muscles. We next looked at the effects of ouabain on BoNT/A-induced muscle atrophy. Administration of ouabain led to a decrease in the myofibrillar cross-sectional area (CSAs) by 14 post-BoNT/A injection. In addition, repeated administration of ouabain increased mRNA expression levels of ubiquitin ligases, which are markers of muscle atrophy, in BoNT/A-injected muscle. These results suggest that ouabain exacerbates BoNT/A-induced neuromuscular paralysis via a marked progression of BoNT/A-induced muscle atrophy.

Key words: Ouabain, Botulinum neurotoxin, Muscle atrophy, MAFbx, MuRF-1

INTRODUCTION

Natural toxins have been used therapeutically in various contexts for many years. Nevertheless, there remains much to learn about how to best exploit natural toxins as therapeutics, including to minimize the risk of immunoresistance. Botulinum neurotoxin serotype A (BoNT/A), one of the most potent toxins known to human, has been the subject of a large number of studies aimed at understanding the acute and long-term effects of exposure to the toxin. At the molecular level, BoNT/A can cleave the C-terminus of the 25-kDa synaptosomal associated protein (SNAP-25) (Blasi *et al.*, 1993), which participates in the fusion of synaptic vesicles with the nerve terminal membrane, resulting in inhibition of neurotransmitter release (Duchen, 1970). Injection of BoNT/A into skeletal muscle prevents the release of acetylcholine from motor nerve terminals, leading to long-term paralysis (Duchen, 1970; Angaut-Petit *et al.*, 1990; Molgo *et al.*, 1990). BoNT/A induces muscle atrophy, which is characterized by a loss of muscle weight, a decrease in the cross-sectional area (CSAs) of the muscle (Morbiato *et al.*, 2007) and up-regulation of ubiquitin ligases in early phases of muscle atrophy (Gomes *et al.*, 2001).

BoNT/A has been utilized clinically as a treatment for hemifacial spasms, cervical dystonia and spasticity (Mahant *et al.*, 2000). Because repeated injections of BoNT/A are necessary in the course of treatment of patients with these disorders, resistance to the toxin may occur as a result of gradual immunization. It is important to find a synergist that can reinforce the effects of BoNT/A, as this may allow use of a lower dose of the toxin, thus

Correspondence: Toshiaki Ishii (E-mail: ishii@obihiro.ac.jp)

reducing the risk of development of BoNT/A immunoresistance.

Several reports have shown that calcium utilization is increased in BoNT/A-treated motor nerves (Santafé *et al.*, 2000; Wang *et al.*, 2004; Fujikawa *et al.*, 2008). Because calcium ions play an important role in the release of neurotransmitter from motor nerve terminals (Crawford, 1974), these observations may reflect adaptation to exposure to BoNT/A. By contrast, ouabain can lead to an increase in intracellular calcium levels and promote neurotransmitter release from motor nerve terminals *in vitro* (Erulkar, 1983; Maeno *et al.*, 1995). Treatment of BoNT/A-treated motor nerve terminals with ouabain has been reported to restore neurotransmitter release in isolated frog muscles (Molgo *et al.*, 1987). We were interested to determine whether ouabain has a similar effect *in vivo*. To address this, we examined the effects of ouabain on BoNT/A-induced neuromuscular paralysis in mice. We found that ouabain exacerbates BoNT/A-induced muscle paralysis *in vivo*.

MATERIALS AND METHODS

Animal care and adherence to accepted ethical standards

Adult male 7- to 8-week-old *ddY* mice were used in all experiments. Animals were maintained in cages at 24°C under a 12 hr light/dark cycle and allowed free access to food and water. All procedures for the care and use of experimental animals received prior approval from the Animal Research Committee in Obihiro University of Agriculture and Veterinary Medicine (OUAVM). The experiments were conducted following the Guidelines for Animal Experiments at OUAVM and the Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology in 1989. At the end of the experiment, the animals were humanely euthanized with an overdose of anesthetic ether.

Injection of botulinum neurotoxin

BoNT/A was purchased from Wako Pure Chemicals (Osaka, Japan) and diluted with 0.05 M acetate and 0.2 M NaCl (pH 6.0). The appropriate dose of BoNT/A, *i.e.*, levels that cause transient but complete paralysis without symptoms of botulism, was determined by testing twitch force and using the digit abduction scoring (DAS) assay (*i.e.*, levels sufficient to induce a score of 2 in the DAS assay). For the experimental DAS assays, each mouse was anesthetized with Avertin® and received a single intramuscular injection of 0.1 ng BoNT/A into the anterolateral region of the left hind leg. The protocol for tension recordings was the same except that 0.05 ng BoNT/A was injected into the central region of the soleus muscle of the left hind leg.

Media

To prepare media, we supplemented Krebs-Ringer solution with the following final concentrations of additional components: 136 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM $NaHCO_3$ and 11 mM glucose. The solution was bubbled with 95% O_2, 5% CO_2 at 37°C and maintained at pH 7.3. The temperature was held constant using an external water jacket and a thermoregulatory device (Thermominder Mini 80; Taiyo, Tokyo, Japan). The preparation was equilibrated in the appropriate bathing solution (20 ml) for at least 30 min prior to use in a treatment.

