FEBS Letters 520 (2002) 18–24 FEBS 26095

Progression of dystrophic features and activation of mitogen-activated protein kinases and calcineurin by physical exercise, in hearts of *mdx* mice

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Received 11 February 2002; revised 15 March 2002; accepted 28 March 2002

First published online 13 May 2002

Edited by Richard Marais

Abstract We have previously demonstrated that calcineurin and p38 mitogen-activated protein kinase (MAPK) are up-regulated in the hearts of *mdx* mice. However, the degree of up-regulation observed was variable, which may reflect variable levels of daily physical activities among the mice. To investigate whether or not exercise affects dystrophic features and activates intracellular signaling molecules in *mdx* hearts, we subjected *mdx* and C57BL/10 mice to treadmill exercise and examined intracellular signaling molecules in cardiac muscles, at the protein level. The heart to body weight ratio was significantly increased in exercised *mdx* mice. Histopathology in exercised *mdx* hearts showed extensive infiltration of inflammatory cells, together with increases in interstitial fibrosis and adipose tissues, all of which were not observed either in exercised C57BL/10 or non-exercised *mdx* hearts. Phosphorylated p38 MAPK, phosphorylated extracellular signal-regulated kinase 1/2 and calcineurin, but not phosphorylated c-Jun N-terminal kinase 1, were up-regulated in exercised *mdx* hearts compared to exercised C57BL/10 or non-exercised *mdx* hearts. These data suggest that physical exercise accelerates the dystrophic process through activation of intracellular signaling molecules in dystrophin-deficient hearts. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Dystrophin-deficient heart; Exercise; p38 mitogen-activated protein kinase; c-Jun N-terminal kinase 1; Extracellular signal-regulated kinase 1/2; Calcineurin

1. Introduction

Duchenne and Becker muscular dystrophies (DMD/BMD) are X-linked recessive diseases characterized by progressive muscle wasting, and both diseases are caused by mutations in the *DMD* gene. In DMD and BMD patients, congestive heart failure with cardiomyopathy is one of the main causes of death. Dilated cardiomyopathy is the most common type of cardiomyopathy, and in some patients it is preceded by hypertrophic cardiomyopathy [1]. BMD patients who are mildly affected, or are in the subclinical stage, often show severe cardiomyopathy [2]. It has been hypothesized that the slow progression of muscular weakness permits a prolonged workload on an impaired myocardium in these BMD patients, eventually resulting in cardiomyopathy [1,2]. However, the hypothesis has not been confirmed.

Dystrophin, the *DMD* gene product, is a structural membrane protein, which maintains the mechanical stability of the muscle fiber membrane during muscle contraction and relaxation, and regulates intracellular calcium homeostasis, possibly by modulation of muscle-specific ion channels [3]. Some reports document that dystrophin-deficient skeletal and cardiac muscles can be more easily damaged than normal muscles by mechanical stresses such as physical exercise [4–7]. Physical exercise or injury is a powerful stimulator of mitogen-activated protein kinase (MAPK) cascades in human skeletal muscle. The c-Jun N-terminal kinases (JNKs), p38 MAPK and extracellular signal-regulated kinase 1 and 2 (ERK1/2) are major components of this cascade [8,9]. Calcineurin, a calcium dependent protein phosphatase, is another intracellular signaling molecule induced by mechanical stress, leading to cardiac and skeletal muscle hypertrophy [10–12]. In fact, we have previously reported that JNK1, p38 MAPK and calcineurin were involved in the pathogenesis of dystrophin deficiency in cardiac muscle [13]. Taken overall, we speculated that physical exercise might directly affect dystrophic features in *mdx* hearts and activate p38 MAPK, JNK1, ERK1/2 or calcineurin signaling in cardiac muscle. To test this hypothesis, we subjected *mdx* and C57BL/10 mice to physical exercise. Here, we show that progression of dystrophic features and significant activation of p38 MAPK, ERK1/2 and calcineurin, but not JNK1, are observed in exercised *mdx* hearts compared to those in both exercised C57BL/10 and non-exercised *mdx* hearts.

2. Materials and methods

2.1. Animals and treadmill exercise

Six-week-old male dystrophin-deficient (*mdx*) and control (C57BL/10) mice were used in this study. Animals were exercised using a treadmill manufactured by Takaramachi Kikai Company (Japan) that had an adjustable belt speed (0–70 m/min), shock bars with adjustable belt amperage (0–1 mA), and an on–off shock switch for each lane. A total of 24 mice were separated into four groups: non-exercised C57BL/10 ($n=6$), exercised C57BL/10 ($n=6$), non-exercised *mdx* ($n=6$) and exercised *mdx* ($n=6$). In the exercised group, the treadmill exercise protocol basically followed that described in the previous report [14]; the mice were placed on the treadmill and run with upstairs inclination of 7°, at 15 m/min for 60 min, twice per week for 5 weeks. Thereafter the speed was increased to 23 m/min (same incli-

