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Dynamic Expression and the Role of BDNF in Exercise-induced Skeletal Muscle Regeneration

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a myokine. However, its role in skeletal muscle has not been well elucidated. In this study, we aimed to investigate its expression profile in skeletal muscle following downhill running and to explore its functions. Male Sprague Dawley rats were assigned to sedentary and downhill running groups. Tail vein blood, total mRNA and protein from soleus muscle was obtained from rats at different time points post-exercise (1d, 3d, 5d, 7d and 14d). We found a significant elevation of BDNF mRNA level 5d and 7d post-exercise ($p < 0.05$), increased BDNF protein level 1d, 3d, 7d and 14d post-exercise ($p < 0.05$), and continuously elevated serum BDNF level ($p < 0.05$). In addition, serum creatine kinase activity was increased 5d following exercise ($p < 0.05$); expression of MyoD was elevated ($p < 0.05$); disruption of myofibers and centralized nuclei in damaged myofibers were clearly observed 1d and 5d post-exercise, respectively. Moreover, AMPK phosphorylation was present 1d post-exercise ($p < 0.05$), while AKT was phosphorylated for 5d post-exercise ($p < 0.05$). In conclusion, downhill running induces a time-dependent up-regulation of BDNF in skeletal muscle, which is involved in exercise-induced skeletal muscle regeneration.

Introduction

Exercise-induced skeletal muscle regeneration is an important physiological process that can lead to positive changes in strength, as well as in the metabolic profile for adaption [4, 6, 7]. Understanding the regulators during this process will help us in designing new treatment strategies for injured, aged or diseased skeletal muscle, in order to obtain better performance and skeletal muscle functions. Myokines are cytokines, and like other peptides, they are produced, expressed and released by muscle fibers and exert autocrine, paracrine or endocrine effects [29]. Brain-derived neurotrophic factor (BDNF) is also a myokine, although it belongs to the neurotrophin family [22] and was first found present in the nervous system, where it regulates neuronal survival, cell growth, synapses formation and transmission plasticity [17, 25, 31]. In addition, exercise has been shown to increase BDNF levels not only in the brain and serum [11, 18] but also in skeletal muscle [23]. Increasing evidence has shown that exercise-induced up-regulation of BDNF in the brain explains the beneficial effects of exercise on memory and cognition [21, 38]. However, exercise-induced BDNF expression in skeletal muscle is less studied, and it is not known whether BDNF is involved in skeletal muscle regeneration following exercise.

Although increased BDNF was found following exercise, results on exercise-induced BDNF expression in skeletal muscle are inconsistent. Some exercise protocols were shown to cause elevation of BDNF in skeletal muscle, while others did not [10, 14, 26]. In addition, the time when BDNF expression was up-regulated in response to exercise remains unclear. Inconsistent results were also observed

in peripheral BDNF levels following exercise. A possible reason for the different results lies in the heterogeneity of the exercise protocols, which include exercise type, intensity, duration and frequency. High-intensity interval training has been shown to evoke larger serum BDNF levels compared to intense continuous exercise [34]; longer duration of exercise was associated with greater increases in BDNF [12]. It is known that different exercise protocols can lead to distinct physiological response. Nevertheless, few studies tested the level of exercise-induced BDNF together with the physiological condition of the skeletal muscle.

Exercise at high intensity can cause skeletal muscle injury and regeneration, which is a dynamic and highly regulated physiological process. Satellite cells are a population of stem cells in skeletal muscle that contribute to postnatal muscle growth and repair [39]. In intact skeletal muscle, satellite cells are mitotically quiescent; upon skeletal muscle injury, they exist from quiescent state and proliferate to produce myoblasts (satellite cell activation); then myoblasts differentiate and fuse with pre-existing myofibers, and with one another to form new myofibers (muscle remodeling) [28]. Individuals with a large satellite cell pool exhibit a robust hypertrophy in response to 16 weeks of exercise [30]; in degenerative skeletal muscle states, such as advanced age and Duchenne muscular dystrophy, the numbers and proliferative potential of satellite cells decreases significantly [20]. As a result, satellite cells are crucial for the growth and regeneration of skeletal muscle. Interestingly, BDNF was found to affect satellite cell function in in vitro studies [9]. Depletion of BDNF in skeletal muscle leads to a delayed regeneration following cardiotoxin injection [9]. It is thus very interesting to learn whether and how BDNF is involved in exercise-induced skeletal muscle regeneration. However, few studies have explored BDNF expression during skeletal muscle regeneration. In this study, we subjected rats to downhill running, tested the time-course of the expression of BDNF following exercise for 14 days; at the same time, we examined the dynamic injury and regeneration of skeletal muscle; we also studied time-dependent activation of AMPK and AKT, in order to explore the roles of exercise-induced BDNF in skeletal muscle.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats (8-week-old, 220 ± 8 g) were purchased from Vital River Laboratories (VRL, China). The animals were housed and bred in the SPF animal facility of the China Institute of Sport Science with free access to rat chow and water under controlled conditions (room temperature 26 ± 2 °C; light/dark cycle 12 h/12 h; relative humidity 40 %–45 %). All animal procedures were conducted in accordance with the protocols approved by national rodent care standards and with the ethical standards of the International Journal of Sports Medicine [15].