Tension recording from the hemidiaphragm muscle

For tension recordings, non-treated mice were first sacrificed and left hemidiaphragm preparations were isolated. Next, the end of the left hemidiaphragm corresponding to the site of insertion into the rib cage (along with the phrenic nerve) was secured. The central tendon was then attached to a force-displacement transducer (TB-652T; Nihon Kohden, Tokyo, Japan). The phrenic nerve was stimulated through a suction electrode connected to an electronic square-pulse generator (SEN-3201; Nihon Kohden) in standard bathing solution. The nerve trunk was stimulated with supermaximal voltage at 0.1 Hz. The resulting isometric twitches were recorded on a thermal array recorder (AD-100F; Nihon Kohden). The control tension was measured 5 min prior to BoNT/A treatment and every 10 min after administration of BoNT/A. Ouabain was added to a final concentration of 10 μM to the organ bath 5 min prior to administration of BoNT/A.

Tension recording of the soleus muscle

Mice were sacrificed 2 weeks after injection of BoNT/A for tension recordings. The soleus muscle was selected for the study because of its small mass and surgical accessibility. Mice injected with vehicle served as controls. Experimental mice were intraperitoneally injected once a day with ouabain (1 μmol/kg/day) from day 0 to day 14. The left soleus muscle (with the sciatic nerve) was removed on day 14. The distal end of the soleus muscle was secured and the other end was attached to a force-displacement transducer (TB-652T; Nihon Kohden). The sciatic nerve or muscle was stimulated with supermaximal voltage through a suction electrode connected to an electronic square-pulse generator (SEN-3201; Nihon Kohden)

in standard bathing solution. A pulse of electrical stimulation was applied to the nerve at 0.1-ms duration to elicit an indirect twitch contraction (nerve stimulation) and to the muscle at 1-ms duration to elicit a direct twitch contraction (muscle stimulation). In some cases, the muscle was repetitively stimulated at 100 Hz to produce tetanus contraction. Mechanical responses were recorded on a thermal array recorder (AD-100F; Nihon Kohden).

DAS assay

For the DAS assay, BoNT/A was injected into the mouse left hind leg and functional recovery was monitored as described previously (Aoki, 2001). In this assay, mice were suspended by their tails to elicit a characteristic startle response in which the animal extends its hind limbs and abducts its hind digits. Following neurotoxin injection, the degree of digit abduction was scored on a five-point scale, wherein a score of 0 indicates normal and a score of 4 indicates maximal reduction in digit abduction and leg extension.

Immunostaining

Soleus and gastrocnemius muscles were dissected and fixed with neutral-buffered 4% paraformaldehyde solution. After fixation, the muscles were cut on an oscillating tissue slicer into 50-μm sections. The sections were permeabilized with 0.5% (v/v) Triton X-100 in phosphate buffered saline (TPBS) for 1 hr and then blocked in TPBS containing 2.0% normal goat serum for 1 hr. After blocking, the sections were incubated for 24 hr at 4°C in mouse anti-dystrophin monoclonal antibody (Novocastra, Newcastle, UK; 1:500 in TPBS). After rinsing in TPBS, the sections were incubated for 24 hr in AlexaFluor® 488 goat anti-mouse IgG (Invitrogen, Tokyo, Japan; 1:1,000 in TPBS). After washing with PBS, sections were mounted onto slides and coverslipped with fluorescent mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). Images were obtained by confocal laser scanning microscopy (Nikon, Tokyo, Japan). CSAs were measured using Image J software (Rasband, W.S., Image J, U. S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997-2010).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Mice were anesthetized and sacrificed, and left gastrocnemius muscles were dissected and frozen in liquid nitrogen for RT-PCR. Total RNA was isolated from the muscle tissue using TRIZOL (Invitrogen) and then quantified by measuring the absorbance at 260 nm. The mRNA expression levels of muscle atrophy F-box (MAFbx), muscle ring finger 1 (MuRF1) and the ubiquitously expressed gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantified by RT-PCR. Total RNA (250 ng) was reverse-transcribed using an oligo dT primer and AMV reverse transcriptase with a Takara RNA LA PCR™ Kit (AMV) v1.1 (Takara Shuzo, Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNA products were amplified using primers for mouse MAFbx (sense, GACTGGACTTCTCGACTGCC; antisense, TCAGCCTCTGCATGATGTTC); MuRF1 (sense, CAACCTGTGCCGCAAGTG; antisense, CAACCTCGTGCCTACAAGATG); or GAPDH (sense, GGGTGGAGCCAAACGGGTC; antisense, GGAGTTGCTGTTGAAGTCGCA). The PCR reactions were carried out in a Bio-Rad I cycler (Bio-Rad, Tokyo, Japan).