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PII: S0014-5793(02)02739-4

nation) and the exercise continued for another 5 weeks. In the non-exercised group, the mice were placed in the treadmill at 2 m/min and 0° incline for the same period as the exercised group. All the mice were completed each exercise without rest and finished a 10-week protocol. Thus, they were exercised for the same total time. The body weight of each mouse was measured every week. After the protocol, mice were anesthetized with diethylether and sacrificed. The hearts were excised, rinsed in saline and weighed. The heart to body weight ratio was calculated. Heart muscle samples were frozen in cooled isopentane, and stored at −80°C until analyzed. This study was carried out in accordance with the Guide for Animal Experimentation, Institute of Experimental Animals, Shinshu University School of Medicine, and all procedures were approved by the Institute Animal Care and Use Committee.

2.2. Microscopic analyses

Transverse cryostat sections (8-μm thickness) of left ventricular myocardium were prepared and stained with hematoxylin and eosin (H&E). To judge the extent of the myocardial damage, the percentage of the areas of dystrophic lesion to the total of the left ventricular was determined using MacSCOPE (Mitani Corporation, Japan).

2.3. Western blot analyses

The heart tissues were homogenized in a gel-loading buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol) and the total protein content was assessed (Bio-Rad DC Assay kit). After the addition of β-mercaptoethanol (final concentration: 5%), 100 μg of each extract was separated on an 8% or 10% SDS–polyacrylamide gel and electroblotted onto a PDEF membrane Immobilon™ (Millipore). The blot was blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% skimmed milk (w/v). Antibodies used for the primary incubation were: anti-GAPDH and anti-calcineurin (MAB374 and MAB1694, respectively, Chemicon International, Inc.), anti-phosphorylated (p-)p38 MAPK, activated form of p38 MAPK (#9211S, New England Biolabs), anti-phosphorylated JNK1, activated form of JNK1 (SC6254, Santa Cruz Biotechnology, Inc.), and anti-phosphorylated ERK1/2, activated form of ERK1/2 (#9252, New England Biolabs). All membranes were incubated with primary antibodies at 4°C overnight. After labeling, the membranes were washed three times in TBST for 10 min each and then incubated with appropriate species-specific horseradish peroxidase-conjugated secondary antibodies for enhanced chemiluminescence (ECL Plus Western blotting detection kit, Amersham Pharmacia Biotech).

2.4. Immunohistochemistry

7-μm sections of frozen tissue were collected on coated slides and allowed to air dry. The slides were washed twice in phosphate-buffered saline (PBS). All polyclonal antibodies against p-p38 MAPK, p-ERK1/2, calcineurin, and GAPDH were diluted 1:500 in PBS with 5% goat serum and applied directly to the sections. The slides were incubated overnight at 4°C in a humidified box, then washed three times with PBS for 10 min. The labeled secondary antibody (anti-rabbit IgG for p-p38 MAPK, p-ERK1/2 and calcineurin, and anti-mouse IgG for GAPDH, fluorescein isothiocyanate (FITC) (Santa Cruz) or biotin-labeled (Dako)), at 1:100 dilutions in PBS with 5% goat serum, was applied and slides incubated for a further 1 h. After washing the slides with PBS three times, the FITC-labeled sections were mounted using glycerol-mounting medium, or the biotin bound antibodies were visualized by avidin–biotin peroxidase kits (Vectastain, Vector Laboratories) using diaminobenzidine (Dako) and the sections were counterstained with hematoxylin.

Table 1
Percentage of area of dystrophic lesion to left ventricle

	Percentage of area (mean ± S.E.M.)
C57BL/10	
Non-exercised ($n=6$)	0.05 ± 0.02%
Exercised ($n=6$)	0.06 ± 0.04%
mdx	
Non-exercised ($n=6$)	1.68 ± 0.31%*
Exercised ($n=6$)	6.23 ± 0.70%**,***

*$P<0.01$, non-exercised C57BL/10 vs non-exercised *mdx*.
**$P<0.01$, non-exercised *mdx* vs exercised *mdx*.
***$P<0.01$, exercised C57BL/10 vs exercised *mdx*.