Downhill running protocol

Thirty-five adult male SD rats were randomly divided into two groups: a sedentary control group (C, n = 5) and a downhill running exercise group (E, n = 30). Rats in the E group were subjected to a bout of motor-driven treadmill running at a speed of 17 m min-1, with a -16 ° incline for 90 min. Following exercise, animals in the E group were divided into six sub-groups (E1-E6, n = 5 in each subgroup). Rats in E1 to E5 were sacrificed at day 1, day 3, day 5, day 7 and day 14 following the downhill running, respectively, for the sample collection. Rats in E6 were subjected to tail vein blood extraction for all the above time points. Rats in the C group were housed and bred under the same conditions but without any exercise.

Sample preparation

Tail vein blood was extracted from the rats in the sedentary and exercise groups at different times following downhill running (day 1, day 3, day 5, day 7 and day 14). After collection, the blood was allowed to clot by leaving it undisturbed for 3–4 h at 4 °C. Then, the clot was removed by centrifuging at 1,000–2,000 × g for 10 min in a refrigerated centrifuge, and the sera were aliquoted and stored at − 80 °C for further use.

Following blood extraction, rats were anesthetized by intra-peritoneal injection of 10 % chloral hydrate (1 mg kg-1). Soleus muscles were dissected from these rats and directly frozen in liquid nitrogen and then kept at − 80 °C for further study.

Serum Creatine Kinase (CK) activity detection

The activity of CK in the rat serum was measured spectrometrically using the kit from KeHua Bio (Shanghai, China) in accordance with the manufacturer's recommendations.

Detection of serum BDNF level by ELISA

The serum BDNF concentration was tested using BDNF commercial ELISA kit from Merck Millipore (Billerica, MA, USA), following the instruction manual. All assays were performed in duplicate.

Histological staining and immunohistochemistry

Skeletal muscles were fixed in 4 % paraformaldehyde and processed to sections (4 µm thickness). After being deparaffinized and hydrated, the slides were processed for Hematoxylin & Eosin (H&E) staining, using the H&E staining kit from BosterBio (Wuhan, China) and following the instruction manual. In addition, basic fuchsine was applied to the paraffin sections of the skeletal muscle to examine muscle injury. Staining results were observed and photographed under light microscope (Leica, Germany).

For immunohistochemistry staining, slides were processed to endogenous peroxidase activity block, using 3 % H_2O_2 solution and heat-induced epitope retrieval and a pressure cooker in Tris-EDTA (pH 9.0) buffer. Primary antibody against MyoD (1:200 dilution) and BDNF (1:100 dilution) were from Abcam (Cambridge, UK), and were incubated in antibody dilution buffer with 0.5 % triton in a humidified chamber at 37 °C for 1.5 h and at 4 °C overnight, respectively. Secondary antibody labeled with HRP was applied to the sections in a humidified chamber at 37 °C for 40 min. Then freshly made DAB substrate solution was applied to the sections on the slides to reveal the color of antibody staining. Hematoxylin was then used for counterstaining. Finally, the slides were dehydrated, cleared, mounted and photographed under light microscope (Leica, Germany).

RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from skeletal muscle in rats using Trizol Reagent (Life Technologies, USA) according to the manufacturer's instruction. The purity of RNA were measured by A260/A280 ratio. One μg of total RNA was reverse-transcribed into complementary DNA (cDNA) using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). Then, target genes were amplified with specific primers for BDNF (forward: 5′-ATGTATCTCCCTGGTCCCCC-3′ and reverse: 5′-GGGACCAAAATGGGAGGAGG-3′) and for HPRT1 (forward: 5′-AAGGACCTCTCGAAGTGTTGGAT-3′ and reverse: 5′-CCACTTTCGCTGATGACACAAC-3′). PCR reaction was performed using KAPA SYBR® FAST qPCR Kit (KapaBiosystems, USA). The amplification was performed in duplicate, using an ABI 7500FAST thermocycler (Applied Biosystems) under the following conditions: 3 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. HPRT1 was used as the endogenous control. Relative expression of the BDNF in the running group compared to the sedentary group was analyzed using the $2^{-\triangle\triangle Ct}$ method. mRNA expression is presented as fold changes relative to control values.

