

**Acute and chronic effects of high-intensity interval training
(HIIT) on postexercise intramuscular lipid metabolism in
rats**

Chen Min^{a&}, Zhou Lei^{a&}, Chen Siyu^a, Shangguan Ruonan^b, Qu Yaqian^a, Sun Jingquan^{a*}

^aInstitute of Sports Science, Sichuan University, Chengdu, China PR

^bCollege of Physical Education, Chengdu University, Chengdu, China PR

[&]These authors contributed equally to this work and should be considered co-first authors

Corresponding author

Jingquan Sun

E-mail: sunjingquan@scu.edu.cn (J.Q.Sun)

Telephone: +028 85418123

Address: Institute of Sports Science, Sichuan University, Chengdu, China PR

Short title: Effects of HIIT on muscular lipid metabolism

Summary

Recovery from exercise refers to the period between the end of a bout of exercise and the subsequent return to a resting or recovered state. It is a dynamic period in which many physiological changes occur. A large amount of research has evaluated the effect of training on intramuscular lipid metabolism. However, data are limited regarding intramuscular lipid metabolism during the recovery period. In this study, lipid metabolism-related proteins were examined after a single bout of exercise in a time-dependent way to explore the mechanism of how exercise induces intramuscular lipid metabolism adaptation. Firstly, all rats in the exercise group underwent a five-week training protocol (HIIT, five times/week), and then performed a more intense HIIT session after 72h of the last-time five-week training. After that, rats were sampled in a time-dependent way, including 0 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h following the acute training session. Our results discovered that five weeks of HIIT increased the

content of intramuscular triglyceride (IMTG) and enhanced the lipolytic and lipogenesis-related proteins in skeletal muscle. Furthermore, IMTG content decreased immediately post HIIT and gradually increased to baseline levels 48h postexercise, continuing to over-recover up to 96h postexercise. Following acute exercise, lipolytic-related proteins showed an initial increase (6-12h) before decreasing during recovery. Conversely, lipogenesis-related proteins decreased following exercise (6-12h), then increased in the recovery period. Based on the changes, we speculate that skeletal muscle is predominated by lipid oxidative at the first 12 hours postexercise. After this period, lipid synthesis-related proteins increased, which may be the result of body recovery. Together, these results may provide insight into how the lipid metabolism-related signaling changes after chronic and acute HIIT and how protein levels lipid metabolism correlates to IMTG recovery.

Keywords: High-Intensity Interval Training₁; Lipid Metabolism₂; Recovery₃; Adaptation₄; Intramuscular Triglycerides.

Introduction

Although exercise is a critical stress that drives the beneficial adaptations associated with routine physical activity, it is during the recovery period in which these adaptations take place. A fundamental and longstanding focus of exercise physiology has been the elucidation of the mechanisms underlying training adaptations. These adaptations are reflected by changes in contractile protein and function(Widrick et al., 2002), mitochondrial function(Spina et al., 1996), metabolic regulation(Green et al., 1992), intracellular signaling(Benziane et al., 2008), and transcriptional responses(Pilegaard et al., 2003). The molecular and cellular events that underpin adaptations to exercise are fundamental aspects of exercise biology(Egan et al., 2013). The responsiveness of signaling pathways to divergent exercise stimuli is essential for understanding the process of how our body adapts to exercise(Hawley et al., 2014). Over the last two decades, many intricacies of recovery have been uncovered through mechanistic studies(Halliwill et al., 2013). Some of these changes observed in recovery may be necessary for long-term adaptation to exercise training, yet some can lead to instability during recovery. Thus, it could be argued that the recovery period is equally important as the exercise stimulus. However, compared with the long-lasting beneficial effects of exercise, the underlying mechanisms of how exercise induces adaptation have not been thoroughly explored(Hughes et al., 2018). Therefore, this study aims to investigate the underlying causes of intramuscular lipid recovery after exercise.