Analysis of MAFbx, MuRF1 and GAPDH cDNAs

Amplified cDNAs were separated by 3.0% agarose gel electrophoresis, stained with SYBR Green (Takara Shuzo), and quantified using an Epi-Light UV FA500 analyzer (Aishin Seiki, Tokyo, Japan) and Image J software. Relative mRNA levels were determined by comparing the fluorescence intensity determined for each gene to that of the control, GAPDH.

Statistical methods

Data were analyzed using the Student's t-test after applying an F test (data reported in Figs. 1, 3 and 5) or using the Mann-Whitney test (data reported in Fig. 2). For the data reported in Fig. 4, data were analyzed using the Kolmogorov-Smirnov test. Probabilities less than 0.05 were considered statistically significant.

RESULTS

To investigate the effects of ouabain on BoNT/A-induced acute paralysis, we first measured the contractility of the phrenic nerve diaphragm *in vitro* following BoNT/A addition in the presence or absence of ouabain. To do this, the phrenic nerve diaphragm preparation was pretreated with 10 μM of ouabain for 5 min and then several doses of BoNT/A were administered. We chose to use 10 μM ouabain as this concentration has been reported as a minimum level that leads to a slow exponential increase in the transmitter release via elevation of intracellular calcium (Maeno *et al.*, 1995). Next, the phrenic nerve was electronically stimulated at 0.1 Hz and muscle contraction was recorded until contraction had stopped completely. BoNT/A inhibited twitch forces elicited by nerve stimula-

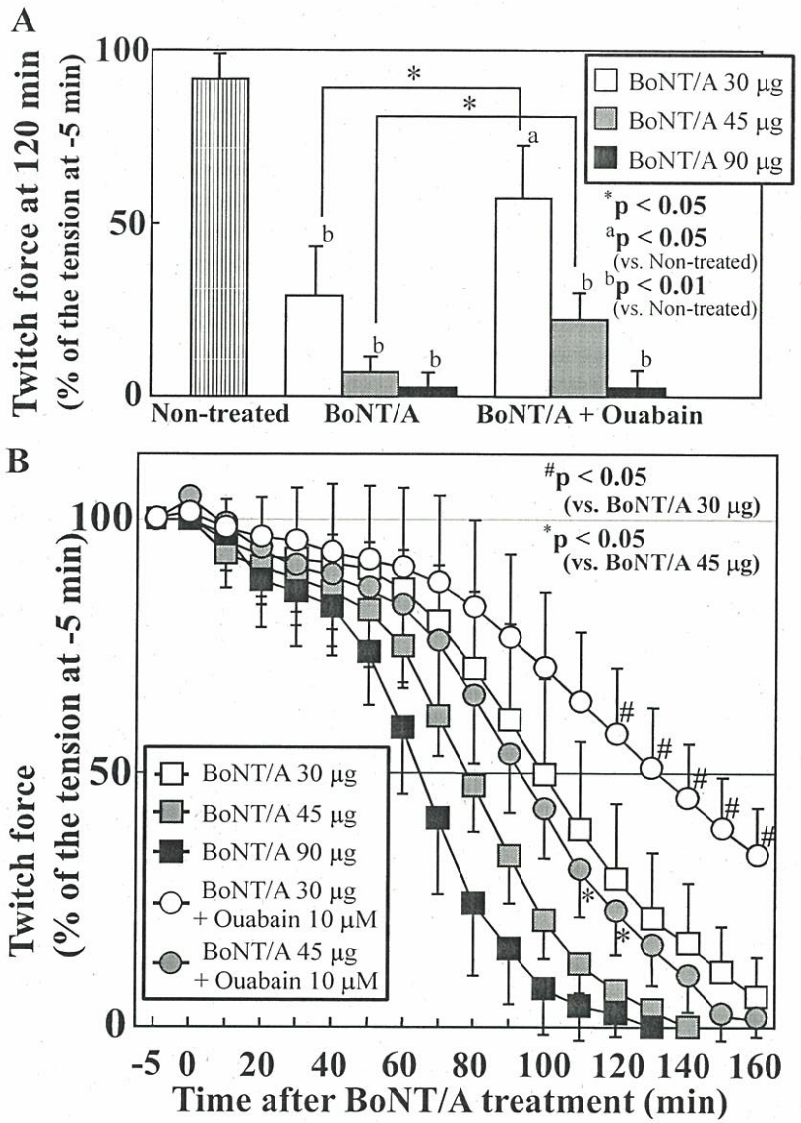

Fig. 1. Effect of ouabain on BoNT/A-induced inhibition of neurotransmission in the murine hemidiaphragm. Dissected left hemidiaphragm samples (see Materials and Methods) were trimmed to 4 mm wide and motor nerves were stimulated at 0.1 Hz. BoNT/A and ouabain were administrated in an organ bath at 0 and -5 min, respectively. Twitch forces were measured at 120 min (A) and every 10 min (B) after various concentrations of BoNT/A was administrated in the presence or absence of 10 μM ouabain. Data are presented as the percentage as compared with levels in pre-treated muscle (-5 min). Results represent means ± S.D. from six preparations. $^{*}P < 0.05$ as compared between BoNT/A treatment in the presence and the absence of ouabain treatment. $^{a}P < 0.05$ and $^{b}P < 0.01$ vs. non-treated.

tion in a dose- and time-dependent manner (Figs. 1A and B). By contrast, pretreatment of ouabain partially blocked the effects of treatment with 30 and 45 μg of BoNT/A but not a higher dose (90 μg; Figs. 1A and B). Thus, ouabain can attenuate BoNT/A-induced inhibition of muscle contraction *in vitro*.

