Full Paper

Liver Hydrolysate Assists in the Recovery From Physical Fatigue in a Mouse Model

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Abstract. It is reported that liver hydrolysate (LH) enhances liver function. However, the effects of LH on physical fatigue are unknown. The aim of this study was to investigate the effect of LH on alterations in locomotor activity and energy metabolism such as 5'-AMP-activated protein kinase (AMPK), glycogen content, and blood lactic acid, after forced walking. Adult male ddY mice were used. Locomotor activity, AMPK phosphorylation, and glycogen content in the liver and soleus muscle, as well as blood lactic acid were determined following LH treatment before and/or after forced walking. The locomotor activity significantly decreased after forced walking for 3 h. Two administrations of LH (30 or 100 mg/kg) significantly increased the locomotor activity, while a single administration either before or after forced walking did not show any specific effect. Administering LH twice activated AMPK in the liver and soleus muscle. Glycogen levels significantly decreased in both the liver and soleus muscle after forced walking, whereas the blood lactate level significantly increased. In contrast, administering LH twice increased muscle glycogen and decreased blood lactic acid. These findings indicate that LH produced an anti-fatigue effect and that this effect appears to involve the efficient glycogen utilization through activation of AMPK.

[Supplementary Figure: available only at <http://dx.doi.org/10.1254/jphs.13084FP>]

Keywords: liver hydrolysate, physical fatigue, 5'-AMP-activated protein kinase, glycogen, lactic acid

Introduction

Fatigue is characterized by a physical and/or mental weariness resulting in negative impacts on work performance and exercise intensity, family life, and social relationships (1). Fatigue can be classified as secondary, physiologic, or chronic. Secondary fatigue results from disturbed sleep, depression, excess exertion, and medication side effect. Physiological fatigue is caused by inadequate rest, physical effort, or mental

strain (2). Chronic fatigue syndrome involves a persistent unexplainable fatigue lasting for more than 6 months, but the etiology remains unclear (3). Long-term physical and mental fatigue leads to health damage and chronic fatigue (4). Physical fatigue is also called peripheral fatigue and may be accompanied by deterioration in performance (5). Recent studies have demonstrated that energy metabolism is involved in the pathophysiology of fatigue (6).

5'-AMP-activated protein kinase (AMPK) is a key regulator of cellular and whole-body energy balance. AMPK phosphorylates and regulates many proteins involved in nutrient metabolism, largely acting to suppress anabolic ATP-consuming pathways while stimulating catabolic ATP-generating pathways (7). The activa-

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tion of AMPK is largely determined by phosphorylation of Thr¹⁷² on the α subunit, which causes a greater than 20-fold increase in activity (8). The role of AMPK has been extensively studied in skeletal muscle and liver where it was demonstrated to increase glycogen breakdown, glycolysis, glucose uptake, and fatty acid oxidation (9). Activation of AMPK by exercise, genetic manipulation, and the AMPK agonist AICAR results in enhanced endurance performance (9). Thus, this may lead to considerable interest in AMPK as a marker for physical fatigue.

Liver hydrolysate (LH) is used as a pharmaceutical agent in Japan. LH is obtained via an enzymatic degradation of livers. It is reported that LH consists primarily of amino acids and peptides (10) and enhances liver functions such as the protection against acetaldehyde- and ethanol-induced toxicity (11, 12). Previously, we have studied the anti-fatigue effect of a liquid nutritive and tonic crude drug including vitamins and amino acids (13). However, the effects of LH on physical fatigue are unknown.

In the present study, we investigated the effects of LH in a forced walking test. This model is also considered useful for evaluating physical fatigue, since locomotor activity counts after forced walking decrease in this test in comparison to those of mice not forced to walk (13). We also investigated the mechanism underlying the anti-fatigue effect of LH.