Exercise-induced adaptations generally respond to changes in exercise volume, which is the combination of exercise intensity (i.e., work per unit time), exercise duration (i.e., time per session), and training frequency (i.e., sessions per week). Cellular stress also occurs in proportion to exercise intensity. There is strong evidence that higher intensities of exercise elicit a more significant metabolic signal than moderate intensities(Egan et al., 2013). In various forms, HIIT is today one of the most effective means of improving cardiorespiratory and metabolic function and, in turn, athletes'

physical performance(Buchheit et al., 2013). Nevertheless, there is less evidence available regarding HIIT's role in mediating lipid recovery in skeletal muscle. Therefore, this study used HIIT as the intervention method to investigate acute and chronic HIIT's effect on intramuscular lipid metabolism.

Skeletal muscle stores a large amount of fat in lipid droplets, especially in physically trained individuals. The adaptation of intramuscular triglyceride (IMTG) storage after exercise may benefit exercising tissue by supplying free fatty acids at the site of the energy demand(Hargreaves et al., 2018). However, our understanding of the regulation of fat metabolism in skeletal muscle during exercise lags behind that of muscular carbohydrate metabolism(Hargreaves et al., 2018). The mechanism controlling the increase in IMTG postexercise has not been fully determined. Therefore, the purpose of this study was to examine the time-course changes of selected IMTG controlling proteins after acute and chronic HIIT. The primary emphasis will be placed on the time-dependent postexercise lipid metabolism during the recovery period. Studies have reported that trained individuals have more efficient and stable intramuscular triglyceride metabolism, and have recommended the use of well-trained subjects when IMTG utilization is examined(Watt et al., 2002). Hence, we trained the rats in the acute training group for five weeks before performing the acute training. Firstly, the content of intramuscular triglyceride was measured after the acute and chronic intervention. Also, several muscular lipolytic proteins were examined, including p-CREB, CREB, p-AMPK, AMPK, and CPT-1B. The activity of CREB and AMPK has been proved to play an important role in skeletal muscle fat metabolism(Thomson et al., 2009). Besides, CPT-1B is responsible for the transfer of free fatty acids into the mitochondria (Ratner et al., 2015) and muscular lipid oxidation(Joseph et al., 2017). In addition to lipolysis, lipid storage also requires numerous other processes, such as lipogenesis. We detected several proteins involved in the regulation of intramuscular lipogenesis(Corbet et al., 2020, Eberle et al., 2004, Knobloch et al., 2013), including PPAR- γ , TGF- β 2, and FASN. We hypothesized that the change of IMTG is likely to be associated with the time-course change of lipid metabolism-related proteins.

Materials and Methods

Animals

The experiment was performed with SD rats (seven weeks of age, obtained from Chengdu DaShuo Biological Technology Co., Ltd. China). Rats were maintained on a standard rodent chow diet and water ad libitum under 12-h light and dark cycles. After the acclimation period, the animals were assigned randomly into two groups: the control group (C, N= 6) and the exercise group (N= 41), which performed a five-week training protocol (HIIT, five times/week). After 72h of the last-time five-week training, rats were challenged with an acute bout of HIIT. The rats in the exercise group were subdivided into 8 groups: control group(CE, N= 6), which only underwent the five-week training, and acute exercise groups(N=35), which were sampled at 0h, 6h, 12h, 24h, 48h, 72h, and 96h after the acute HIIT session.

Training protocols

All animals were initially familiarized with a motor-driven treadmill (Duan Animal Treadmill Co.Ltd, Huangzhou, China) for four days to avoid novelty and/or stress effects. Studies have highlighted the importance of utilizing well-trained individuals when IMTG utilization is examined (Watt et al., 2002). Hence, we trained the rats in the acute group for five weeks before performing the acute training. The five-week HIIT protocol consisting of running on a treadmill (6 sets at 25 m.min⁻¹ for 3min followed by 3min at 14.5 m.min⁻¹ with, five days/week). Each set of training was preceded by a warm-up (5 min at 14.5 m.min⁻¹). After this chronic training, the rats in the acute exercise group performed a more intense HIIT session (6 sets at 29.5 m.min⁻¹ for 3min followed by 3min at 16.5 m.min⁻¹) after 72h of the five-week protocol. After that, rats in the acute exercise group were sampled in a time-dependent way, including 0 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h after the acute training session.