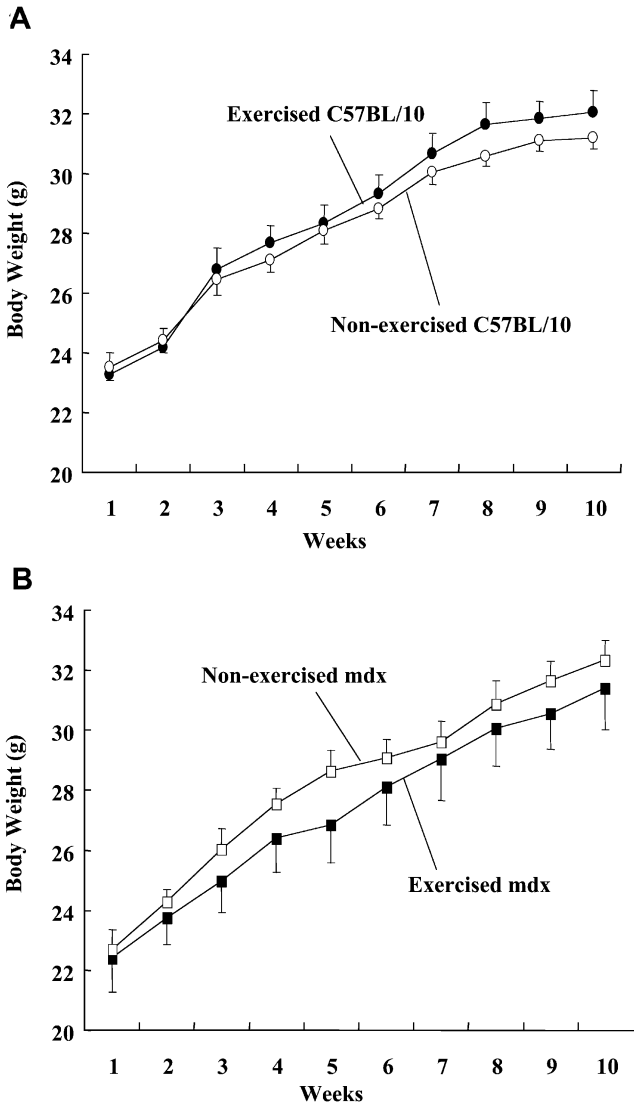

Fig. 1. Timetable showing body weight changes in C57BL/10 and *mdx* mice in exercised or non-exercised groups ($n=6$ for each group). Bar shows mean+S.E.M.

2.5. Statistical analyses

Semi-quantitative assessment of band intensity was performed by using the NIH Image software package, and the level of each protein relative to that of GAPDH was calculated. The unpaired Student's *t*-test was performed to compare two groups (non-exercised C57BL/10 vs exercised C57BL/10, non-exercised C57BL/10 vs non-exercised *mdx*, exercised C57BL/10 vs exercised *mdx*, and non-exercised *mdx* vs exercised *mdx*). A P value <0.05 was considered statistically significant. All values are expressed as mean ± S.E.M.

3. Results

3.1. Body weight during the treadmill exercise and heart to body weight ratio

We found no significant difference in body weight between non-exercised C57BL/10 and exercised C57BL/10 mice; however, the weight of exercised C57BL/10 mice was slightly heavier than that of non-exercised C57BL/10 at each time point (Fig. 1A). Furthermore, the body weight of exercised *mdx* mice was slightly less (but not significantly) than that of non-exercised *mdx* (Fig. 1B). The difference in final body

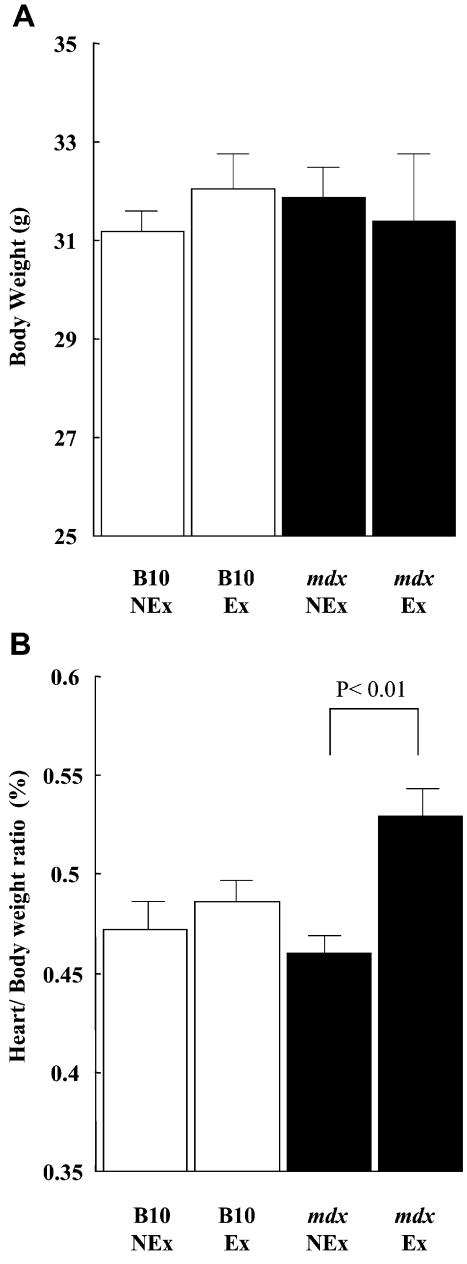

Fig. 2. A: Final body weight of C57BL/10 and *mdx* mice in exercised or non-exercised groups ($n=6$ for each group). Bar shows mean+S.E.M. B: Heart to body weight ratio of C57BL/10 and *mdx* mice in exercised or non-exercised groups ($n=6$ for each group). Bar shows mean+S.E.M.