Materials and Methods

Animals

Male mice from the strain ddY weighing 26–28 g were used in all experiments. Animals were kept in plastic cages with free access to food and tap water in an animal room maintained under a controlled environment (23°C $\pm$ 1°C, 55% $\pm$ 5% humidity, 12 h/12 h alternate light/dark cycles with lights on at 8:00 a.m.). Groups of 5–28 mice for behavioral experiments, 4–5 mice for western blotting experiments, 3–5 mice for glycogen assay, and 6–7 mice for lactate assay were used only once. All experiments were performed following the approval of the Ethics Committee for Animal Experiments at Tohoku Pharmaceutical University and in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Compound

The AMPK agonist AICAR (5-aminoimidazole-4-carboxamide-1- β -D-ribofuranosyl 5'-monophosphate) was purchased from Toronto Research Chemicals, Inc. (North York, Canada). AICAR was dissolved in saline. AICAR

Table 1. Main components of liver hydrolysate

	(%)
Total Amino Acids, Peptides and Protein	75.00
Amino Acid	(34.89)
Peptides and Protein	(40.11)
Total Saccharides	5.7
Lipids	0.02
Total Nucleic Acid Bases	1.35
Inorganic Compounds	2.89
Moisture	2.08
Vitamins	0.10
Glutathione	0.42
Amino Acid	(mg/g)
Ala	31.8
Arg	1.2
Asp	17.6
Cystine	2.5
Glu	35.0
Gly	19.7
His	9.0
Ile	26.0
Leu	48.3
Lys	27.3
Met	0.6
Phe	23.7
Pro	18.8
Ser	22.5
Thr	22.4
Trp	7.0
Tyr	2.9
Val	32.6

was administered in a volume of 10 ml/kg body weight by intraperitoneal (i.p.) injection. The composition of the LH used in the present study, and supplied from Zeria Pharmaceutical Co., Ltd., Tokyo, is shown in Table 1. LH is obtained via an enzymatic degradation of livers (10). The LH was dissolved with distilled water. Mice were orally given either vehicle (distilled water) or LH at 10 ml/kg mouse body weight.

Forced walking exposure

The method for applying forced walking has been described in a previous report (13, 14). Briefly, forced walking was imposed on 10 mice at room temperature in a cylindrical cage (37-cm diameter), which was rotated on the horizontal axis at 2.0 rpm by an electric motor, giving a walking speed of 2.3 m/min for 3 h. Control mice were placed in a cylindrical cage without rotation.

Protocol and evaluation of physical fatigue

Single treatment groups: Vehicle or LH (30 or 100 mg/kg)

Fig. 1. Effect of LH on locomotor activity after forced walking for 3 h. A: Experimental time course, see “Experimental protocol (A)” on lower right. B: Experimental time course, see “Experimental protocol (B)” on lower right. C: Experimental time course, see “Experimental protocol (C)” on lower right. Each column shows the locomotor activity for 90 min. Error bars represent the S.E.M. Statistical differences were determined using the Tukey-Kramer test with one-way ANOVA [A: $F(3, 46) = 12.01$, $P < 0.01$; B: $F(3, 61) = 13.22$, $P < 0.01$; C: $F(3, 62) = 8.89$, $P < 0.01$]. ** $P < 0.01$ vs. Control/Water group. # $P < 0.05$, ### $P < 0.01$ vs. FW/Water group.

was orally administered before (protocol A) or 15 min after (protocol B) forced walking for 3 h. Following forced walking, the mice were placed in a multichannel Supermex activity box (Muromachi Kikai, Co., Tokyo) and allowed to adapt for 15 min before locomotor activity was measured every 15 min for 90 min. This instrument can monitor even minute movements in all 3 planes of motion (sagittal, coronal, and horizontal) as one movement, owing to its infrared sensor with multiple Fresnel lenses that can be moved close enough to the cage to capture multidirectional locomotor alterations in a single mouse. The Supermex instrument was connected to a behavioral analyzing system (CompACT AMS) (Muromachi Kikai), which can interpret each movement as one count. Therefore, vertical movements such as jumping, as well as horizontal movements such as walking and running, could be counted. Measurements

were performed between 12:00 and 16:00 h, during the light phase.

Double treatment groups: Vehicle or LH (30 or 100 mg/kg) was orally administered before the start of the 3-h forced walking. After forced walking, the mice were placed in the activity-monitoring box as above and allowed to adapt for 15 min. Then, either vehicle or LH (30 or 100 mg/kg) was orally administered a second time (protocol C). Locomotor activity was measured every 15 min for 90 min. Protocols A to C are illustrated in Fig. 1. AICAR (6.25 – 50 mg/kg, i.p.) was administered before forced walking. Following forced walking for 3 h, mice were individually placed in activity cages and allowed to adapt for 15 min before a second i.p. injection of AICAR or saline. Thus, the time course for AICAR injection is the same as shown in protocol C.