Western blot

Firstly, the entire gastrocnemius was homogenized, with different muscle types mixed. Then, approximately 180 mg of mixed whole gastrocnemius muscle each group was homogenized again in ice-cold RIPA buffer and then centrifuged at 12000 RPM for 30 min at 4°C. The supernatant's protein concentration was determined by BCA assay (Thermo) and trimmed by PBS for western blotting analysis. 40-50µg protein for each lane was separated on a 10/12% SDS-PAGE gel and transferred onto a PVDF membrane. Then, blocked with 5% skimmed milk for 30-60min. Antibodies used for western blotting were p-CREB (Abcam, ab7540), PPAR-γ (Abcam, ab272718), p-AMPK α 1 α 2 (Abcam, ab133448), AMPK α 1 α 2 (Abcam, ab207442), TGF- β 2 (Abcam, ab113670), FASN (CST, 31805), CPTIB (Abcam, 5-79065); GAPDH (Affinity, AF7021), CREB (Affinity, AF6188). Blots were developed using Western Lightning ECL (Affinity). All the bands were analyzed with Image J. GAPDH was used for the normalization of each protein to ensure the loading of equal quantities of protein.

Triglyceride assay

Intracellular triglycerides were assayed using a triglyceride assay kit (GPO-POD; Applygen Technologies Inc., Beijing, China). According to the manufacturer's recommended protocol. 50mg±5 of the gastrocnemius muscle of each rat was weighed and lysed on ice. After a 70°C heating for 10mins, each sample was placed in a 96-well plate with two duplicates and then mixed with the kit's A+B solution. After a 15mins incubation at 37°C and cooling to room temperature, the resultant purple color is measured using a spectrometer at 492 nm. Then the final values are normalized by each sample's protein concentration measured by the BCA assay.

Ethical approval

All procedures in the present study were approved by the Sichuan University animal ethics committee and carried out according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals.”

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8.0; GraphPad Software, Inc., La Jolla, CA, USA). The data are presented as the mean \pm standard deviation. Significant differences between the two groups were analyzed with the unpaired student test. The comparison among the multiple groups was assessed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The chronic and acute effect of HIIT on intramuscular triglycerides abundance

As shown in Fig. 1A, the five-week HIIT intervention induces significant increases in the abundance of triglycerides in rats' gastrocnemius. After the acute training, the content of IMTG significantly declines immediately after the training and gradually over-recovered to a level beyond pre-exercise in the gastrocnemius of trained rats (Fig. 1B).

The chronic and acute effect of HIIT on muscular lipolytic-related proteins

As shown in Fig. 2A, compared to the control rats, the five-week HIIT intervention induces significant increases in the expression of lipolytic-related proteins, including p-CREB/CREB, p-AMPK/AMPK, and CPT-1b. As for the acute training group, the expression of lipolytic-related proteins, including p-AMPK/AMPK, p-CREB/CREB, are upregulated after the acute HIIT and then gradually decline at the recovery period in the gastrocnemius of trained rats (Fig. 1B). Simultaneously, the expression of CPT-1b remains unchanged after exercise but suppresses at the recovery period and recovered at 72h after the acute training.

The chronic and acute effect of HIIT on muscular lipogenesis-related proteins

As shown in Fig. 3A, compared to the control rats, five-week HIIT intervention induces significant increases in lipogenesis-related proteins, including PPAR- γ , FASN, and TGF- β 2. As for the acute training group, the expression of lipogenesis-related proteins, including PPAR- γ and TGF- β 2, declines after acute HIIT and then gradually increases at the recovery period in the gastrocnemius of trained rats (Fig. 2B). At the same time, the expression of FASN increases after acute training and gradually returns to the baseline level after the peak is reached. This tendency is in line with the changes of postexercise IMTG.

