Myalgic Encephalomyelitis/Chronic Fatigue Syndrome Induced by Repeated Forcing Swimming in Mice

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INTRODUCTION

Chronic fatigue syndrome (CFS), a heterogeneous disorder, is characterized by unexplained general malaise and persistent fatigue. CFS is also called myalgic encephalomyelitis (ME) and has an estimated minimum prevalence rate of approximately 0.2%. In addition to fatigue, ME/CFS includes tent fatigue. CFS is also called myalgic encephalomyelitis is characterized by unexplained general malaise and persist -

Pyruvate dehydrogenase (PDH) catalyzes the formation of acetyl-CoA, CO₂ and nicotinamide adenine dinucleotide (NADH) (H⁺) by oxidative decarboxylation of pyruvate in the mitochondria. Acetyl CoA is transferred to tricarboxylic acid (TCA) cycle and used for cell respiration. PDH is a crucial enzyme localized in the mitochondria matrix linking glycolysis to TCA cycle and oxidative phosphorylation. PDH is inhibited by phosphorylation, which is dependent on the pyruvate dehydrogenase kinase (PDK) activity. PDH activity is important for muscle function and exercise. Reduction of oxidative decarboxylation of PDH caused by up-regulation of PDK impairs muscle function in peroxisome proliferator-activated receptor (PPAR)-α agonist injected rodent model. Moreover, glucose metabolism and exercise function are inhibited by impairments in insulin-stimulated dephosphorylation of skeletal muscle PDH in a sedentary aging population.

Recent studies have clarified a good relation between ME/CFS and PDH. In ME/CFS patients, it has been reported that PDH activity was impaired by up-regulation of PDK mRNA expression in peripheral blood mononuclear cells. Moreover it has been reported that sodium dichloroacetate (DCA) inhibits PDK, activates PDH, and was effective for ten patients suffering from ME/CFS. However, the effect of DCA was evaluated by Fatigue Severity Scale (FSS), which is a subjective assessment in which patients select from 7 degrees of severity. Thus, it is critical to evaluate the effect of DCA with objective indicators such as motor function. Additionally, few studies have showed the relevance of ME/CFS and PDH in vivo. Studying ME/CFS in vivo is important for elucidating the pathophysiological mechanism of ME/CFS.

The purpose of the present study was to establish a repeated forced swimming ME/CFS model in mice and to investigate the function of PDH in ME/CFS and the potential efficacy of DCA in treatment of ME/CFS.
METHODS

Animals All experiments were performed on adult male (7-weeks-old; Japan SLC Ltd., Hamamatsu, Japan) ICR mice. The temperature of the animal facilities was 24 ± 2°C, and the lighting was under a 12h/12h light/dark cycle (lights on at 8:00h). Food and water were available ad libitum. All procedures related to animal care and treatment conformed to the animal care guidelines issued by the Gifu Pharmaceutical University Animal Experiment Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Gifu Pharmaceutical University.

Induction of ME/CFS Model with Forced Swimming Test The forced swimming test was conducted as previously described with some modifications. Each mouse (with tail loaded of 5% of body weight) was placed in a plastic cylinder (14-cm diameter) filled with water (3000 mL, 23–25°C) for 6 min for 25 d. Water was exchanged for each mouse. The movements of the mice were recorded by a video camera, and mice was judged to be immobile when they remained floating passively in the water, making only small movements to keep their heads above the water. The measurement of immobility time was performed in a blind manner by a single observer (SD). Control mice were not received any treatment.

Post-swim Fatigue Post-swim fatigue was measured immediately after the forced swimming test. Each mouse was removed from the forced swimming apparatus and placed in clear observation cage. We measured the elapsed time until mice start to grooming (licking and rubbing of the skin/fur). The post-swim fatigue time was noted on days 1, 2, 3, 4, 5, 10, 15, 20 and 25.

Open Field Test The open field test was performed to assess locomotor activity at day 8 as described in detail with some modification. The test was performed using a open field apparatus (30 cm long × 30 cm wide × 30 cm high). Each mouse was placed into the apparatus and allowed to move freely for 1 h. The movements of the mice were recorded by a video camera. The total distance moved in the area was recorded using a computer-operated EthoVision XT system (Noldus, Wageningen, the Netherlands). The total distance moved in the area was used as index of locomotor activity.