Protein extraction and western blot analysis

For protein extraction, skeletal muscle samples were dissected from rats on ice, and homogenized in RIPA buffer containing protease and phosphatase inhibitors for 40 min at 4 °C with gentle shaking and centrifuged at 13000 g for 20 min to eliminate debris. Determination of the protein concentration was performed using BCA assay. Lysates containing the same amount of protein were loaded and resolved in SDS–PAGE electrophoresis, followed by transferring to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Membranes were then blocked (2 % BSA, 0.1 % Tween-20 in TBS, pH 8.0) for 1 h and incubated with anti-BDNF (1:500) from Abcam (Cambridge, UK), anti-phospho-AMPK (Thr172) (1:1000) or anti-phospho-AKT (Ser473) (1:2000) antibodies from Cell Signaling (Danvers, MA, USA). β-actin (1:1000) from ZSGB-BIO (Beijing, China) was used as loading control. Anti-rabbit and anti-mouse horseradish peroxidase secondary antibodies from ZSGB-BIO (Beijing, China) were diluted at 1:10000. Blots were revealed by homemade ECL, and the results were analyzed using Image J software.

Statistical analysis

Data were presented as mean ± SEM. Student's t test was applied to compare the differences of means between two groups. Values of $p < 0.05$ were considered statistically significant.

Results

Dynamic increase of BDNF in response to downhill running

To explore the dynamic BDNF expression in skeletal muscle following downhill running, we tested the mRNA level and protein level of BDNF in skeletal muscle at different time points following downhill running through qRT-PCR and Western blot, respectively. We observed a significant increase of BDNF mRNA levels 5d and 7d post-exercise (▶ **Fig. 1a**, $p < 0.05$). A significant increase of BDNF protein level in skeletal muscle was observed 1d, 3d, 7d and 14d post-exercise (▶ **Fig. 1b,c**, $p < 0.05$). Besides, a sustained increase of serum BDNF was found (▶ **Fig. 1d**, $p < 0.05$). In addition, we studied the location of BDNF in skeletal muscle. Positive BDNF staining was found in the regenerated myofibers (▶ **Fig. 1f–h**). Therefore, downhill running induces a time-dependent expression of BDNF in skeletal muscle.

Skeletal muscle injury in response to downhill running

To study the role of BDNF in skeletal muscle, we first tested the physiological condition of skeletal muscle following downhill running. Histological study showed focal fiber disruption and positive staining of basic fuchsine one day post-downhill running (▶ **Fig. 2a,b**), indicating the damage of myofibers. Moreover, we tested dynamic activity of serum creatine kinase (CK). Being a intracellular enzyme, circulating CK activity is raised when the membrane permeability of myofibers is increased. Through continuously monitoring serum CK activity in five rats at different time points for 14 days, we found that serum CK activity was significantly increased 5d following exercise (▶ **Fig. 2c,d**, $p < 0.05$), indicating the increased permeability of myofibers.