Western blotting

Double-treated mice were used for western blotting experiments. Twenty mice were randomly divided into four groups. The mice were sacrificed by decapitation 15 min after the last vehicle or LH administration. After sacrifice, the liver and soleus muscle were immediately removed on an ice-cold glass plate. Individual tissue samples were homogenized in 150 μ l of CellLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at $15,000 \times g$ for 15 min at 4°C. Supernatants were collected and their protein concentration determined using the Advanced Protein Assay Reagent (Cytoskeleton, Inc., Denver, CO, USA). Supernatants were then diluted in 4 $\times$ Laemmli sample buffer (300 mM Tris-HCl (pH 6.8), 8% SDS, 40% glycerol, 12% 2-mercaptoethanol, and 0.012% bromophenol blue) and incubated at 95°C for 5 min. All dissections and sample preparation were performed on the same day. Aliquots of the obtained extracts (30 μ g of protein/well for each extract) were loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane, which was then incubated with blocking solution [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.01% Tween 20, and 5% skim milk] for 1 h and probed with antibodies against AMPK α (2532; Cell Signaling Technology, Beverly, MA, USA) and p-AMPK α (Thr¹⁷²) (2531, Cell Signaling Technology) overnight at 4°C. The membrane was then washed with blocking solution without milk, incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 2 h followed by visualization of the immunoreactive species with the ECL select western blotting detection reagent (GE Healthcare, Little Chalfont, UK).

Glycogen and blood lactate assays

The liver and soleus muscle were stored at -80°C until glycogen content was assayed using a commercial kit (Glycogen Assay Kit; Bio Vision, Inc., Milpitas, CA, USA).

Lactate was measured in blood samples collected from the heart using Lactate Pro2 (Arkrey, Kyoto).

Statistical analyses

Results are expressed as means $\pm$ standard errors of the mean (S.E.M.). The significance of differences was determined by the Student's *t*-test for two-group comparison and by a one-way analysis of variance (ANOVA), followed by Newman-Keuls Multiple Comparison test for multigroup comparisons. $P < 0.05$ represented a significant difference.

Results

As shown in Fig. 1, A – C, the locomotor activity in water-treated mice after 3 h of forced walking was significantly decreased compared to the unexercised control group ($P < 0.01$). LH at 30 or 100 mg/kg given either before or after forced walking did not show any effects on locomotor activity different from water after forced walking (Fig. 1: A and B). In contrast, the mice given LH at 30 or 100 mg/kg both before and after forced walking showed significantly higher locomotor activity than the mice given water [LH (30 mg/kg); $P < 0.01$, LH (100 mg/kg); $P < 0.05$, Fig. 1C]. However, administering LH twice had no effect on locomotor activity in unexercised mice (Supplementary Fig. 1, available in the online version only). We noted that LH significantly increased locomotor activity 15 min after the last LH administration (data not shown). Therefore, samples of liver, soleus muscle, and blood were collected from a subset of mice from this time point for analyses.

Forced walking for 3 h significantly increased AMPK phosphorylation at Thr¹⁷² in the liver but not in the soleus muscle when compared with control mice (Student's *t*-test, $t = -2.4$, $P < 0.05$; not illustrated in Fig. 2A since the graph presents multiple group comparisons). Compared to water, the administration of LH (100 mg/kg) increased AMPK phosphorylation at Thr¹⁷² in both the liver and soleus muscle after forced walking without changes in the total amount of AMPK protein (Fig. 2: A and B).

The mice given the AMPK agonist AICAR at 12.5 or 25 mg/kg both before and after forced walking showed a significantly higher locomotor activity than mice given saline [AICAR (12.5 mg/kg); $P < 0.01$, AICAR (25 mg/kg); $P < 0.05$, Fig. 3].

The glycogen content in both the liver and soleus muscle of mice given water after forced walking was significantly decreased compared with the unexercised control group (Fig. 4). Muscle glycogen of mice given LH at 100 mg/kg after forced walking was significantly increased compared with the mice who received water after forced walking (Fig. 4B). Although there was a trend towards an increase in the liver, differences in glycogen content did not reach significance between mice given LH at 100 mg/kg or water after forced walking (Fig. 4A, $P = 0.10$). The blood lactate level significantly increased after forced walking for 3 h in mice given water. In contrast, the increase was significantly suppressed by LH treatments (30 mg/kg, $P < 0.01$; 100 mg/kg, $P < 0.05$; Fig. 5).

