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Continuous administration of poloxamer 188 reduces overload-induced muscular atrophy in dysferlin-deficient SJL mice

Naoki Suzuki[a,*], Tetsuya Akiyama[a], Toshiaki Takahashi[b], Hazuki Komuro[a], Hitoshi Warita[a], Maki Tateyama[a], Yasuto Itoyama[c], Masashi Aoki[a]

[a] Department of Neurology, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Japan
[b] Department of Neurology, National Nishitaga Hospital, Sendai, Japan
[c] National Center Hospital, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan

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ABSTRACT

Dysferlin-deficient SJL mice are commonly used to study dysferlinopathy. We demonstrated that poloxamer 188 (P188), a membrane sealant, is effective in reducing the loss of muscle mass in SJL mice when administered using an osmotic pump for 6 weeks. We did not observe significant changes over a 2-week administration period, suggesting that longthier observation is necessary to determine the effectiveness of P188. We also examined exercise endurance in P188-administered SJL mice using a rolling cage. Phosphorylated p38 was found to be reduced in P188-administered SJL mice; additionally, using microarray analysis, we found diminished expression of atrogin-1, an E3 ubiquitin ligase, as the effector of muscular atrophy. Chronic infusion of P188 to dysferlin-deficient SJL mice reduced muscular atrophy, and administering p38 and atrogin-1 in the gastrocnemius muscle improved its motor function. These results provide a basis for potential treatments for dysferlin-deficient skeletal muscle fibers.

1. Introduction

Mutations in the dysferlin gene cause several phenotypes of muscular dystrophy, called "dysferlinopathies"; these include limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy (MM), and distal anterior compartment myopathy (DACM) (Aoki et al., 2001; Bashir et al., 1998; Illa et al., 2001; Klinge et al., 2010; Liu et al., 1998; Paradas et al., 2010; Rosales et al., 2010; Takahashi et al., 2003). Dysferlinopathies are autosomal recessive diseases, and different phenotypes are observed even within the same family (Illarioshkin et al., 2000; Saito et al., 2007). Dysferlin deficiency has been proven to lead to defective membrane resealing in skeletal muscle and muscle necrosis (Bansal et al., 2003).

SJL mice, which display dysferlin deficiencies (Bittner et al., 1999) and inflammatory muscle changes (Suzuki et al., 2005), are commonly used to study dysferlinopathy. The spontaneous myopathy of SJL mice begins at 4–6 weeks of age and is nearly complete by eight months of age. The mutation in SJL mice is an in-frame deletion of 171 base pairs of the dysferlin gene. Assuming that the structure of the gene is similar in mice and humans, this in-frame deletion predicts that in human mutations 57 amino acids of dysferlin protein, including most of the fourth C2 domain, would be absent.

Poloxamer 188 (P188) is a stable 8.4 kDa amphiphilic polymer that localizes in lipid monolayers (Wu et al., 2005) and damaged regions of membranes (Maskarinec et al., 2005). When applied to injured cells, P188 repairs disrupted membranes and enhances the recovery of skeletal muscle (Lee et al., 1992), fibroblasts (Merchant et al., 1998), and the spinal cord (Borgens et al., 2004) following a variety of injury-inducing protocols. Chemically based membrane sealants have been acutely tested in mdx mice and have recently been proposed as a new therapeutic approach for cardiac membrane stabilization in muscular dystrophy (Yasuda et al., 2005). This treatment modality aims to directly seal the membrane tears that occur in the absence of dystrophin. Cases of Duchenne muscular dystrophy (DMD) in mdx mice showed that application of the membrane sealant poloxamer 188 (P188) confers acute cardiac protection. Additionally, the chronic infusion of severely affected dystrophic dogs with membrane-sealing poloxamer reduced myocardial fibrosis and fully prevented left ventricular remodeling (Townsend et al., 2010). The effects of P188 on other types of muscular dystrophy have not been examined.

Our aim was to determine the effects of P188 when administered with an osmotic pump for 6 weeks in a mouse model of dysferlinopathy. Using SJL mice, we found that p38 and atrogin-1 played

* Corresponding author. Tel.: +81 22 717 7189; fax: +81 22 717 7192.
E-mail address: naoki@med.tohoku.ac.jp (N. Suzuki).

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key roles in the influence of P188 on the progression of muscular atrophy induced by overloading.

2. Materials and methods

2.1. Animals

All mice were handled according to approved animal protocols in our institution. Gastrocnemius and tibialis anterior muscles were dissected from female control mice (SWR) and SJL (SJL/JOrllcoCrj) mice (Charles River Japan Inc., Yokohama, Japan) ($n = 10$ and 50, respectively). Prior to this study, we had examined the muscles of both strains of mice to investigate the muscular pathology and progression of myopathy (Suzuki et al., 2005). In 2-month-old SJL mice, there were slight or no muscle changes compared with the control mice. In contrast, 9-month-old SJL mice had an increased variety of fiber diameters, degenerating fibers, regenerating fibers, and increased inflammatory responses. With those results in mind, mice within the 19- to 25-week age range were chosen for use.