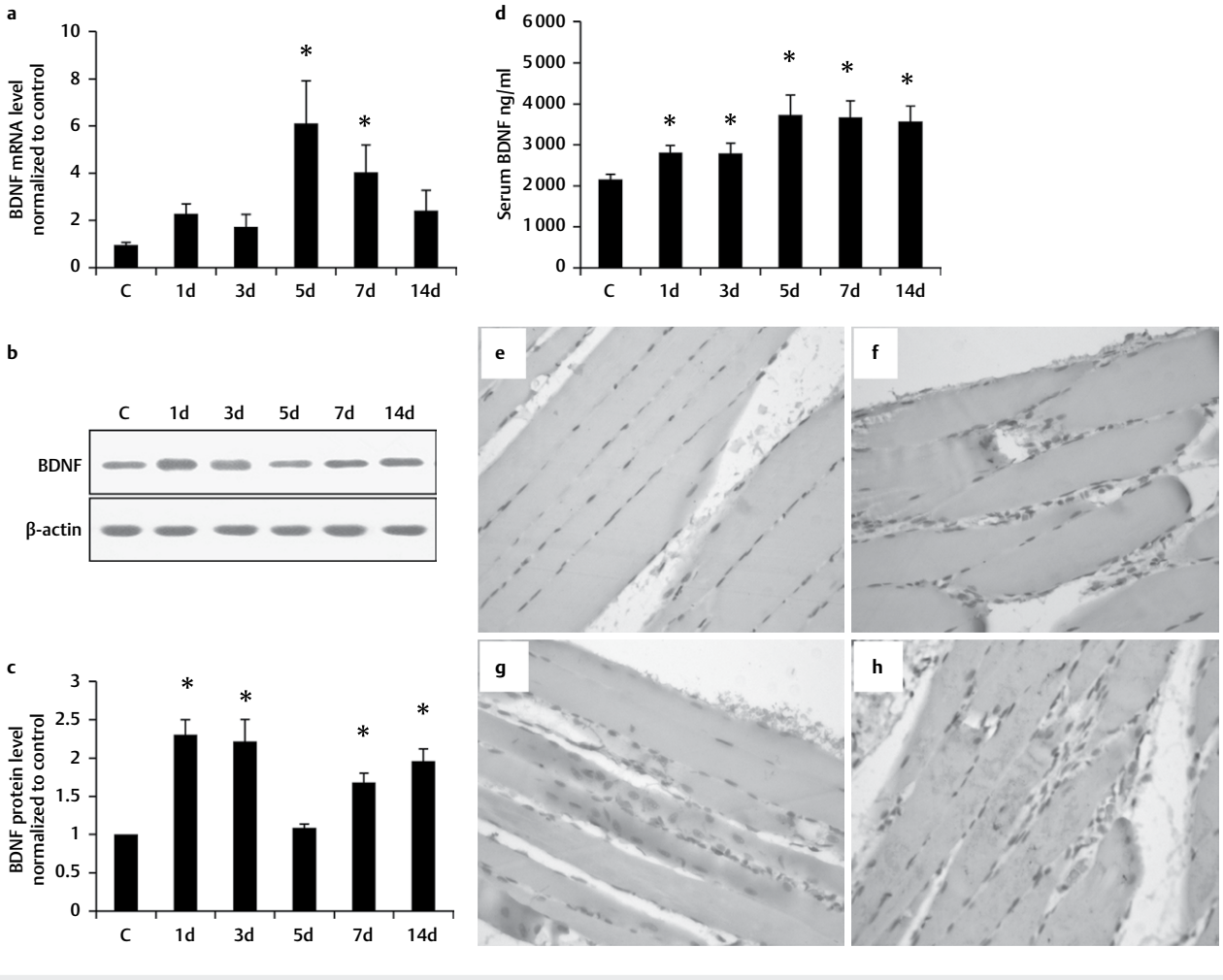
Dynamic regeneration of skeletal muscle following downhill running

Satellite cells contribute to the postnatal skeletal muscle growth and repair. After observing increased expression of BDNF in skeletal muscle following downhill running, we investigated the time-dependent expression of MyoD, a marker of activated satellite cells. We found an increase in the number of cells expressing MyoD in skeletal muscle from the first day following exercise (▶ **Fig. 3**, $p < 0.05$), indicating the satellite cell activation during the regeneration of skeletal muscle.

To further study the regeneration of skeletal muscle, we investigated the histological condition of skeletal muscle in response to downhill running through H&E staining. Focal fiber disturbances one day post-downhill running indicate the injury of skeletal muscle cells following downhill running (▶ **Fig. 4a,d**). Subsequently, aggregated nuclei were observed in the disrupted skeletal muscle cells three days post-downhill running, suggesting migration and fusion of activated satellite cells (▶ **Fig. 4b,e**); Next, centrally located nuclei were present five days post-downhill running, indicating newly formed skeletal cells by satellite cells (▶ **Fig. 4c,f**). Finally, centralized nuclei were found moving to the periphery of myofibers 7 days and 14 days following exercise and most fibers are neatly located (▶ **Fig. 4g,h**). The histological evidence confirms the skeletal muscle injury and regeneration following downhill running.

Time-dependent activation of AMPK and AKT in skeletal muscle in response to downhill running

AMPK has been reported to be activated by BDNF in skeletal muscle in vitro [23]. Elevation of BDNF expression in skeletal muscle following downhill running led us to test the activation of AMPK. A significant phosphorylation of AMPK was found one day following exercise compared with control (▶ **Fig. 5a,b**). However, there was no activation of AMPK when BDNF expression was elevated in skeletal muscle 7d and 14d post-exercise. In addition, we tested the activation of AKT, which is crucial in regulating protein synthesis for the adaption of skeletal muscle in response to exercise. Activa-



▶ **Fig. 1** Dynamic BDNF level in response to downhill running. mRNA level **a** and protein level **b** and **c** of BDNF in skeletal muscle, as well as serum BDNF level **d** in rats at different time points following downhill running. Staining of BDNF was also shown in sedentary **e** and exercised skeletal muscle **f-h**. Data are Mean ± SEM. *, $p < 0.05$ compared to control group.