weight between the groups was not statistically significant (Fig. 2A). The heart weight of each exercised group was slightly greater than that of each non-exercised group, but the differences were also not statistically significant (data not shown). However, the ratio of heart to body weight in exercised *mdx* mice was slightly but significantly higher than that in non-exercised *mdx* or exercised C57BL/10 (Fig. 2B).

3.2. Histopathology of heart

We examined the left ventricular myocardium histopathologically. Macroscopically, the hearts in exercised *mdx* mice seemed to be slightly larger than those in non-exercised *mdx*; however, dilatation of left ventricle and thickness or thinness of left ventricular wall were not evident (data not shown). H&E staining of the samples showed no remarkable change in either non-exercised or exercised C57BL/10 mice (Fig. 3A,B), while in *mdx* mice, non-exercised cardiac muscle showed a slightly dystrophic change such as fiber necrosis and atrophy, together with slight infiltration of inflammatory cells (Fig. 3C). In exercised *mdx* cardiac muscle, the amount of interstitial fibrosis and adipose tissue was increased; infiltration of inflammatory cells was prominently enhanced and fiber disorganization was observed (Fig. 3D). We semi-quantitatively evaluated the lesion of fibrosis, adipose tissue and infiltration of inflammatory cells in the myocardium of the left ventricle. The difference of the percentage of dystrophic lesion in the myocardium between non-exercised and exercised C57BL/10 was not significant. While the percentage in non-exercised and exercised *mdx* groups was much higher than that in C57BL/10 groups. When compared between non-exercised and exercised *mdx* hearts, the percentage was more greatly increased in exercised *mdx* (Table 1).

3.3. Up-regulation of p-p38 MAPK, p-ERK1/2 and calcineurin in the hearts

We next examined levels of the activated forms of p38 MAPK (p-p38 MAPK), JNK1 (p-JNK1) and ERK1/2 (p-ERK1/2), and calcineurin in cardiac muscles by Western blotting. p-p38 MAPK was increased two-fold in exercised C57BL/10 hearts compared to non-exercised C57BL/10 hearts (Figs. 4 and 5), while the level in non-exercised *mdx* hearts was elevated three-fold, compared to non-exercised C57BL/10 hearts. These data were consistent with our previous report [13]. Moreover, p-p38 MAPK levels displayed a more dramatic increase in exercised *mdx* hearts than in exercised C57BL/10 or non-exercised *mdx* hearts. However, the level of p-JNK1 was not always up-regulated by exercise (Figs. 4 and 5). The relative level of p-ERK1/2 and calcineurin to GAPDH showed a similar profile. The basal level of these proteins was up-regulated in *mdx* hearts compared to C57BL/10 hearts, and physical exercise increased the level of these proteins, especially in *mdx* mice (Figs. 4 and 5).

We also investigated the localization of p-p38 MAPK, p-ERK1/2 and calcineurin by immunohistochemistry. Immunoreactivities with antibodies against p-p38 MAPK, p-ERK1/2 and calcineurin were increased in degenerative fibers around the fibrotic change or the infiltration of inflammatory cells in non-exercised and exercised *mdx* hearts (Figs. 6 and 7). And the signals seemed to be stronger in the exercised groups than non-exercised groups.

4. Discussion

We have shown that the heart to body weight ratio of exercised *mdx* mice was significantly increased; this might partly reflect the fact that the body weight was slightly less in exercised *mdx* mice than in non-exercised *mdx*. But compared to non-exercised *mdx* mice, the heart weight itself was increased in exercised *mdx*. It has recently been reported that the heart to body weight ratio of *mdx* mice was 45% higher at 6 months of age, and that *mdx* heart showed hypertrophic changes by this age [15]. More importantly, we are the first to have demonstrated that physical exercise induces dystrophic features in cardiac muscle of *mdx* mice. As for the involvement of cardiac muscle in the changes observed in *mdx*