Fig. 2. LH treatment increased AMPK phosphorylation in the liver (A) and soleus muscle (B) after forced walking. Representative images of western blots probed with antibodies against phosphorylated AMPK α (Thr¹⁷²) and total AMPK α in the liver and soleus muscle (upper panels). Densitometric quantitation of AMPK α (Thr¹⁷²) phosphorylation is shown (lower panels). Data are expressed as fold expression relative to the control. Error bars represent the S.E.M. The numbers in parentheses indicate the number of mice in each group. Statistical differences were determined by the Tukey-Kramer Test with one-way ANOVA for multigroup comparisons [A: $F(3, 16) = 4.15$, $P < 0.05$; B: $F(3, 12) = 2.41$, $P = 0.11$], and by Student's *t*-test for two group comparison (between control and FW/Water groups). * $P < 0.05$ vs. control group. # $P < 0.05$ vs. FW/Water group.

Fig. 3. Effect of AICAR on locomotor activity after forced walking for 3 h. Each column shows the locomotor activity for 90 min. Error bars represent the S.E.M. Statistical differences were determined using Tukey-Kramer test with one-way ANOVA [$F(5, 72) = 2.52$, $P < 0.05$]. ** $P < 0.01$ vs. Saline group. # $P < 0.05$, ## $P < 0.01$ vs. FW/Saline group.

Discussion

This is the first study to show that LH can induce a recovery from fatigue and activate AMPK in the liver and muscle. LH treatment also increased muscle glycogen and reduced blood lactic acid. The underlying mechanism

of the anti-fatigue action of LH is not known, but AMPK activation may be involved.

LH is produced via an enzymatic degradation in the liver. This LH contains various components, such as amino acids, peptides, minerals, nucleic acids, glutathione, and vitamins, but some components remain to be identified (Table 1). First, we investigated the anti-fatigue effect of LH on decreased locomotor activity after forced walking for 3 h. In another physical fatigue animal model, the anti-fatigue effect was evaluated by the use of a swimming pool rigged as a forced swimming apparatus for measuring maximum swimming time. It is well known that the anti-fatigue effect compounds prolong the swimming time in this forced swimming test (15–19). In addition, this forced swimming model shows decreased glycogen in the liver and skeletal muscle, and increased blood lactic acid (19, 20). The forced walking model has also been used to evaluate the anti-fatigue effects of various compounds including nutritive and tonic crude drugs (13, 21), and bonito extract (14). After forced walking for 3 h, the mice in the present study were found to have a decreased glycogen content in their liver and soleus muscle and an increased level of lactic acid in their blood, indicators of physical fatigue (Figs. 4 and 5). A combined administration of LH before and after the 3-h forced walking increased locomotor activity following the exercise, indicating

Fig. 4. Effect of LH on the liver (A) and soleus muscle (B) glycogen after forced walking. Error bars represent the S.E.M. The numbers in parentheses indicate the number of mice in each group. Statistical differences were determined the Tukey-Kramer test with one-way ANOVA [A: $F(3, 11) = 3.81, P < 0.05$; B: $F(3, 16) = 2.70, P = 0.08$]. * $P < 0.05$ vs. Control/Water group. # $P < 0.05$ vs. FW/Water group.

Fig. 5. Effect of LH on blood lactate after forced walking. Error bars represent the S.E.M. Statistical differences were determined using the Tukey-Kramer test with one-way ANOVA [$F(3, 22) = 8.22, P < 0.01$]. ** $P < 0.01$ vs. Control/Water group. # $P < 0.05$, ## $P < 0.01$ vs. FW/Water group.

that LH possesses an anti-fatigue effect (Fig. 1).