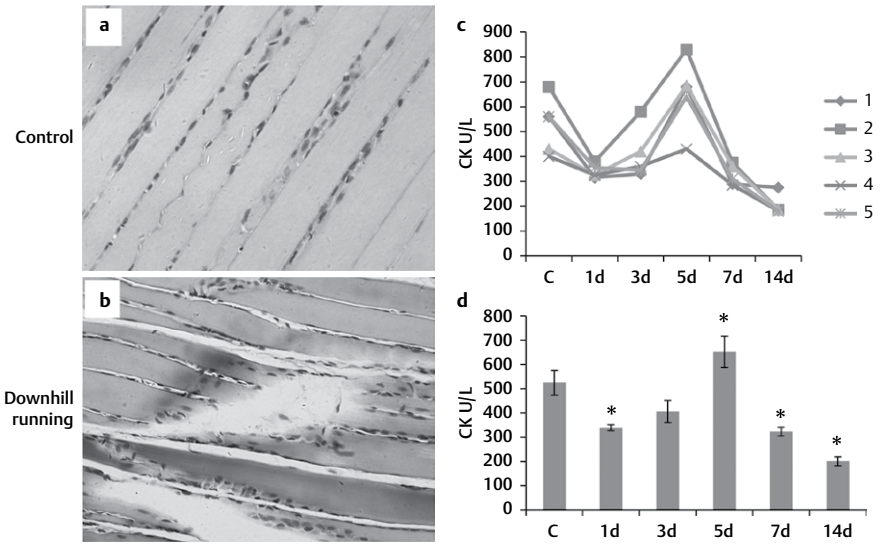
tion of AKT was found present 1d, 3d and 5d in skeletal muscle in rats following downhill running (▶ **Fig. 5a,c**). These results indicate that a bout of downhill running induces an acute activation of AMPK and a sustained activation of AKT following exercise.

Discussion

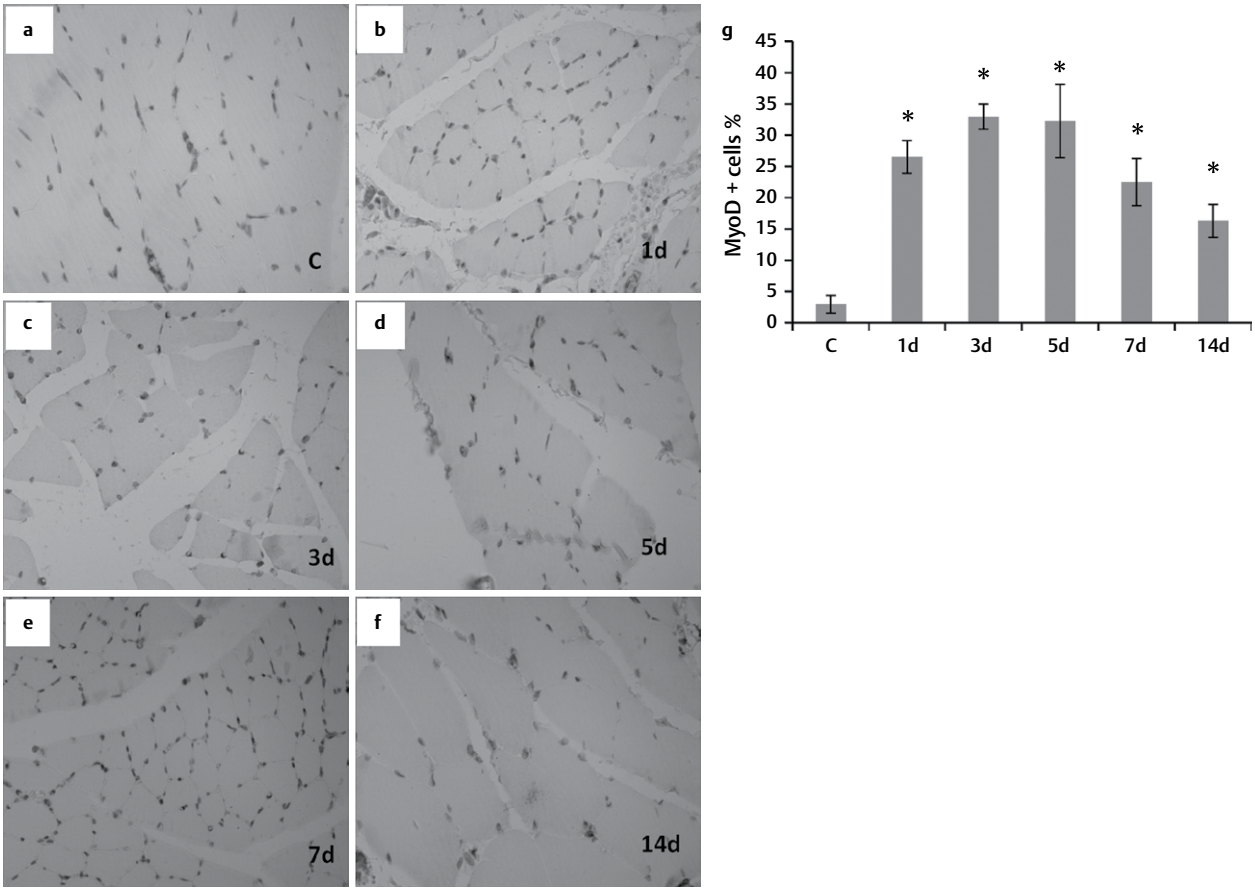
In the present study, we measured time-dependent expression of BDNF in skeletal muscle during downhill running-induced dynamic skeletal muscle injury and regeneration, together with time-dependent activation of signaling pathway. We showed for the first time that exercise-induced BDNF is involved in skeletal muscle regeneration.