Mitochondria Isolation Mitochondria were isolated according to the manufacturer’s instructions of Mitochondria Isolation Kit for Tissue (Abcam # ab110169, Tokyo, Japan). At the end of experiments for behavioral testing, mice were sacrificed. Gastrocnemius muscles were removed and minced in isolation buffer. Gastrocnemius muscles were homogenized by dounce homogenizer (30–40 dounce strokes). The homogenates were centrifuged at 1000 × g for 10 min at 4°C. The supernatants were centrifuged at 12000 × g for 15 min at 4°C. The pellets were washed by resuspending in 1.0 mL of isolation buffer supplemented with 10 μL protease inhibitor cocktail and centrifuged at 12000 × g for 15 min at 4°C (repeated again).

PDH Enzyme Activity PDH enzyme activity was measured according to the manufacturer’s instructions of PDH enzyme activity microplate assay kit (Abcam # ab109902, Tokyo, Japan). We determined the sample protein concentration of isolated mitochondria (using bicinchoninic acid (BCA)). Samples were loaded on 96 well plate and incubated for 3 h at room temperature. Assay solutions were added to each well. We measured optical density (OD 450 nm) in a kinetic mode at room temperature for 30 min and calculated the rate between two time points for all the samples where the increase in absorbance is the most linear with the microplate reader (Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Drug Treatment DCA was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Mice were randomly divided into DCA treatment group (50 mg/kg) and saline treatment group (10 mL/kg). DCA was dissolved in saline and administered intraperitoneally at 30 min before each forced swimming test. Saline was administered as vehicle instead of DCA at the same time point.

Statistical Analysis Data are represented as the means ± standard error of the means (S.E.M.s). Statistical comparisons were made by Student’s t-test, Dunnnett’s test, and χ² test. The statistical analyses were performed with the SPSS Statistics (IBM, Armonk, NY, U.S.A.) software. A p value <0.05 was considered to be statistically significant.

RESULTS

Repeated Forced Swimming Did Not Affect Body Weight We measured the body weight for 25 d after exposure to repeated forced swimming. The body weight of the mice exposed to the repeated forced swimming for 6 min/d for 25 d did not differ significantly from control mice (Fig. 1B). This result indicates that body weight change did not affect behavioral tests.

Repeated Forced Swimming Increased the Fatigue-Like Behavior in Mice We investigated whether repeated forced swimming (load 5% of body weight, 6 min/d, 25 d) increased the fatigue-like behavior via behavioral tests. (Fig. 1A) We
measured the immobility period during the forced swimming test to assess the fatigue-like behavior. An increase in the immobility period caused by repeated forced swimming was considered to be a behavior related to ME/CFS. The immobility period was significantly increased on days 5, 10, 15, 20 and 25 compared to day 1. (Fig. 2A) (degree of freedom; 8, $F = 13.415$) Next, we measured post-swim fatigue time immediately after the forced swimming test. The number of mice with post-swim fatigue time over 30 s was increased each day. (Fig. 2B) These results indicate that the repeated forced swimming induced ME/CFS-like behavior in mice.

Repeated Forced Swimming Decreased Locomotor Activity in Open Field Test To investigate the effect of repeated forced swimming on locomotor activity, we conducted an open field test at 8d after exposure to repeated forced swimming. Representative images are provided in Fig. 3A. Total distance moved was lower in mice exposed to repeated forced swimming than in control mice (Fig. 3B). This result indicates that repeated forced swimming decreases locomotor activity.

Pyruvate Dehydrogenase Activity Was Impaired by Repeated Forced Swimming At the end of experiments, gastrocnemius muscles were removed and isolated mitochondria for measuring. PDH activity was measured. Values are expressed as the means ± S.E.M.s, Control ($n = 10$), ME/CFS ($n = 11$). **; $p < 0.01$ vs. Control. (Student’s $t$-test).

Syndrome We examined the efficacy of DCA as an activator of PDH in ME/CFS. We administered DCA (50 mg/kg, intraperitoneally (i.p.)) to mice during repeated forced swimming test (load 5% of body weight, 6 min/d, 15 d). The body weight of the DCA-treated mice did not differ significantly from saline treated-mice. (Fig. 5A) The immobility period of the DCA-treated mice was significantly decreased on days 5, 10 and 15 compared to the saline-treated mice. (Fig. 5B) DCA had no effect on immobility period on day 1. (Fig. 5C) These results indicate DCA suppresses the emergence of fatigue-like behavior. The level of PDH activity of DCA treated-mice was significantly increased compared to the ME/CFS group. (Fig.
Maximum velocity of mice, which is used as an indicator of exercise capacity, was improved by DCA administration. (Fig. 6B) These results indicate that DCA might be beneficial for preventing fatigue-like behavior in this condition.

