Intramuscular Mitochondrial Adaptation and Lipid Metabolic Alteration in Rats after Chronic High-intensity Interval Training (HIIT) of Different Training Periods

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Research Article

Keywords: Exercise, Lipid metabolism, Mitochondria, Skeletal muscle, Exercise training period

Posted Date: June 16th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1733332/v1

Original Research

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Acknowledgment

Thanks to all the peer reviewers for their opinions and suggestions.

Funding

This work was funded by a grant from the Fundamental Research Funds for the Central Universities of China and the National Natural Science Foundation of China (No. 31801002) grant. The authors are appreciative for Dr. Jingquan Sun`s help in obtaining the education grant.

Conflict of Interest

The authors declare no conflict of interest.

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Abstract

Background: High-intensity Interval Training (HIIT) is a time-efficient form of exercise and has gained popularity in recent years. However, at molecular level, the understanding about the effects of HIIT is not comprehensive, and even less is elucidated about HIIT of different training duration cycles, although different durations always lead to different post-training consequences.

Method: In this study, by training SD rats using HIIT protocols lasting for different training duration cycles, we investigated the adaptive response of intramuscular triglyceride abundance and mitochondria enhancement after HIIT training (2, 4, 6, 8, and 10 weeks). We selected 72h after the last session of training as the time point of sacrifice.

Results: The suppressed activation of the cAMP-PKA pathway indicates that skeletal muscle was in the recovery phase at this time point. Intramuscular triglyceride abundance was significantly elevated after 2, 4, and 10 weeks of HIIT. However, the lipid metabolism-related proteins inconsistently changed in a chaotic trend (see Table. 1). The expression levels of PGC1-α and COX IV decreased after 2 and 4 weeks of training and began to rise when the training duration reached 6 weeks. Interestingly, the variation tendency of PGC1-α and COX IV is very similar to that of CPT-1B.

Conclusion: Given the fact that CPT-1B is responsible for the transfer of free fatty acids into the mitochondria to facilitate muscular lipid oxidation, there is a great possibility that: A) the IMTG accumulation observed within 0-4 weeks [defined as phase 1 here] of HIIT might be primarily attributed to damaged mitochondria oxidation capacity. B) when the training duration reached 6 weeks [defined as phase 2], mitochondria function and biogenesis began to be improved by training stimulus and might result in the disappearance of IMTG increase. We believe the phase 2 is rather similar to the condition of athletes` paradox, which should be a healthy adaptation induced by exercise training. Also, for HIIT Rat Modelling, intervention duration cycles longer than 4 weeks are recommended if similar HIIT frame of this study is used.

Keywords

Exercise; Lipid metabolism; Mitochondria; Skeletal muscle; Exercise training period

Introduction

Mitochondrial free fatty acid (FFA) oxidation has long been recognized as a predominant energy contributor during aerobic exercise[1-4]. Despite the fact that skeletal muscle uptakes FFA from the circulation, the mitochondrial FFA consumption during aerobic exercise is largely dependent on IMTG (intramuscular triglyceride) hydrolysis[4]. It was observed that, when exercise intensity reached 65% of VO_{2max}, the contribution of plasma FFA significantly decreased and the rate of IMTG hydrolysis increased and provided about half of the FFA used for total fat oxidation[5-7]. These findings evidence the indispensable roles of IMTG and intramuscular mitochondria in exercise capacity enhancement. Actually, IMTG accumulation and mitochondria oxidation capacity are always promoted after different kinds of long-term aerobic training[8-11], which is regarded as positive post-training adaptation. For instance, IMTG storage and mitochondrial biogenesis were promoted after long-term aerobic exercise with different training intensities[12-14]. Also, higher IMTG content and strengthened mitochondria were observed in endurance athletes regardless of their training patterns[10, 15, 16].

Among various modes of aerobic training, HIIT is a time-efficient exercise strategy with similar training frameworks, which makes HIIT a very feasible training protocol for health promotion[17], and its positive effects on skeletal muscle have been previously reported in both animal studies and human studies. Jonathan`s group has found that regular HIIT increased maximal activities and total protein content of cytochrome c oxidase (COX) in human skeletal muscle, and the abundance of peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) and SIRT1 were also elevated after HIIT[18]. In animal studies, it has been reported that HIIT could increase the rates of mitochondrial fatty acid oxidation in fast-twitch and slow-twitch skeletal muscle fibers[19].