Previous studies have tested BDNF expression in skeletal muscle for three days post exercise [10, 23]. However, little is known about the expression of BDNF in skeletal muscle during longer periods following exercise. In this study, we investigated BDNF expression for 14 days following downhill running and observed a trend of increase of BDNF mRNA level 1d post-exercise and a significant increase 5d and 7d following exercise (▶ **Fig. 1a**, $p < 0.05$). Interestingly, a significant increase of BDNF protein levels was observed later than the increase of mRNA level (▶ **Fig. 1b,c**). Similarly, Cuppini et al. found increased BDNF mRNA levels 24 h and 48 h post-exercise. Nevertheless, a significant elevation of BDNF protein levels was observed 48 h and 72 h post-exercise, suggesting the delay of the appearance of increased BDNF protein levels from the up-regulated mRNA levels [10]. In addition, elevation of BDNF expression appeared in two phases: an early time increase (1d and 3d post-exercise) and a late time increase (7d and 14d after downhill running). In vitro and ex vivo studies showed that BDNF increases fat oxidation in skeletal muscle cells [23], which is an early physiological event in skeletal muscle in response to exercise. As a result, the increase of BDNF protein levels at later time points (7d and 14d) following exercise suggests that the role of BDNF in skeletal muscle post-exercise is more than regulating energy metabolism.

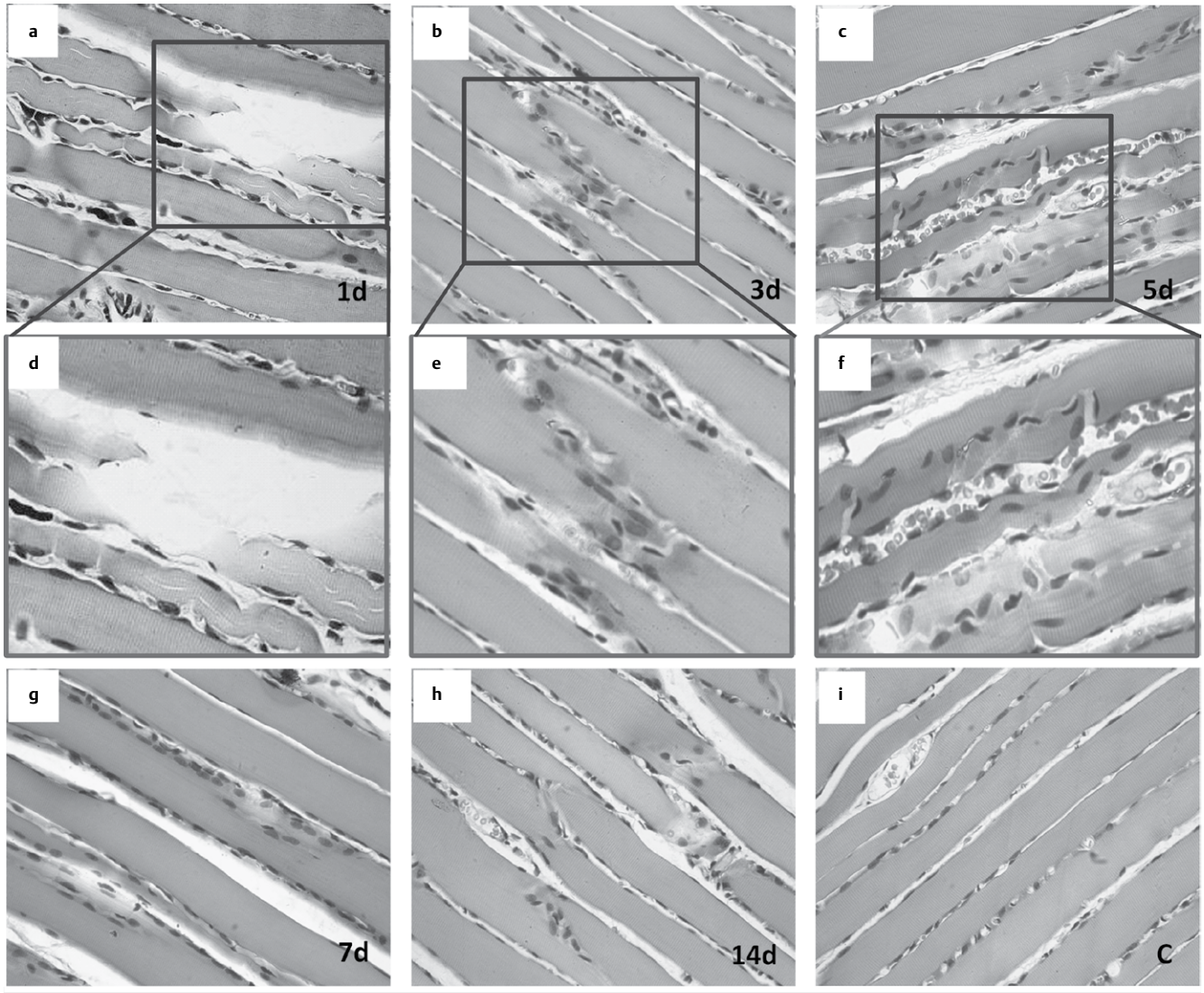
We also studied the serum level of BDNF in rats following downhill running and found a sustained elevation of serum BDNF (▶ **Fig. 1d**), which is different from a transient increase of peripher-