Discussion

In the present study, we have examined, in rats' skeletal muscle, the effects of a single bout of HIIT and chronic HIIT on the expression of proteins involved in lipid metabolism, as well as the content of IMTG. We discovered that five weeks of HIIT increased the content of IMTG and enhanced the lipid metabolism-related proteins in skeletal muscle. Furthermore, the content of IMTG was declined after acute HIIT and gradually over-recovered to a level beyond pre-exercise. Besides, intramuscular lipid metabolism-related proteins changed differently after acute HIIT training. Based on the changes, we speculate that skeletal muscle is predominated by lipid oxidative at the first 12 hours postexercise. After this period, lipid synthesis-related proteins increased, and lipolytic-related proteins declined, which may be the result of body recovery. Together, these results may provide insight into how the lipid metabolism-related signaling changes after chronic and acute training, and how lipid metabolism-related proteins correlate to IMTG.

Fatty acid derived from intramuscular triglycerides is a significant energy source during exercise. Moderate-to-high intensity exercise results in higher energy demand in the exercising muscle. Thus, the adaptation of intramuscular triglyceride storage after exercise may benefit exercising tissue by supplying free fatty acids at the site of the energy demand(van Loon et al., 2003). However, the molecular mechanism underlying this adaptation is unknown (Bergman et al., 2020). It is established in previous studies that chronic HIIT induces intramuscular triglyceride storage(Zacharewicz et al., 2018). Our data is consistent with these studies, which show a significant increase in IMTG after five weeks of HIIT. Also, it is proved that exercise induces the consumption of intramuscular triglyceride storage during exercise and the recovery to the pre-exercise level usually takes 3-7 days(Kiens et al., 1998). Our data indicate that intramuscular triglyceride content declined immediately after exercise and gradually over-recovered to a level beyond pre-exercise at 72h after the acute training.

IMTG replenishment via skeletal muscle lipogenesis is influenced by intramuscular lipid metabolism, including lipolytic and lipogenesis(Lundsgaard et al., 2020). This necessitates metabolic recovery contributes to restoring substrate stores in recovery. To achieve that, a plethora of metabolic changes will occur to regain substrate homeostasis(Lundsgaard et al., 2020, Tsiloulis et al., 2015). However, the molecular mechanisms involved herein have not been subject to a thorough evaluation. To determine the intramuscular lipid metabolism condition during recovery, several proteins involved in the regulation of lipolysis and lipogenesis in skeletal muscle were examined after acute and chronic training. Lipolysis-related protein includes CPT-1B, which is responsible for the transfer of free fatty acids into the mitochondria(Ratner et al., 2015), AMPK, which is involved in regulating intramuscular triglyceride breakdown during exercise and is thought to be a crucial rate-limiting enzyme in intramuscular triglyceride breakdown(Kiens, 2006), and CREB, which plays an indispensable role in maintaining lipid homeostasis via protein kinase A-mediated phosphorylation of CREB and positively associate with lipolysis(Altarejos et al., 2011). It is well established that regular exercise increases the expression of lipolytic-related