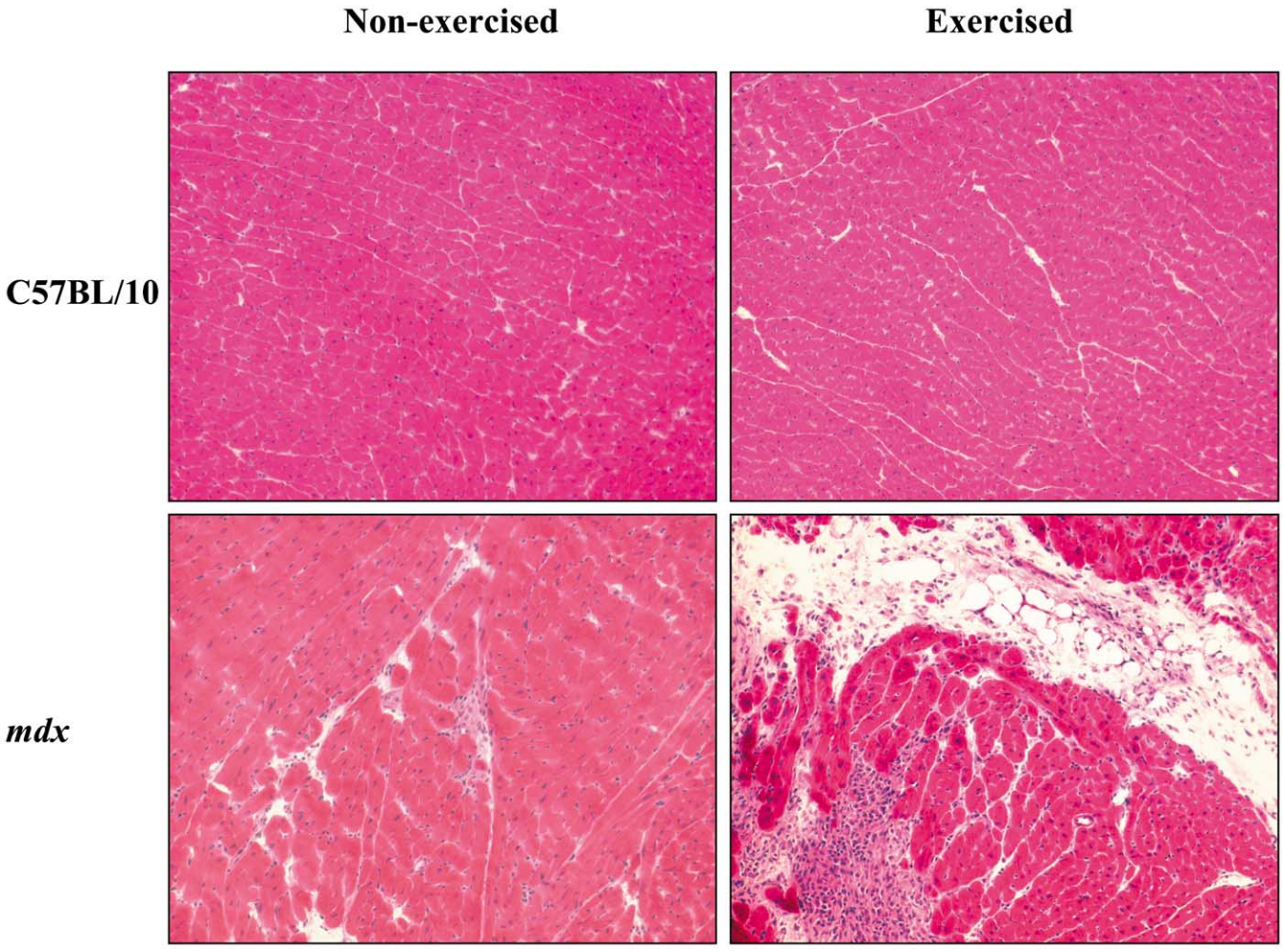

Fig. 3. H&E staining of left ventricular myocardium of exercised or non-exercised C57BL/10 and *mdx* mice (×100).

mice, previously reported findings have been controversial: some papers reported extensive necrosis and fibrosis in *mdx* cardiac muscle [16,17], while others indicated the absence of a pathologic lesion [18,19]. The difference observed was considered to be associated with the physical activity of the mice [20], and our data support this hypothesis.

In the next step, we focused on some stress-activated protein kinases and calcineurin as key molecules mediating exercise-induced cardiac involvement. MAPKs constitute a conserved family of serine/threonine protein kinases that regulate many cellular responses, including cell proliferation, differentiation and survival [21]. Activation of MAPK signaling also mediates changes in expression of muscle genes in response to exercise or injury [8,9]. Calcineurin is a protein phosphatase that is activated by a sustained increase in intracellular free calcium concentration, and which can be induced by mechanical stress [10]. Since dystrophin-deficient muscle membranes are vulnerable to mechanical stress [4–7], and since mechanical stress is a powerful stimulator of stress-activated protein kinases and of calcium influx, we hypothesized that MAPKs and calcineurin may be strongly activated in dystrophin-deficient cardiac muscle by physical exercise. We also hypothesized that up-regulation of these signaling pathways may modulate the dystrophic process. As expected, the results from the Western blots showed that p-p38 MAPK, p-ERK1/2 and calcineurin registered a significant increase in exercised *mdx* hearts compared to the exercised C57BL/10 or non-exercised *mdx* hearts. Moreover, the immunohistochemical studies showed that the immunoreactivities of p38 MAPK, ERK1/2 and calcineurin were strong, especially in the degenerative fibers around or in the dystrophic lesion in *mdx* hearts.