▶ **Fig. 2** Injury of skeletal muscle following downhill running. Representative basic fuchsin staining of skeletal muscle from sedentary rats **a** and rats 1d following downhill running **b**; magnification, 400X. **c** Dynamic serum CK activity post-exercise in 5 rats. **d** Statistical analysis of **c**; Data are Mean ± SEM. *, $p < 0.05$ compared to control group.



▶ **Fig. 3** MyoD staining in skeletal muscle at different time points following downhill running. MyoD expression in skeletal muscle of sedentary rat **a** and rats 1 day **b**, 3 days **c**, 5 days **d**, 7 days **e**, and 14 days **f** following downhill running; magnification, 400X. **g** Quantitative analysis of the percentage of cells with positive MyoD staining (n = 3). Data are Mean ± SEM. *, $p < 0.05$ compared to control group.

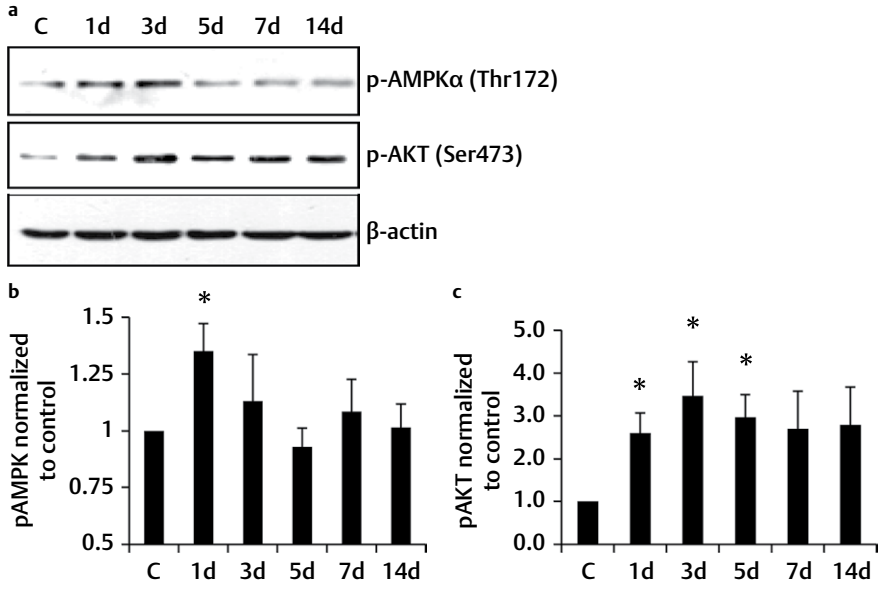


▶ **Fig. 4** Dynamic histological study of skeletal muscle following downhill running. Representative H&E staining of skeletal muscle from exercise rats **a-h** and sedentary rats **i**. **a-c**, **g** and **h** represent staining of the skeletal muscle 1 day **a**, 3 days **b**, 5 days **c**, 7 days **g** and 14 days **h** following downhill running in rats (magnification 400X). **d, e** and **f** are the magnified images from **a, b** and **c**, respectively.

al BDNF level following exercise intervention reported by several studies [16, 33, 36]. Since platelets were found to store and release BDNF [13], the transient increase of peripheral BDNF may be due to the release of BDNF from platelets in response to exercise. On the other hand, BDNF could pass through the blood-brain barrier (BBB) [27], and brain-derived BDNF is regarded as a major contributor to the peripheral level of BDNF following exercise [32]. Berchtolk et al. found that BDNF protein levels in hippocampus remains elevated until two weeks after exercise ends [3], suggesting the long-lasting increase of BDNF caused by exercise. Accordingly, the continuously increased BDNF may pass through BBB to the blood, which may explain the long lasting increase of serum BDNF levels following exercise. Our data on the different expression pattern of BDNF levels in serum and in skeletal muscle further indicate that skeletal muscle-originated BDNF may play a local role in skeletal muscle in response to exercise.