proteins in skeletal muscle(Ray Hamidie et al., 2015, Ziaaldini et al., 2017). Our results are in accordance with previous research and showed that five-week HIIT is effective in increasing intramuscular lipolytic-related proteins. As for the acute protocol, since endurance exercise induces transient upregulation of the pro-oxidative intracellular mediators involved in stimulating fat oxidation and energy expenditure, it is well recognized that whole-body fatty acid oxidation remains increased for several hours following aerobic endurance exercise(Lundsgaard et al., 2020). In line with previous studies, our data show that in immediate and early recovery (0-12h), intramuscular lipid oxidative-related proteins, including the activity of AMPK and CREB, are sustained at a high level, which seems mainly to be a result of the increased energy demand at the post-exercise oxygen consumption (EPOC) period. After this period, the suppressed activity of AMPK and CREB may be the result of body recovery. However, the expression of CPT-1b has not been changed immediately after acute HIIT but suppressed during the recovery period. In summary, the declined expression of CPT-1b, AMPK, and CREB may favor the postexercise body recovery. Notably, data regarding the mechanisms underlying EPOC after HIIT is limited(Borsheim et al., 2003). Here, this study provides some evidence about the molecule changes in the HIIT-induced EPOC period. Our data show that the increased lipolytic-related proteins within the muscle may be one mechanism of the increased oxidation post-exercise. In terms of duration, some studies have shown that EPOC may last for several hours after exercise, others have concluded that EPOC is transient and minimal(Borsheim et al., 2003). The conflicting results may be resolved if differences in exercise intensity and duration are considered, since this may affect the metabolic processes underlying EPOC. In our study, the protein-level changes of lipid metabolism proteins indicate that the HIIT-induced EPOC period lasts for 12h. After the EPOC period, lipolytic-related proteins were suppressed, this change is conducive to the post-exercise energy recovery in skeletal muscles.

In addition, we also detected several key lipogenesis-related proteins including PPAR- γ , FASN, and TGF- β 2. They have been reported to play a crucial role in the muscular lipogenesis and the formation of lipid droplets (Corbet et al., 2020, Eberle et al., 2004, Knobloch et al., 2013). The changes in lipogenesis-related proteins after regular training is still controversial(Huang et al., 2017, Morifuji et al., 2005, Zheng et al., 2019). On the one hand, exercise has the effect of promoting weight loss, but on the other hand, there are also studies reporting that exercise upregulates lipogenesis-related proteins in skeletal muscle. Our results show that chronic HIIT is able to induce an increase in lipogenesis-related proteins. It was reported that after regular exercise, the upregulated expression of PPAR γ and FASN correlate with the increase of IMTG (Dobrzyn et al., 2010). This is in line with our result in the chronic training group. Studies regarding how TGF- β 2 responds to regular exercise are limited(Takahashi et al., 2019). Our chronic group's results show that five weeks of HIIT increases the expression of TGF- β 2 in skeletal muscle. As for the acute group, our time-point data provide evidence that two major lipogenesis-related proteins, TGF- β 2 and PPAR γ , declined immediately after exercise and then gradually over-recovered to a pre-exercise

level in the recovery period. It is reported that PPAR- γ negatively regulates lipid oxidation (Yan et al., 2016). Therefore, the decrease of lipogenesis-related proteins during the EPOC period coupled with the increases of lipolytic-related proteins together may act as a signaling pathway for the increased post-exercise oxidation. After the EPOC period, these lipid synthesis-related proteins increased, which may be a result of intramuscular lipid recovery. Besides, the expression of FASN slightly increased after exercise and reached a peak level at 12h after training. Previous studies have shown that the increase of lipogenesis-related proteins is able to promote the recovery of intramuscular triglycerides (Moseti et al., 2016). Therefore, the physiological significance of the maintained elevation in lipogenesis-related proteins might favor the resynthesis of skeletal muscle triglyceride stores.