**DISCUSSION**

We showed that repeated forced swimming for 25 d increased the immobility period. (Fig. 2) The impact of repeated forced swimming is classified in the category of physical fatigue, which is a core presentation of ME/CFS. The repeated forced swimming test has previously been used to test exercise endurance or as a means to identify fatigue. Moreover, the prolongation of the immobility period in repeated forced swimming is defined as a situation related to ME/CFS. However, there is a possibility that increasing immobility period reflects learning of harmful effects. We performed post-swim fatigue and open field test in order to evaluate the degree of fatigue in various ways. We showed that the post-swim fatigue period was increased and distance moved was reduced in the open field test in this ME/CFS rodent model. (Figs. 2, 3) These results are consistent with previous studies showing that chronic swimming stress induced rodent models of ME/CFS are associated with impaired locomotor activity, anxiety-like behavior, oxidative stress, and other functional deficits in mice.

We confirmed that the time of staying central did not change in the open field test. It has been reported that staying central in the open field test is reduced in some depression models. Therefore, unchanged staying central may be clue to distinguish between depression-like behavior and fatigue-like behavior. Therefore, we considered that the similar pathology of ME/CFS may occur in the ME/CFS rodent model.

During mitochondrial respiration, a small fraction of the electrons leaks from complexes I and III of the mitochondrial electron transport system and produces superoxide and other reactive oxygen species (ROS). In particular, exercise increases the productions of superoxide and ROS in muscles. Superoxide and ROS have been reported to be important contributors to the development of fatigue. Moreover, ROS activates PDK. Muscle cells activate endogenous cellular antioxidant mechanisms and protect against oxidative stress of ROS. It is known that these endogenous cellular antioxidant mechanisms are inhibited by chronic fatigue.
Therefore, chronic fatigue makes mitochondria vulnerable to oxidative stress. Moreover, we found that PDH activity was reduced by chronic fatigue in the ME/CFS rodent model (Fig. 4). Pyruvate, the final product of glycolysis, enters mitochondria via the mitochondrial pyruvate carrier and has two major metabolic fates: conversion to acetyl-CoA by PDH (oxidative metabolism), or conversion to lactate by lactate dehydrogenase (LDH; anaerobic metabolism).\(^9\) The lowering of the PDH activity accumulates pyruvate, which leads to the overproduction of lactate, even in the presence of adequate oxygen levels. These dysfunctions in PDH activity decrease the production of ATP.\(^3\) Therefore, energy depletion may be caused by PDH dysfunction and mitochondrial abnormality as a part of ME/CFS pathogenesis. We demonstrated a crucial role of the PDH activity in fatigue-like behaviors following repeated forced swimming and its underlying mechanism in an ME/CFS rodent model. Targeting PDH to improve energy production status may lead to therapies that provide long-term benefits.

Finally, we showed that DCA treatment improved immobility period in the repeated forced swimming test (Fig. 5). DCA treatment was beneficial in preventing fatigue-like behavior in ME/CFS. PDH activity is suppressed by reversible phosphorylation at Ser232, Ser293, Ser300 for four types of PDK1–4. In contrast, phosphorylated PDH is dephosphorylated by PDH phosphatase 1 and 2, after which PDH activity is restored.\(^3\) DCA is known to increase the PDH activity by inhibiting PDK2 and PDK4.\(^3\) However, previous reports demonstrated that treatment with DCA caused adverse effects with oxidative stress in hepatic tissues, hepatomegaly, and decreased the locomotor activity.\(^3\) Further studies of DCA will be needed to clarify effective blood concentration and provide a long-term evaluation of its effects.

In conclusion, we demonstrated that impairment of the PDH activity was caused in ME/CFS rodent model. Furthermore, treatment with DCA is effective for the PDH activity after chronic fatigue stress. These findings indicate that PDH might be an important therapeutic target for treatment of ME/CFS.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**


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