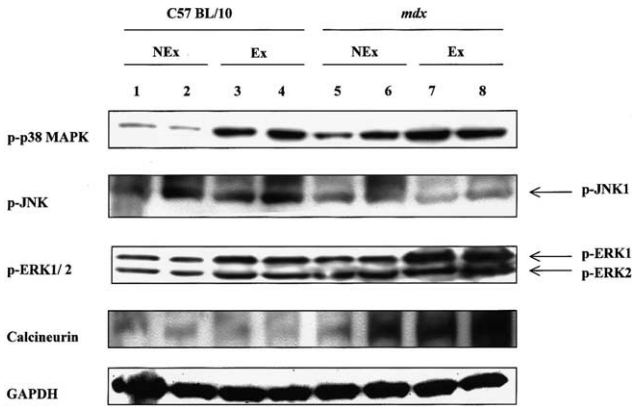

Fig. 4. Representative Western blotting analyses of p-p38 MAPK, p-JNK1, p-ERK1/2, calcineurin and GAPDH in left ventricular myocardium of exercised or non-exercised C57BL/10 and *mdx* mice ($n = 6$ for each group).

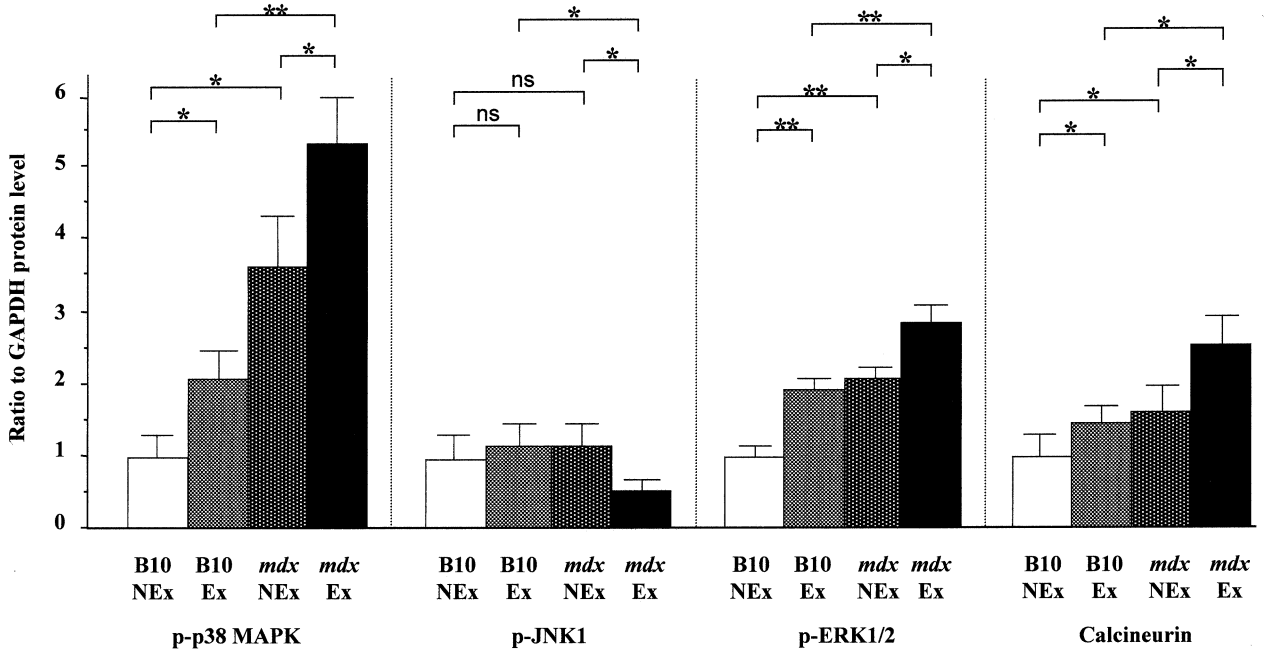

Fig. 5. Relative (to GAPDH) protein levels of p-JNK1, p-p38 MAPK, p-ERK1/2, calcineurin in C57BL/10 and *mdx* in left ventricular myocardium of exercised or non-exercised groups ($n=6$ for each group). Bar: mean+S.E.M.; ns: not significant; $^{*}P<0.05$; $^{**}P<0.01$.