We found that LH activated AMPK in the liver and muscle (Fig. 2), suggesting that LH may act as an AMPK agonist. It is noteworthy that LH can activate AMPK in the liver and muscle. AMPK is known to play a major role in energy homeostasis and is considered a key master switch regulating glucose and lipid metabolism (22). AMPK activation switches on ATP-producing processes and inhibits ATP-consuming anabolic processes (23). In the liver, activated AMPK inhibits energy-consuming biosynthetic pathways, including fatty acid and cholesterol biosynthesis, yet switch on catabolic

pathways that generate ATP, such as fatty acid oxidation, glucose uptake, and glycolysis (24 – 26). AMPK activation changes skeletal muscle myofiber type composition, which mimics the fiber type switch induced by endurance training (27). Additionally, it is reported that treatment with the AMPK agonist AICAR could induce metabolic genes expression and enhance running endurance (28). Therefore, all the above findings suggested that AMPK might act as a key mediator of endurance training-induced changes, which was also supported further by the result that twice AICAR treatment produced an anti-fatigue effect (Fig. 3). It is well accepted that the most important physiological effect of fatigue is on the energy metabolism from muscular activity, and the development of exercise endurance is the most powerful representation of anti-fatigue enhancement (29).

In the present study, LH treatment was found to increase glycogen content in muscle, which may well contribute partly to the LH related recovery from fatigue. It is well known that exercise-induced fatigue affects performance, but fatigue can be attributable to many factors, including accumulation of metabolites, and depletion of muscle glycogen (30 – 32). At intermediate exercise intensity, exhaustion is due mostly to depletion of muscle glycogen (30, 33). The saved glycogen could become an available energy source for the following phases of exercise, which would delay the onset of fatigue. So increasing the muscle glycogen store is conducive to recovery from fatigue. The glycogen-sparing effect of LH could provide an important survival advantage in situations requiring extended periods of prolonged endurance exercise because glycogen depletion is associated with physical exhaustion, and slower

utilization of glycogen results in improved endurance exercise performance.

Finally, LH treatment was found in the present study to decrease blood lactic acid. The liver converts lactate back to glycogen and releases glycogen into the blood. The muscle produces plenty of lactic acid when it obtains enough energy from anaerobic glycolysis almost simultaneously, when doing high-intensity exercise. Furthermore, the increase in lactic acid level will bring about a reduction of pH in muscle tissue and blood, as well as induce many side effects from various biochemical and physiological processes. Therefore, a rapid removal of lactic acid from blood is beneficial for relieving fatigue.

In conclusion, we have demonstrated that LH induces recovery from fatigue in a forced walking test, at least in part, by activating AMPK in liver and muscle. LH treatment also increased muscle glycogen and decreased blood lactic acid. As the present study demonstrates that LH induces recovery from fatigue in a force walking test, it may prove beneficial for preventing fatigue. Further pharmacological research and identification of the active constituents in LH will be required.