To evaluate BoNT/A-induced paralysis *in vivo*, we assessed muscle paralysis using a DAS assay. To do this, we injected 0.1 or 0.2 ng BoNT/A into the left gastrocnemius muscle at day 0 and measured the DAS daily. We

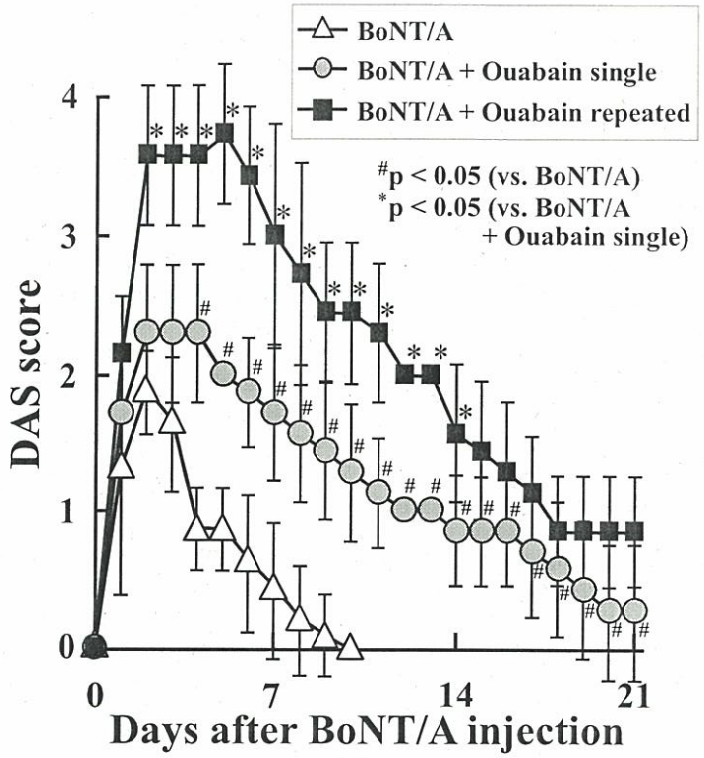

Fig. 2. Effect of ouabain on muscular paralysis following BoNT/A injection. Mice were anesthetized and 0.1 ng BoNT/A was injected into the left gastrocnemius muscle on day 0. Paralysis of the left hind limbs was assessed by the DAS assay once a day. Mice were also injected intraperitoneally with 1 μmol/kg ouabain on day 0 (ouabain single dose) or daily starting on day 0 and continuing through day 6 (ouabain repeated doses). Results represent means ± S.D. from eight mice. $^{\#}P < 0.05$ as compared with treatment with only BoNT/A. $^{*}P < 0.05$ as compared with BoNT/A + ouabain single dose treatment.

found that injection of 0.1 ng BoNT/A induced incomplete paralysis of the hind limb muscle. The DAS score reached level 2 on day 2 following a single injection of 0.1 ng BoNT/A, decreased over time, and finally reached level 0 on day 10 (Fig. 2). On the other hand, a single injection of 0.2 ng BoNT/A induced severe paralysis in which the DAS score reached a maximum of 4 on day 2, decreased to 2 by day 7, and thereafter remained at a value of 2 until day 21 (data not shown). A representative DAS dose-response curve obtained in this study is consistent with data presented in a previous report (Aoki, 1999, 2001).

To assess the effects of ouabain on BoNT/A-induced muscle paralysis *in vivo*, 1 μmol/kg of ouabain was injected intraperitoneally following BoNT/A injection and DAS scores were determined daily. Because administration of 1 μmol/kg ouabain alone did not influence the DAS score or result in general changes in behavior, we chose 1 μmol/kg ouabain treatment for these studies. A single administration of ouabain following intramuscular injection of BoNT/A significantly prolonged BoNT/A-induced neuromuscular paralysis and delayed recovery from paralysis, whereas the maximal DAS score was not significantly changed (Fig. 2). In contrast, consecutive daily injection of ouabain for 7 days significantly increased the maximal DAS score and prolonged paralysis (Fig. 2). Moreover, the DAS score remained at a peak level during the period of ouabain administration. Recovery following paralysis was not significantly different between mice treated with a single injection and consecutive daily injections of ouabain. Thus, ouabain exacerbates BoNT/A-induced muscle paralysis.