In contrast to p-p38 MAPK, p-ERK1/2 and calcineurin, p-JNK1 was slightly down-regulated by exercise in *mdx* cardiac muscles. At present, we do not have satisfactory explanation for this, however, this is partially consistent with our previous report [13]. Together with the reports that p-JNK1 was increased in 5-month-old *mdx* hearts [22] and that activation of multiple cardiac MAPK pathways largely depends on the stages of cardiac damage [23], the regulation of p-JNK1 may depend on the developmental stage of cardiomyopathy in *mdx*. Further study will be needed to see how JNK1 is regulated in the development of dystrophic change in heart.

These MAPK molecules or calcineurin might presumably be involved in the dystrophic feature, thus we here discuss how the activation of these molecules modulates progression of the dystrophic process. MAPKs and calcineurin are thought to share two main roles. Firstly, these signaling mol-

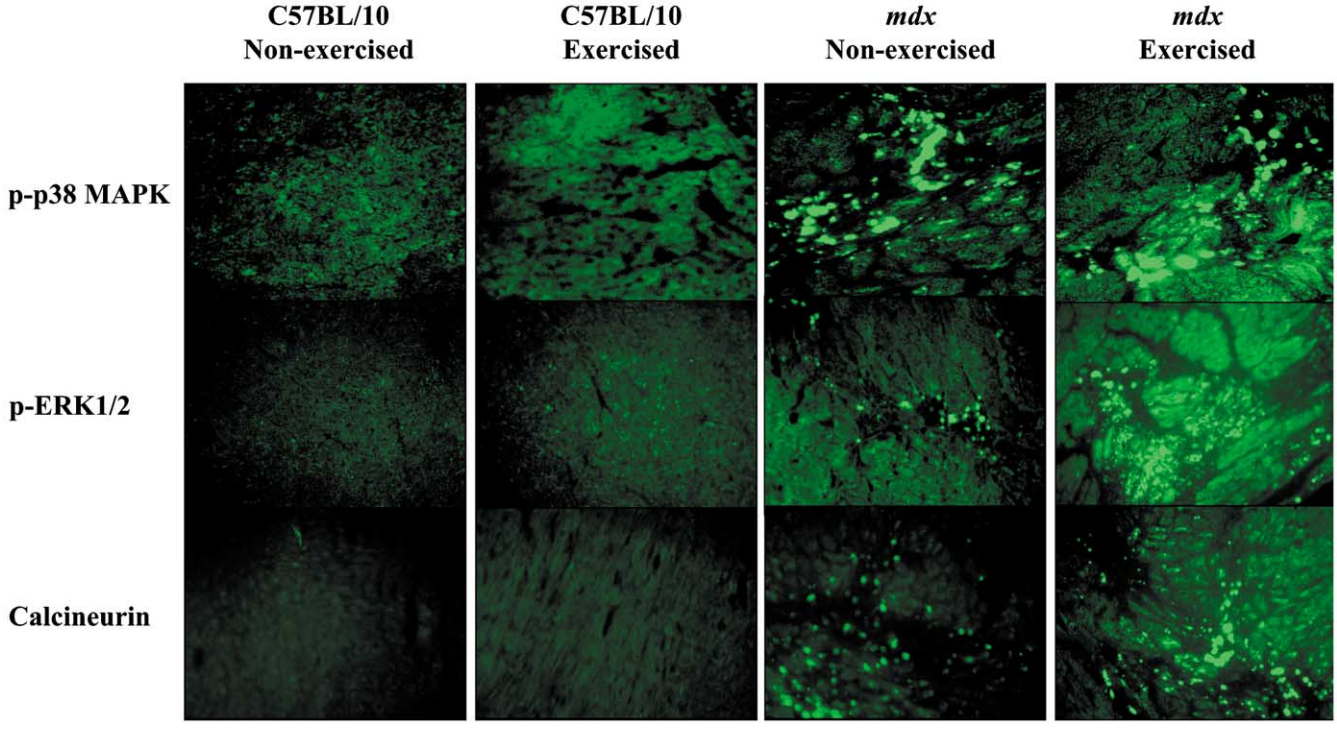

Fig. 6. Immunohistochemistry of cardiac muscle in exercised or non-exercised C57BL/10 and *mdx* mice (×100).