2.2. Creatine kinase (CK) determination

Blood (200 μL) was collected in Eppendorf tubes using cardiac puncture under deep anesthesia and allowed to clot at room temperature prior to centrifugation and serum collection. CK determination was performed according to the manufacturer's instructions using a standard spectrophotometric method. The data were expressed as units per liter.

2.3. Reagents and osmotic pump

19-Week-old mice were operated upon. Sterile saline was injected into the control mice. The total dose of poloxamer 188 (P188; Sigma–Aldrich, MO) was 20 mg, infused continuously for 42 days. The dosage was modified from that used in previous reports (Ng et al., 2008; Quinlan et al., 2006; Yasuda et al., 2005). The osmotic pumps (Alzet osmotic pump 2006, Alzet, CA) were incubated in sterile saline at 37 °C for 40 h to attain a constant flow rate prior to use. The pumps were filled to capacity with either P188 or sterile saline using a filling needle. SJL and SWR mice (19 weeks old) were anesthetized using intraperitoneal Nembutal. The skin between the scapulas was incised, and the pumps were implanted in the subcutaneous pocket. The normal pumping rate was 0.15 μL/h for 6 weeks. The reservoir volume was 200 μL, and the weight of each osmotic pump was 1.1 g. The mice were sampled at 25 weeks old, with body weights between 25 and 30 g.

2.4. Functional tests

Grip strength of the forearms was assessed using a grip strength meter (GPM-100, MelQuest, Japan) according to the manufacturer's instructions. 25-Week-old mice were used. Three successful forelimb strength measurements ($n = 5$) were recorded in the morning by an investigator blinded to treatment conditions. The average grip strength measurement from each day was used for the subsequent analysis. Motor endurance was measured using a rolling cage (RS-204-5, Kori-Seiki, Japan). The number of rotations per day was recorded, and the average number of rotations for three consecutive days was calculated ($n = 5$).

2.5. Tissue preparation

Both body and wet muscle weight were recorded. The gastrocnemius and tibialis anterior muscles were collected individually using standard dissection methods and cleaned of excess fat, connective tissue, and tendons. Several of the muscles were frozen in isopentane that was cooled by liquid nitrogen for histological and immunohistochemical analysis, whereas the other muscles were frozen directly in liquid nitrogen for either RNA isolation or protein extraction and stored at −80 °C.

2.6. RNA extraction

RNA was extracted from the gastrocnemius, tibialis anterior, and soleus muscles using Qiagen RNeasy Fibrous Tissue Mini Kits (Qiagen, CA). RNA concentrations were determined using a spectrophotometer, and the RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA). All RNA samples had RNA Integrity Numbers (RIN) of 9.0 or higher.

2.7. Real time PCR

For RT-PCR, first strand cDNA was synthesized using oligo-dT primers. The expression levels of selected genes (atrogin-1/MAFbx and beta-actin) were analyzed using CFX96 (BioRad, CA) following the manufacturer's instructions. The primers were 5′-TCGCAGCCAAGAAGAGAAAG-3′, 5′-GGCAGTCGAGAAGTCCAGTC-3′ for atrogin-1 and 5′-CTGGCTCCTAGCACCATGAAGAT-3′, 5′-GGTGGACAGTGAGGCCAGGAT-3′ for beta-actin.

2.8. Western blotting

Skeletal muscle protein was extracted from mouse hindlimb muscle samples for Western blot analysis. We used the Bradford method and Coomassie Brilliant Blue G-250 (Bio-Rad) to determine the protein concentrations. Protein fractions were then extracted using a reducing sample buffer containing 10% SDS, 70 mM Tris–HCl, 5% β-mercaptoethanol and complete inhibitor cocktail (Roche, Basel, Swiss). Protein (15 or 30 μg per lane) was separated on a SDS-polyacrylamide gel, and the resulting gel was subsequently transferred to a polyvinylidene difluoride membrane (Millipore) using 250 mA for 1 h. The blot was later incubated with the primary antibodies. The signals were detected using the enhanced chemiluminescence plus method (GE, NJ). Relative quantities of proteins in the western blots were determined using scanning densitometry and expressed in arbitrary units.

2.9. Nuclear fractionation

2.9.1. Nuclear and cytosolic protein extraction

Nuclear extracts were taken from mouse skeletal muscle as previously described (Suzuki et al., 2007). The cytosolic extract was obtained from the first supernatant of the nuclear extract preparation. The supernatant was placed in Millipore Ultrafree-4 centrifugal columns that had been pre-wetted with 0.5 mL of dilution buffer (20 mM HEPES, 40 mM KCl, 10% glycerol, 0.2 mM EDTA, 1 mM DTT) and centrifuged ($7500 \times g$) at 4 °C for 30 min. Dilution buffer (0.8 mL) was added to the column, and the 30-min spin was repeated.

2.10. Statistical analysis

Significant differences were determined with either the unpaired Student's t-test or the Mann–Whitney test using Excel. All data are expressed as the means ± SEM. Statistical significance is defined as $^{*}p < 0.05$.