To further analyze the effects of ouabain on BoNT/A-induced muscle paralysis, we next measured the contractile responses elicited by electrical stimulation of motor nerves or muscles in isolated soleus muscles treated with

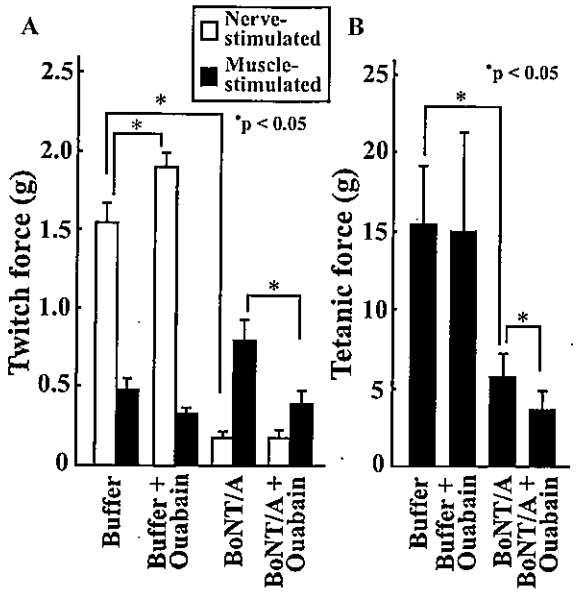

Fig. 3. Effect of ouabain on the twitch force of BoNT/A-treated soleus muscles. Mice were anesthetized and 0.05 ng BoNT/A was injected into the left soleus muscle. Ouabain (1 μmol/kg) was injected intraperitoneally once a day for 2 weeks. Next, the soleus muscle was dissected and twitch forces elicited by electric stimulation on motor nerve or muscle at 0.1 Hz were measured (A). Tetanic forces were measured with electric stimulation of the muscle at 100 Hz (B). Sodium acetate buffer was injected into the soleus muscle as a control. Results represent means ± S.E. of six preparations (A) and means ± S.D. of six preparations (B). $*P < 0.05$.

BoNT/A, with or without ouabain treatment. Toward that goal, we injected 0.05 ng BoNT/A, a dose sufficient to induce paralysis, into the left soleus muscles and then intraperitoneally injected ouabain once a day for a total period of 2 weeks. BoNT/A-treated soleus muscles were isolated after the course of the treatment, and contraction induced by an electrical stimulation to the muscles and motor nerves was then measured. In some experiments, we conducted electrical stimulation with a high frequency (100 Hz) in order to induce a response that made it possible to measure the tetanic force. We found that a single injection of BoNT/A leads to a significant decrease in muscle contraction induced by nerve stimulation and that only administration of ouabain for 14 days increases muscle contraction (Fig. 3A). However, daily administration of ouabain for 14 days after a single injection of BoNT/A did not prevent a BoNT/A-induced decrease in contraction (Fig. 3A). In contrast, a single injection of BoNT/A led to a significant increase in muscle contraction induced by direct muscle stimulation, the reason of which remains unknown, and repeated administration of ouabain prevented a BoNT/A-induced increase in contraction (Fig. 3A). However, ouabain alone did not affect induction of contraction by direct muscle stimulation (Fig. 3A). In addition, a single injection of BoNT/A also led to a significant decrease in contraction in response to tetanic muscle stimulation (Fig. 3B). Although administration of ouabain alone did not affect muscle contraction induced by tetanic stimulation, repeated administration of ouabain after a single injection of BoNT/A resulted in a further decrease in muscle contraction (Fig. 3B).

Based on these data, we hypothesized that ouabain promotes muscle atrophy by decreasing muscle contractions. To test this hypothesis, we next investigated the effects of ouabain on BoNT/A-induced structural changes in skeletal muscles. CSAs of BoNT/A-treated soleus and gastrocnemius muscles were assessed using immunostaining of dystrophin (Figs. 4A and B). Dystrophin is a cytoskeletal protein located on the intracellular side of the plasma membrane of muscle fibers. Comparisons of the frequency and distribution of CSA in soleus and gastrocnemius muscles are shown in Figs. 4C and D, respectively. A single injection of BoNT/A significantly decreased the CSA in both soleus and gastrocnemius muscles, and repeated treatment of ouabain led to a further decrease in the CSA in both BoNT/A-injected soleus and gastrocnemius muscles (Figs. 4C and D). However, ouabain did not exert any effects on control, buffer-injected muscles.

Next, we measured the mRNA expression levels for the genes that encode MAFbx and MuRF1 ubiquitin ligases. These two genes are up-regulated with a progression of muscle atrophy (Gomes *et al.*, 2001) and the increase in mRNA expression levels of the genes is used as markers of muscle atrophy (Glass, 2005; Velders *et al.*, 2008). We tested mRNA levels of the genes in BoNT/A-injected gastrocnemius muscles with or without an intraperitoneal injection of ouabain. Treatment with BoNT/A led to an increase in expression levels of MAFbx but not MuRF1 mRNA 1 week after injection (Figs. 5A and C). However, the BoNT/A-induced increase in MAFbx mRNA levels was not observed 2 weeks after BoNT/A injection (Figs. 5B and D), suggesting that BoNT/A injection exerts a transient response. By contrast, treatment with ouabain led to an increase in mRNA levels of both MAFbx and MuRF1 in BoNT/A-injected gastrocnemius muscles 1 week after injection. Treatment with ouabain alone did not affect MAFbx or MuRF1 mRNA levels. Moreover, we saw no significant differences among the control and experimental groups 2 weeks after BoNT/A