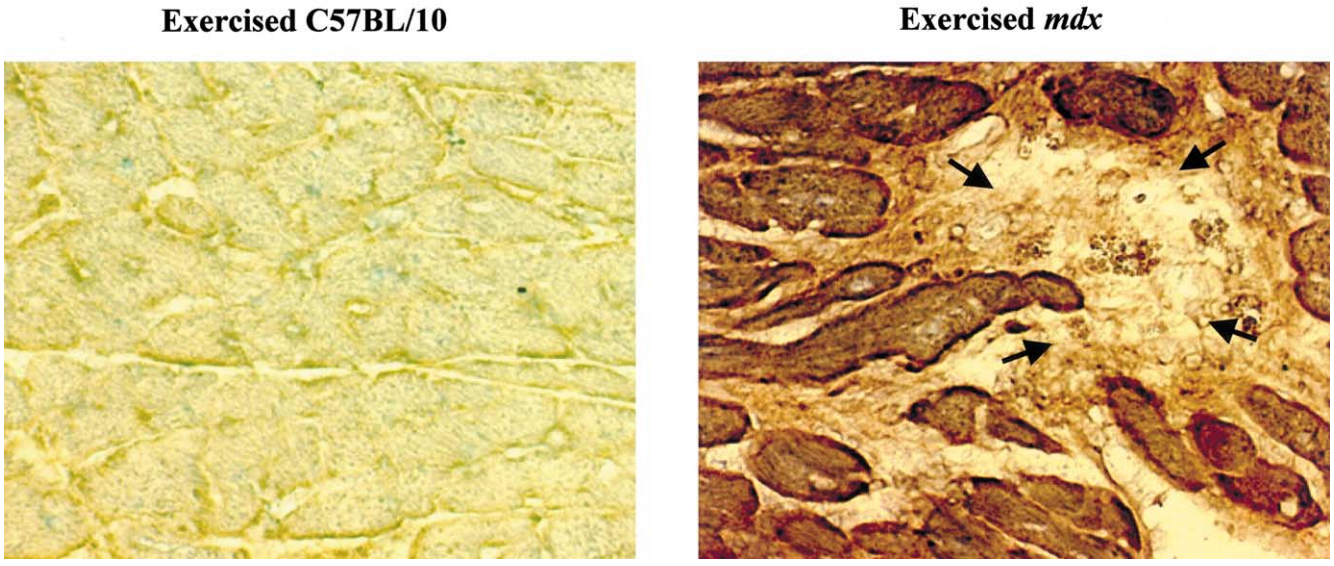

Fig. 7. P-p38 MAPK immunoreactivity of cardiac muscle fibers in exercised C57BL/10 and exercised *mdx* mice (×200). Diaminobenzidine signals were strong in the degenerative fibers around the fibrosis (arrows) in exercised *mdx* heart (B) compared to those in exercised C57BL/10 heart (A).

ecules may be involved in the process of muscle fibrosis and/or regeneration. Several growth factors, for example transforming growth factor-β, basic fibroblast growth factor, platelet-derived growth factor and insulin-like growth factor, may be important players in the process of fibrotic proliferation and muscle regeneration [24]; in addition calcineurin and MAPK are involved in activation of these growth factors [25–27]. Furthermore, considering the fact that muscle-specific enhancer factor MEF2 activity is increased in regenerating skeletal muscle [28] and that both calcineurin and MAPKs (especially p38 MAPK) can activate MEF2 DNA binding activity [29,30], the process of muscle fibrosis and regeneration in dystrophin-deficient muscle may be influenced by calcineurin and MAPKs.

Secondly, MAPKs and calcineurin may be associated with apoptosis in dystrophin-deficient muscle. A study of muscle biopsies from DMD patients confirmed the presence of apoptosis, together with an up-regulation of the pro-apoptotic gene *bax* and a down-regulation of the anti-apoptotic gene *bcl-2* [31]. In *mdx* mice, apoptotic nuclei were seen only in degenerating muscle fibers [32]; apoptotic nuclei in the fibers were increased in number and expression of the *bcl-2* gene was down-regulated in exercising *mdx* muscles [33]. Calcineurin induces apoptosis through dephosphorylation of Bad, which is a pro-apoptotic member of the Bcl-2 family [34], and MAPKs are also directly involved in regulation of the apoptotic cascade [35]. Therefore, both pathways could take part in apoptosis in dystrophin-deficient muscle.

We have previously reported that calcineurin, JNK1 and p38 MAPK signaling pathways are up-regulated in cardiac muscle of utrophin–dystrophin-deficient mice, and that calcineurin and p38 MAPK are also up-regulated in *mdx* hearts [13]. In the present study, we showed that physical exercise accelerated dystrophic features and that p38 MAPK, ERK1/2 and calcineurin were significantly activated in the exercised *mdx* hearts. In the future, it will be necessary to clarify in more detail how the up-regulation of these signaling molecules is associated with the progression of dystrophic features in the heart. For this purpose, it is informative to see the effects of the inhibitors of MAPKs [36–38] or calcineurin on *mdx* hearts. Based on our present data, we are now planning a further trial of the inhibitors of p38 MAPK, ERK or calcineurin on exercised *mdx* mice in order to better understand the mechanism of progression of the dystrophic change in heart.

Acknowledgements: This work was supported by Research Grant (11B-1) for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare, Japan.

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