Despite a large number of studies on HIIT`s effects on skeletal muscle, the “time-related” features of HIIT have not been fully understood yet. Our group has previously focused on one side of the “time-related” features — different intramuscular physiological changes at different time points after one bout of HIIT exercise[11]. According to our results, intramuscular triglycerides content decreased immediately after acute HIIT and gradually recovered to baseline at 48h after exercise, and over-recover up to 96h post-exercise. Lipolytic proteins showed an initial increase (6-12h) before decreasing during recovery, while lipogenesis-related proteins decreased following exercise (6-12h post-exercise), then increased in the recovery period. Based on these changes, we hypothesized that lipid oxidation is predominant in skeletal muscle during the first 12 hours after exercise. After this period, lipogenesis is promoted, which might lead to energy substrate recovery[11].

Another side of the “time-related” features of HIIT is: the different physiological adaptations after HIIT of different training duration cycles. Training protocols of different durations always have different impacts on skeletal muscle[20]. Existing research pays more attention to the duration of each training session[21-23] instead of training duration cycles. Hence, systematically exploring the role of training duration cycles in exercise-induced skeletal muscle adaptation would not only enhance our understanding of training theory but also facilitate animal modeling in future sports science research. Here, SD rats were subjected to HIIT of different duration cycles and sacrificed at 72h after the last session of training to investigate the effects of training duration cycles on intramuscular triglyceride (IMTG) accumulation and mitochondria enhancement (mitochondrial biogenesis and oxidation capacity).

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 10 groups: 2-week exercise group (2E), 2-week control group (2C), 4-week exercise group (4E), 4-week control group (4C), 6-week exercise group (6E), 6-week control group (6C), 8-week exercise group (8E), 8-week control group (8C), 10-week exercise group (10E), and 10-week control group (10C). Rats in 2E, 4E, 6E, 8E, and 10E groups were subjected to regular HIIT lasting for 2, 4, 6, 8, and 10 weeks respectively and sacrificed at 72h after the last HIIT session. Rats in 2C, 4C, 6C, 8C, and 10C groups were given no intervention and sacrificed together with their corresponding exercise groups.

Training Protocol

To avoid excitement and stress, all animals were initially familiarized with a motor-driven treadmill (Duan Animal Treadmill Co.Ltd, Huangzhou, China) for four days. Consequently, we trained rats according to our previously established HIIT protocol, which consists of 6 sets of running (each set includes running at a constant speed of 25 $m.min^{-1}$ for 3min followed by a 3-minute intermittent phase at a constant speed of 14.5 $m.min^{-1}$[23]. Each training session was preceded by a warm-up (5 min at 14.5 $m.min^{-1}$). Using this protocol, rats in 2E, 4E, 6E, 8E, and 10E groups were trained for 2, 4, 6, 8, and 10 weeks respectively.

Western Blotting

After the animals were sacrificed, the gastrocnemius of each rat was quickly excised on ice and then stored at −80°C. Approximately 100mg of gastrocnemius was homogenized in ice-cold RIPA buffer and then centrifuged at 12,000RPM for 30min at 4°C. The supernatant's protein concentration was determined by BCA assay (Thermo) and trimmed by PBS for western blotting analysis.

About 40–50μg protein was loaded in each lane and 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 Anti-Rabbit Secondary Antibody (31,460, Invitrogen), Anti-Mouse Secondary Antibody (31,460, S0002), GAPDH (AF7021, Affinity), P-CREB (AB32096, Abcam), CREB (AB32515, Abcam), P-PKA (AF7246, Affinity), PKA (AF7746, Affinity), sterol regulatory element-binding protein 1c (SREBP-1C; AF4728, Affinity), FASN (CST, 31,805), P-ACC (Abcam, ab68191), acetyl-CoA carboxylase (ACC; CST, 3676s), CPT-1B (PA5-79065, Invitrogen), adipose triglyceride lipase (ATGL; AB207799, Abcam), P-ATGL (AB135093, Abcam), P-HSL (AF2206, Beyotime), hormone-sensitive lipase (HSL; AF6403, Affinity), PGC-1α (AB188102, Abcam), and COX IV (3E11, CST). 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 of Gastrocnemius