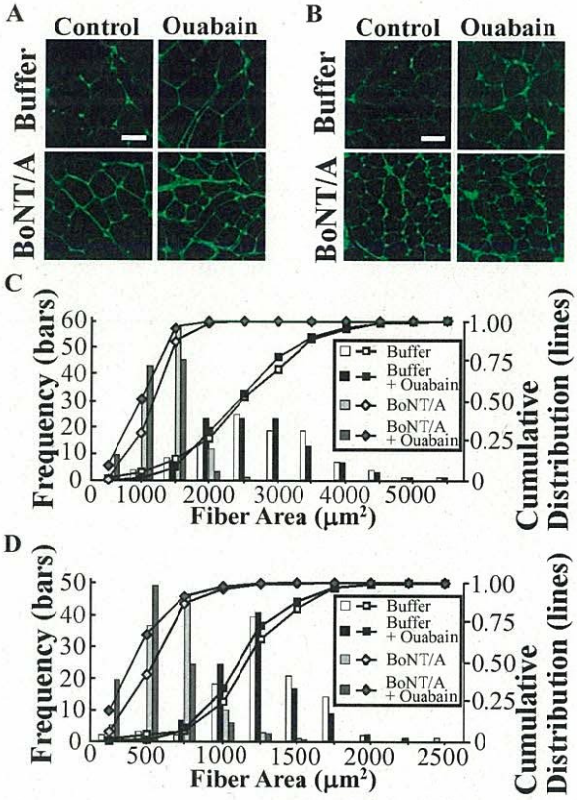

Fig. 4. Effect of ouabain on BoNT/A-induced muscle atrophy of the gastrocnemius (A, C) and soleus muscle (B, D). Mice were anesthetized, and 0.1 ng BoNT/A was injected into the left gastrocnemius muscle or 0.05 ng BoNT/A was into the left soleus muscle. Ouabain (1 μmol/kg) was injected intraperitoneally once a day for a week. Two weeks after BoNT/A injection, the gastrocnemius and/or soleus muscles were dissected, fixed and immunolabeled with anti-dystrophin antibodies. Images were obtained by confocal laser scanning microscopy (A and B). Scale bar, 50 μm. Image J software was used to determine the frequency and cumulative distribution of positive signal and the total area in tissue cross-sections of the gastrocnemius (A, C) and soleus muscle (B, D). The cumulative distribution is reported as the sum of the frequency of CSA smaller than a specific area for each muscle. A single injection of BoNT/A significantly decreased the CSA in both soleus and gastrocnemius muscles ($P < 0.05$, by the Kolmogorov-Smirnov test for a difference in the distribution), and repeated treatment of ouabain led to a further decrease in the CSA in both BoNT/A-injected soleus and gastrocnemius muscles ($P < 0.05$, by the Kolmogorov-Smirnov test for a difference in the distribution).

injection (Fig. 5D). Thus, ouabain appears to transiently stimulate expression of the two ubiquitin ligases, MAFbx and MuRF1, within 1 week after a single injection of BoNT/A.

DISCUSSION

Here, we have shown that ouabain exacerbates BoNT/A-induced muscle paralysis via a marked progression of BoNT/A-induced muscle atrophy, a result that may impact approaches to treatment of muscle-related disorders with BoNT/A.

As we found that *in vitro*, pretreatment with ouabain partially blocks the effects of BoNT/A in the hemidiaphragm muscle, it seemed reasonable to propose that ouabain might increase intracellular calcium concentrations in motor nerves and help the recovery of neurotransmitter release in BoNT/A-treated motor nerve terminals. In contrast to the *in vitro* results, a single administration of ouabain significantly prolonged BoNT/A-induced neuromuscular paralysis and consecutive daily injection of ouabain exacerbated the BoNT/A-induced neuromuscular paralysis *in vivo* (Fig. 2). Moreover, consecutive daily injection of ouabain led to a significant decrease in both twitch and tetanic forces as assayed in isolated BoNT/A-injected muscles (Fig. 3). These results suggest that ouabain acts as a synergist that reinforces the effect of BoNT/A *in vivo*. Therefore, it is possible that ouabain could be useful as a co-treatment that allows for administration of lower levels of and/or less frequent administration of BoNT/A.