Unaccustomed eccentric exercise, such as downhill running has been widely reported to result in acute muscle damage, whereas concentric exercise does not [1, 19]. In response to downhill running, we observed myofiber disruption (▶ **Fig. 4a,d**) and positive staining of basic fuchsine (▶ **Fig. 2a,b**) in skeletal muscle one day following downhill running, indicating the injury of skeletal muscle cells. Besides, an increase of serum CK activity, a widely used marker for skeletal muscle injury [8], was found in rats five days following downhill running (▶ **Fig. 2c,d**). Our data is in accordance with several studies in which the eccentric exercise has been characterized with skeletal muscle injury [1, 5] and with a delayed increase of CK activity (between two and seven days) [35, 37]. It has been proposed that the delayed appearance of serum CK activity may be linked to inflammation, which leads to the increased permeability of sarcolemma [2]. As a result, the BDNF up-regulation five days post-exercise may be induced by a delayed injury in skeletal muscle following downhill running.

Regeneration is normally triggered following injury. Skeletal muscle cells are poorly capable to regenerate, and the repair of damaged skeletal muscle is undertaken by satellite cells. Upon skel-



▶ **Fig. 5** Time-dependent activation of AMPK and AKT in skeletal muscle following downhill running. **a** Representative results of phosphorylation of AMPK and AKT at different time points following downhill running. Quantification of activated AMPK **b** and AKT **c** compared to corresponding β-actin level (n = 3). Data are presented as means ± SEM. * , $p < 0.05$ compared to sedentary group.

etal muscle injury, satellite cells start to proliferate (satellite cell activation), a process characterized by expressing myogenic transcription factor, such as MyoD and Myf5 [24]. We found up-regulation of MyoD expression in skeletal muscle from the first day following downhill running (▶ **Fig. 3**), which is in accordance with previous data that eccentric exercise results in muscle damage accompanied by increased satellite cell activity 24 h post-exercise [19]. Our results indicate that the regeneration process has been triggered in soleus muscle by downhill running. At the same time, we observed increased BDNF protein levels in skeletal muscle (▶ **Fig. 1b,c**). However, previous studies showed that BDNF knockout in skeletal muscles did not lead to any significant difference in the number of satellite cell-derived myoblasts in culture [9], suggesting that BDNF could not facilitate the proliferation of satellite cells. We next tested the activation of AMPK, as BDNF was found to activate AMPK in L6 myotubes in vitro [23]. A significant increase of AMPK activation was found one day following downhill running (▶ **Fig. 5a,b**). The elevation of BDNF in skeletal muscle at the early time points post-exercise may therefore be related to energy metabolism.

After being activated, satellite cells differentiate and fuse with pre-existing myofibers and with one another to form new myofibers (muscle remodeling) [28]. To explore the role of BDNF in skeletal muscle, we next performed histological studies to examine the repaired and newly-formed myofibers, which can be identified by their centrally located nuclei [39]. Centralized myonuclei were found in skeletal muscle 5d and 7d following downhill running, showing newly-formed myofibers (▶ **Fig. 4c,f**). At the same time, we observed elevation of BDNF mRNA level and increased expression of BDNF protein in skeletal muscle (▶ **Fig. 1a,b,c**). We further confirmed the location of BDNF expression in regenerated skeletal muscle through immunohistochemistry study (▶ **Fig. 1e–h**). In addition, Western blot analysis showed that activation of AMPK was disappeared five days post exercise when AKT was maintained activated (▶ **Fig. 5**), indicating the anabolic status of skeletal muscle during regeneration. Moreover, an in vitro study showed that myotubes from BDNF MKO myoblasts contained fewer myonuclei and exhibited an increase in the number of unfused myocytes compared with controls [9], indicating that BDNF is required for the formation of new myofibers. Therefore, all these data indicate that exercise-induced BDNF at the late time points participates in regulating the remodeling of damaged skeletal muscle.

In summary, our study showed early and late elevations of BDNF in skeletal muscle following downhill running. Late increase in BDNF is involved in regulating the remodeling of damaged skeletal muscle during regeneration. Our results may contribute to further understanding the mechanism of exercise-induced skeletal muscle regeneration, which will help us in turn to design better strategies for improving skeletal muscle functions, as well as for treating aged and diseased skeletal muscle.

Acknowledgements

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Conflict of interest

We declare that there are no conflicts of interests, whether financial or otherwise.

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