The highlight of this study is that during exercise-induced recovery, the change tendency of lipid metabolism-related proteins is consistent with the change of IMTG. At the first 12h after HIIT, IMTG declined with the predominance of EPOC. After this period, IMTG gradually recovered with the increase of lipogenesis-related proteins and suppression of lipolytic-related proteins. Together, these results may provide insight into the timeline information for studies examining aspects of lipid metabolism and IMTG with HIIT. Such knowledge could aid the timing of biopsies for future human-based exercise studies and provide a greater understanding of the molecular responses to exercise. However, it is essential to mention that the specific time points in the current study may not have caught the actual peak inductions due to the transient nature of the responses. The timing of the peak may have been different between protocols (Brandt et al., 2016) and our finding only provided the effects of HIIT on postexercise intramuscular lipid metabolism in rats.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Figure legends

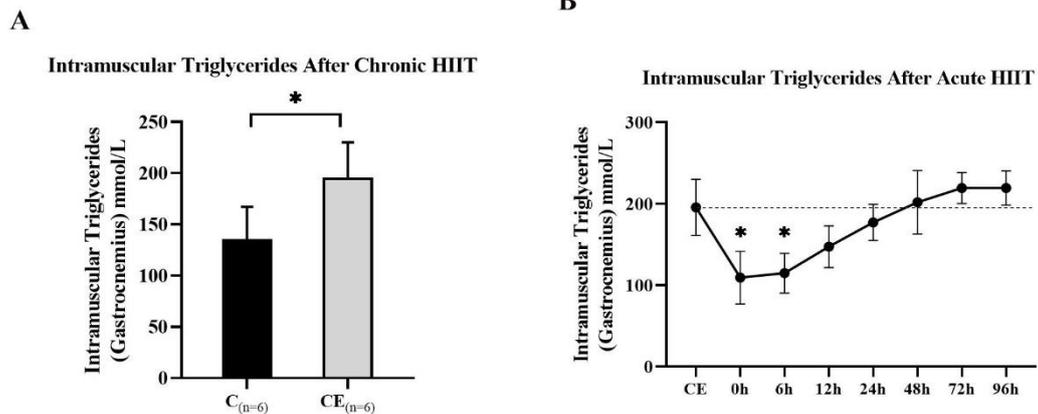


Fig. 1 The chronic and acute effect of HIIT on intramuscular triglycerides abundance in gastrocnemius. Experiments were performed with control group (C, n=6), five-week HIIT group (CE, n=6), and acute training group (0h, 6h, 12h, 24h, 48h, 72h, and 96h; n=5/each time-point). Two replicates were used for each sample. The data were presented as the mean \pm standard deviation. Each postexercise timepoint data are compared with CE and significant differences were analyzed with one-way ANOVA. *P<0.05.

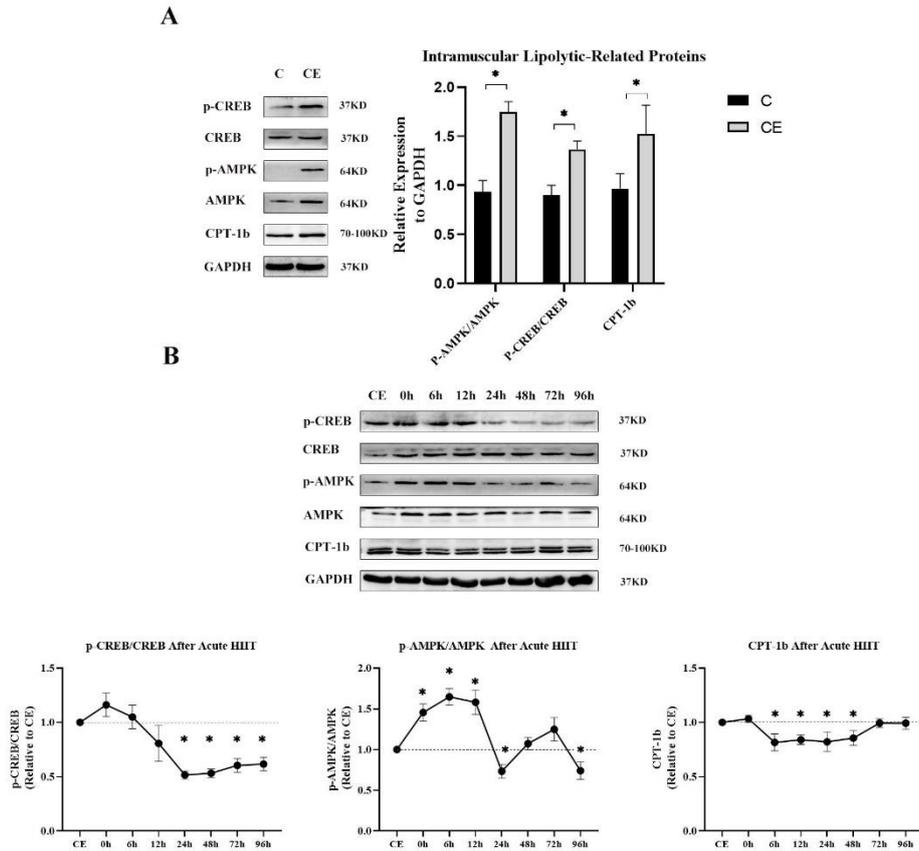
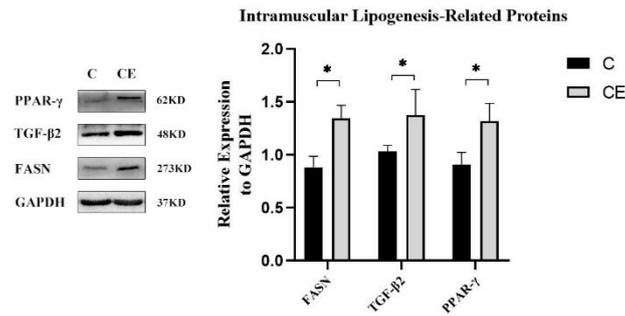