Ouabain leads to an increase in neurotransmitter release from motor nerve terminals (Maeno *et al.*, 1995). Therefore, ouabain might also lead to an increase in the neurotransmitter release from motor nerve terminals, resulting in the increase of the contractility diaphragm *in vitro* (Fig. 1). On the other hand, repeated administration of ouabain increased nerve-stimulated but not muscle-stimulated twitch force in buffer-injected soleus muscle (Fig. 3A). This result also indicates that ouabain leads to an increase in the neurotransmitter release from motor nerve terminals. In contrast to *in vitro*, ouabain did not attenuate BoNT/A-induced inhibition of twitch force by nerve stimulation (Fig. 3). It is considered that the inhibitory effect of BoNT/A prevails over the stimulatory effect of ouabain on the neurotransmitter release from motor nerve terminals. Repeated administration of ouabain after a single injection of BoNT/A resulted in a further decrease in tetanic force induced by muscle stimulation (Fig. 3B). The result implies that ouabain may affect muscle other than motor nerve terminals and lead to a promotion of muscle atrophy in BoNT/A-injected soleus muscle

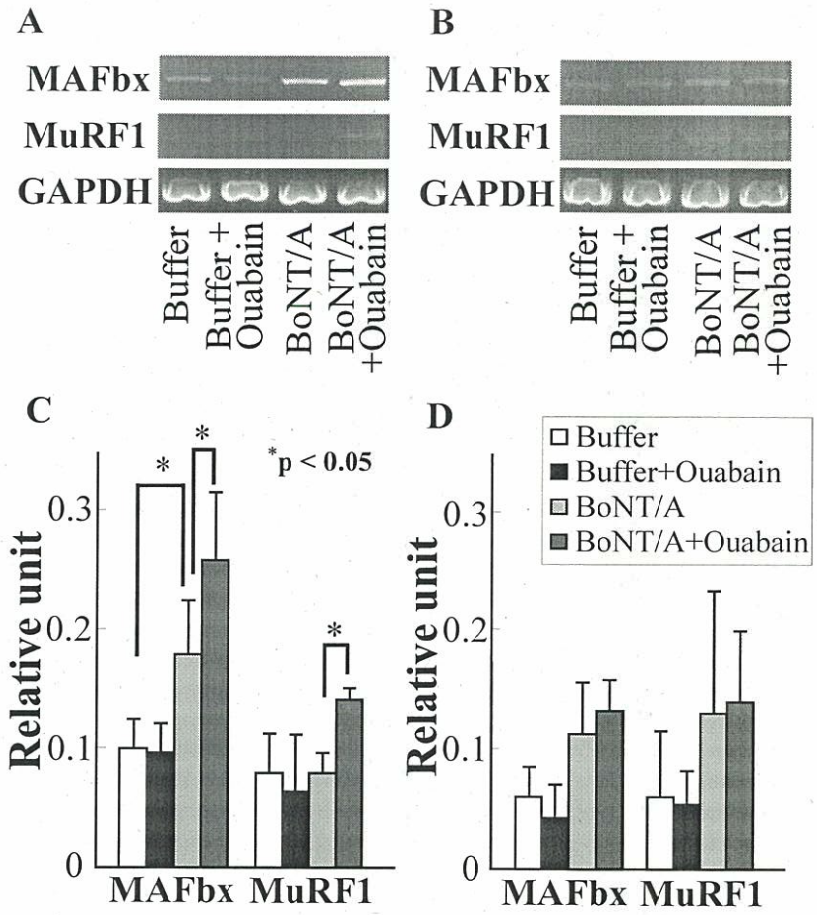

Fig. 5. Effect of ouabain on the expression levels of MAFbx and MuRF1 mRNAs in BoNT/A-injected gastrocnemius muscle. Mice were anesthetized and 0.1 ng of BoNT/A was injected into the left gastrocnemius muscle. Ouabain (1 μmol/kg) was injected intraperitoneally once a day for 1 week. The levels of MAFbx and MuRF1 mRNAs, which are indicators of muscle atrophy, were measured in BoNT/A-injected gastrocnemius muscles by RT-PCR 1 week (A) or 2 weeks (B) after injection. The mRNA levels of these ubiquitin ligases are shown as the signal relative to that for GAPDH. Results represent means ± S.D. from three preparations. $*P < 0.05$.

(Figs. 4 and 5), resulting in a decrease in tetanic contraction (Fig. 3).

On the contrary to tetanic force, a single injection of BoNT/A increased twitch force in soleus muscle (Fig. 3A). The reason remains unknown. However, it has been reported that BoNT/A-injection or denervation increases twitch force in diaphragm muscle (Zhan and Sieck, 1992; Shindoh *et al.*, 1994; Fujikawa *et al.*, 2008). Moreover, denervation decreases fast-twitch muscle fibers and increases slow-twitch muscle fibers in diaphragm muscle (Lewis *et al.*, 1996). We previously reported that the proportion of muscle fibers may affect twitch force in diaphragm muscle (Fujikawa *et al.*, 2008). BoNT/A is also known to decrease fast-twitch muscle fibers and increases slow-twitch muscle fibers in skeletal muscles (Dodd *et al.*, 2005). Therefore, BoNT/A-induced change in the proportion of slow- and fast-twitch muscles may lead to an increase in twitch force in soleus muscle (Fig. 3A).