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References

- Mehta RK, Agnew MJ. Influence of mental workload on muscle endurance, fatigue, and recovery during intermittent static work. *Eur J Appl Physiol*. 2012;112:2891–2902.
- Brown RF, Schutte NS. Direct and indirect relationships between emotional intelligence and subjective fatigue in university students. *J Psychosom Res*. 2006;60:585–593.
- Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, Komaroff A. The chronic fatigue syndrome: a comprehensive approach to its definition and study. *Ann Intern Med*. 1994;121:953–959.
- Lowry TJ, Pakenham KI. Health-related quality of life in chronic fatigue syndrome: predictors of physical functioning and psychological distress. *Psychol Health Med*. 2008;13:222–238.
- Fitts RH. Cellular mechanisms of muscle fatigue. *Physiol Rev*. 1994;74:49–94.
- Myhill S, Booth NE, Howard JM. Chronic fatigue syndrome and mitochondrial dysfunction. *Int J Clin Exp Med*. 2009;2:1–16.
- Winder WW, Hardie DG. AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol*. 1999;277:E1–E10.
- Corton JM, Gillespie JG, Hardie DG. Role of the AMP-activated protein kinase in the cellular stress response. *Curr Biol*. 1994;4:315–324.
- Hardie DG, Sakamoto K. AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology (Bethesda)*. 2006;21:48–60.
- Inoue N, Hamasaki A, Hidaka S, Miura N, Fukahori M, Maruyama M, et al. [Analysis of the components of porcine liver hydrolysate and examination of the antioxidant activity and angiotensin converting enzyme (ACE)-inhibiting activity]. *Yakugaku Zasshi*. 2013;133:107–115. (text in Japanese with English abstract)
- Washizuka M, Hiraga Y, Furuichi H, Izumi J, Yoshinaga K, Abe T, et al. [Effect of liver hydrolysate on ethanol- and acetaldehyde-induced deficiencies]. *Nihon Yakurigaku Zasshi*. 1998;111:117–125. (text in Japanese with English abstract)
- Kishimoto R, Hasegawa E, Shirakawa Y. Effect of liver hydrolysate on serum alcohol and its metabolite levels in a mouse model for acute alcohol intoxication. *Jpn Pharmacol Ther*. 2011;39:199–207.
- Tadano T, Nakagawasai O, Nijima F, Tan-No K, Hanawa MA, Sakata Y, et al. Effect of nutritive and tonic crude drugs on physical fatigue-induced stress models in mice. *Pharmacol Res*. 2003;47:195–199.
- Nozawa Y, Yamada K, Okabe Y, Ishizaki T, Kuroda M. The anti-fatigue effects of the low-molecular-weight fraction of bonito extract in mice. *Biol Pharm Bull*. 2009;32:468–474.
- Takii H, Ishihara K, Kometani T, Okada S, Fushiki T. Enhancement of swimming endurance in mice by highly branched cyclic dextrin. *Biosci Biotechnol Biochem*. 1999;63:2045–2052.
- Kamakura M, Mitani N, Fukuda T, Fukushima M. Antifatigue effect of fresh royal jelly in mice. *J Nutr Sci Vitaminol (Tokyo)*. 2001;47:394–401.
- Koh JH, Kim KM, Kim JM, Song JC, Suh HJ. Antifatigue and antistress effect of the hot-water fraction from mycelia of *Cordyceps sinensis*. *Biol Pharm Bull*. 2003;26:691–694.
- Murase T, Haramizu S, Shimotoyodome A, Tokimitsu I, Hase T. Green tea extract improves running endurance in mice by stimulating lipid utilization during exercise. *Am J Physiol Regul Integr Comp Physiol*. 2006;290:1550–1556.
- Takeda K, Machida M, Kohara A, Omi N, Takemasa T. Effects of citrulline supplementation on fatigue and exercise performance in mice. *J Nutr Sci Vitaminol (Tokyo)*. 2011;57:246–250.
- Ikeuchi M, Koyama T, Takahashi J, Yazawa K. Effects of astaxanthin supplementation on exercise-induced fatigue in mice. *Biol Pharm Bull*. 2006;29:2106–2110.
- Hanawa M, Asano T, Akiyama K, Yabe K, Tsunoda K, Tadano T, et al. Effect of Zena F-III, a liquid nutritive and tonic drug, on the neurochemical changes elicited by physical fatigue in mice. *Pharmacol Biochem Behav*. 2000;66:771–778.
- Lin CL, Huanug HC, Lin JK. Theaflavins attenuate hepatic lipid accumulation through activating AMPK in human HepG2 cells. *J Lipid Res*. 2007;48:2334–2343.
- Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol*. 2007;8:774–785.
- Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*. 2002;109:1125–1131.
- Hardie DG. The AMP-activated protein kinase pathway—new players upstream and downstream. *J Cell Sci*. 2004;117:5479–5487.

- 26 Yin HQ, Kim M, Kim JH, Kong G, Kang KS, Kim HL, et al. Differential gene expression and lipid metabolism in fatty liver induced by acute ethanol treatment in mice. *Toxicol Appl Pharmacol.* 2007;223:225–233.
- 27 Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, et al. Transcriptional co-activator PGC-1 α drives the formation of slow-twitch muscle fibres. *Nature.* 2002;418:797–801.
- 28 Narkar VA, Downes M, Yu RT, Embler E, Wang YX, Banayo E, et al. AMPK and PPAR δ agonists are exercise mimetics. *Cell.* 2008;134:405–415.
- 29 Belluardo N, Westerblad H, Mudó G. Neuromuscular junction disassembly and muscle fatigue in mice lacking neurotrophin-4. *Mol Cell Neurosci.* 2001;18:56–67.
- 30 Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. *Acta Physiol Scand.* 1967;71:140–150.
- 31 Hermansen L, Hultman E, Saltin B. Muscle glycogen during prolonged severe exercise. *Acta Physiol Scand.* 1967;71:129–139.
- 32 Sahlin K. Metabolic factors in fatigue. *Sports Med.* 1992;13:99–107.
- 33 Saltin B. Muscle glycogen utilization during work of different intensities. *Adv Exp Med Biol.* 1971;11:289–299.