Intracellular triglycerides were assayed using a triglyceride assay kit (GPO-POD; Applygen Technologies Inc., Beijing, China). About 25±5mg of the gastrocnemius muscle of six mice of each

group were weighed and lysed on ice. After 70°C heating for 10min, each sample was placed in a 96-well plate with two duplicates and then mixed with the kit's A+B solution. After a 15min incubation at 37°C and cooling to room temperature, the resultant purple color is measured using a spectrometer at 492nm. Then the final values are normalized by each sample's protein concentration measured by the BCA assay.

Statistical Analysis

Data is presented as mean ± SD. The comparison between the means of two groups was assessed by the independent-samples t-test. We assigned * for values below 0.05. Statistical graphs and statistical analyses were carried out using Prism 8 (GraphPad).

Results

The activation state of the cAMP-PKA pathway at the sacrifice time point (72h post-exercise).

According to the previous study, we selected the ratios of P-CREB/CREB and P-PKA/PKA to indicate the activation state of the cAMP-PKA pathway[24]. Generally, the intramuscular cAMP-PKA signaling pathway was in a suppressed state when rats were sacrificed. As presented in Figure 1, the ratios of P-CREB/CREB (Thr198) were decreased in each exercise group compared with corresponding control groups. A similar changing trend could also be observed in the ratios of P-PKA/PKA (Ser133), although data in a few groups was not significant statistically.

Intramuscular triglyceride accumulation after HIIT of different training duration cycles.

As presented in Figure 2, intramuscular triglyceride (IMTG) content was significantly elevated after 2, 4, and 10 weeks of regular HIIT, while the IMTG changes after 6 and 8 weeks of training were not apparent compared with their corresponding control groups.

Changes in lipogenesis-related proteins after regular HIIT of different training duration cycles

The intramuscular changing trend in lipogenesis-related proteins after HIIT is rather chaotic. As presented in Figure 3, the expression levels of SREBP-1C decreased after 2 weeks of training, and a statistically insignificant upward trend was observed after 4 weeks of training. The expression levels of FAS decreased after 4 weeks of training and were elevated after 6 and 8 weeks of training. The ratio of P-ACC/ACC (phospho S79) was not significantly influenced.

Changes in lipolysis-related proteins after regular HIIT of different training duration cycles

As presented in Figure 4, the ratio of P-HSL/HSL (phospho S853) was almost decreased by all the training protocols. The ratio of P-ATGL/ATGL (phospho S406) was elevated after 2 and 4 weeks of

training. The expression levels of CPT-1B decreased after 2 and 4 weeks of training, and an upward trend was observed after 6, 8, and 10 weeks of training. The opposite changing trends in the ratio of P-HSL/HSL and P-ATGL/ATGL exhibit a quite chaotic change in lipolysis-related proteins.

Mitochondrial adaptations after regular HIIT of different training duration cycles

As shown in Figure 5, the expression levels of PGC1-α and COX IV decreased after 2 and 4 weeks of training and showed an upward trend after 6, 8, and 10 weeks of training except for the change of PGC1-α after 10 weeks of training.

Discussion

In this study, efforts have been made to establish a series of Rat HIIT Models using protocols of different training duration cycles (2, 4, 6, 8, and 10 weeks). We selected 72h post-exercise as the time point of sacrifice, and, according to our previous study, we assume that the energy substrate storage would be in the recovery phase at this time point[11]. This view is also validated in this present research when we observed the inhibited intramuscular cAMP-PKA pathway(a pathway that is believed to favor the post-exercise energy substrate recovery)[11, 25]. Afterward, the intramuscular triglyceride (IMTG) accumulation after HIIT of different training duration cycles was detected and showed an upward trend after 2, 4, and 10 weeks of training, while the changes after 6 and 8 weeks of HIIT is not apparent. Unexpectedly, the lipid metabolism-related proteins changed in a chaotic trend except for CPT-1B, while mitochondrial biomarkers decreased after 2 and 4 weeks of training and showed an upward trend after 6, 8, and 10 weeks of training. Based on the abovementioned results, this study attempts to support the creation of HIIT Rat Models and propose a possible explanation for chronic HIIT-induced IMTG accumulation.