Because BoNT/A causes muscle atrophy (Morbiato *et al.*, 2007), we further examined the effect of ouabain on BoNT/A-induced muscle atrophy. Histological characteristics of muscle atrophy include decrease in muscle fiber size, increase in plasma lamella, and decrease in the number of muscle fibers (Morbiato *et al.*, 2007; Chockalingam *et al.*, 2002). The data presented here show that, consistent with previous reports (Morbiato *et al.*,

2007; Chockalingam *et al.*, 2002), intramuscular injection of BoNT/A leads to a significant decrease in the size of muscle fibers of both the soleus and gastrocnemius muscles. Although ouabain treatment alone did not affect CSAs in buffer-injected muscles, consecutive daily injection of ouabain in BoNT/A-injected muscles did lead to a further decrease in CSAs. These results suggest that ouabain exacerbates BoNT/A-induced muscle atrophy.

Several previous studies suggest that down-regulation of insulin-like growth factor (IGF-1)/PI3K/Akt signaling can lead to muscle atrophy (Bodine *et al.*, 2001). Indeed, inhibition of PI3K and expression of a dominant-negative Akt reduced the mean size of myotubes in differentiated C2C12 cells (Rommel *et al.*, 2001). Moreover, muscles from mice lacking Akt1 and Akt2 showed muscle hypoplasia (Peng *et al.*, 2003). On the other hand, in cultured myotubes, inhibition of PI3K stimulates protein degradation and expression of MAFbx mRNA, which is invariably induced in muscle atrophy (Sacheck *et al.*, 2004). These reports suggest that activation of the IGF-1/PI3K/Akt pathway suppresses protein degradation and MAFbx mRNA expression, leading to protection from muscle atrophy. Members of the forkhead box O (Foxo) class of transcription factors, downstream targets of the PI3K/Akt pathway, are thought to help up-regulation of MAFbx and MuRF1 mRNA transcription (Sandri *et al.*, 2004; Zhao *et al.*, 2005; Waddell *et al.*, 2008). The MAFbx and MuRF1 genes encode E3 ubiquitin ligases, which mediate ubiquitination of distinct protein substrates (Winston *et al.*, 1999; Yamao, 1999; Bodine *et al.*, 2001). Ouabain reportedly induces hypertrophy by activating the PI3K/Akt and MEK/Erk pathways in cultured cardiac muscles and myotubes (Kometiani *et al.*, 1998; Liu *et al.*, 2007). In this study, however, we showed that ouabain leads to a decrease in the size of muscle fibers of BoNT/A-injected muscles and an increase in the expression levels of both MAFbx and MuRF1 mRNAs. These results suggest that ouabain might have different effects on BoNT/A-treated versus untreated muscles. Muscle atrophy can occur for a variety of reasons, including denervation and disuse (Lecker *et al.*, 1999). However, it is currently not known if ouabain exacerbates muscle atrophy induced by sources other than BoNT/A treatment.

BoNT/A-induced up-regulation of MAFbx mRNA and ouabain-induced increases in both MAFbx and MuRF1 mRNAs were observed at 1 week after treatment with BoNT/A but not at 2 weeks post-injection (Fig. 5C). On the other hand, there were no significant differences in the mRNA levels of both MAFbx and MuRF1 among the control and experimental groups 2 weeks after BoNT/A injection (Fig. 5D). Moreover, we found that injection of BoNT/A alone and repeated injection of ouabain result in muscle atrophy with a decrease in the size of muscle fibers 2 weeks after BoNT/A injection (Figs. 4A and B). It has been reported that expression levels of both MAFbx and MuRF1, which play key roles in the generation of muscle atrophy, are up-regulated only in the early phases of the muscle atrophy process, prior to muscle weight loss (Gomes *et al.*, 2001). This suggests that a transient increase in MAFbx and MuRF1 mRNA levels in the early phases of muscle atrophy process (*i.e.*, 1 week after BoNT/A injection) recovers to basal levels in later stages (*i.e.*, by 2 weeks after BoNT/A injection). In addition, a single injection of BoNT/A alone significantly increased MAFbx but not MuRF1 mRNA by 1 week after injection. Although ouabain treatment alone did not affect these mRNAs, administration of ouabain led to significant increases in both MAFbx and MuRF1 mRNAs 1 week after BoNT/A injection. These results suggest that ouabain might promote the progression of muscle atrophy via further stimulation of BoNT/A-induced mRNA induction of MAFbx and MuRF1. However, why upregulation of MuRF1 mRNA was not observed 1 week after injection with BoNT/A alone remains unclear.

Repeated administration of BoNT/A is used as a clinical treatment for muscle-related disorders. This can unfortunately lead to gradual immunization and resistance; indeed, therapeutic injection of BoNT/A has been reported to induce immunological responses (Doellgast *et al.*, 1997). Our results suggest that co-treatment with ouabain might minimize the number and/or levels of BoNT/A injection necessary to elicit the same therapeutic result, thus also minimizing the risk of developing BoNT/A immunoresistance.

In summary, we have shown that ouabain exacerbates BoNT/A-induced muscle paralysis, likely via increasing the expression of ubiquitin ligases. Further studies are required to elucidate how ouabain can stimulate expression of ubiquitin ligases.

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