Fig. 2 Western blot analysis and relative fold protein expression of p-CREB/CREB, p-AMPK/AMPK, and CPT-1b. Relative expression levels were normalized to GAPDH. The acute group's protein level is normalized to GAPDH at first and then relative to the group of CE, which did not perform the acute training. Three bands are used for statistics. The data were presented as the mean \pm standard deviation. Each postexercise timepoint data are compared with CE and significant differences were analyzed with one-way ANOVA. * $P < 0.05$.

A



B

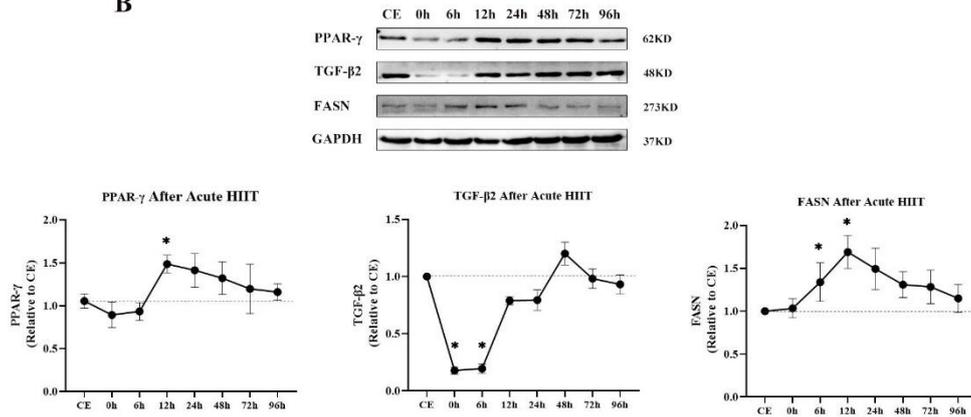


Fig. 3 Western blot analysis and relative fold protein expression of PPAR- γ , FASN, and TGF- β 2. Relative expression levels were normalized to GAPDH. The acute group's protein level is normalized to GAPDH at first and then relative to the group of CE, which did not perform the acute training. Three bands are used for statistics. The data were presented as the mean \pm standard deviation. Each postexercise timepoint data are compared with CE and significant differences were analyzed with the one-way ANOVA. * $P < 0.05$.