It has long been established that lipid metabolism is regulated by cAMP-PKA signaling pathway. The activation of the cAMP-PKA pathway would promote lipolysis and suppress lipogenesis. For instance, the cAMP-PKA signaling pathway is activated during exercise, which would promote lipolysis and provide energy substrates for skeletal muscle contraction[25]. While the inhibition of the cAMP-PKA pathway would promote lipogenesis, suppress lipolysis, and consequently contributes to lipid accumulation[26-28]. In skeletal muscle, such a lipogenesis process is validated by a recent study reporting suppressed cAMP-PKA signaling pathway is involved in IMTG (one of the main energy substrates in skeletal muscle) storage[29]. In the present study, it was observed that the cAMP-PKA signaling pathway was in a suppressed state at 72h after the last session of regular HIIT. This finding aligns with our previous research indicating that skeletal muscle was in the recovery phase 72h after HIIT[11], which also makes the results of this study specifically applicable for skeletal muscle in the *recovery phase* after regular HIIT.

Intramuscular triglyceride (IMTG) could be broken down to free fatty acid (FFA) for intramuscular energy supply through mitochondrial oxidation[30-32]. Therefore, increased IMTG content means more energy substrate is available for skeletal muscle contraction and would improve muscle function if accompanied by promoted lipid oxidation capacity[29, 32-36]. Our IMTG results indicate that intramuscular triglyceride abundance was significantly elevated after 2, 4, and 10 weeks of HIIT, and it seems that a superficial conclusion could be obtained: 2, 4 and 10 weeks of HIIT might be effective in HIIT Rat Modelling from the aspect of IMTG storage. However, the lipometabolic

proteins changed in a chaotic trend (see Table. 1), which is rather inconsistent with the changing trend of IMTG. Similarly, other laboratories also reported some confusing post-exercise changing trends regarding lipid metabolic regulators. Hossein et al. found that long-term exercise improved PPAR-γ expression. Shinobu et al. reported that intramuscular SREBP-1c expression was promoted after regular training. Conversely, in Rebecca`s research, PPAR-α, PPAR-γ, and SREBP-1c all failed to increase either after an acute exercise bout or after long-term exercise training. These inconsistent and controversial results call into question the reliability of the superficial conclusion proposed above and aroused our interest for further research.

TABLE 1 | Changing trends of lipid metabolism-related proteins after training

	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
SREBP-1C	↓	↑	→	→	→
FAS	→	↓	↑	↑	→
P-ACC/ACC	→	→	→	→	→
P-HSL/HSL	↓	↓	→	↓	↓
P-ATGL/ATGL	↑	↑	→	→	→
CPT-1B	↓	↓	↑	↑	↑

Note: ↑ represents an upward trend; ↓ represents a downward trend; → represents an unapparent trend.

Indeed, the increased IMTG content would be a positive symbol only if accompanied by mitochondrial promotion. For example, high triglyceride content in type 2 diabetes is always accompanied by mitochondrial dysfunction[37-39], and this damaged mitochondria function would contribute to *insulin resistance* (a main distinguishing feature of type 2 diabetes)[35, 40]. Conversely, trained athletes, with strengthened mitochondria, would be able to combine high IMTG stores with superior insulin sensitivity, which has been termed the *athletes` paradox*[32, 41]. These facts inspired us to further probe changes in mitochondria, which might also explain the aforementioned inconsistent results and determine optimal training duration cycles for HIIT animal modeling. In fact, mitochondrion is a crucial target for manipulating intramuscular triglyceride metabolism[42] because the impaired lipid oxidation caused by mitochondria dysfunction would reduce FFA consumption and promote the accumulation of intramuscular triglycerides[43, 44]. Interestingly, however, a few researchers revealed that IMTG increased along with strengthened mitochondria (mitochondrial biogenesis, maintenance, and content, etc.)[29, 45, 46]. Actually, these researchers used long-term intervention instead of single bout of stimulus and selected only one observation time point. The IMTG content might decreased before the observation time point but was not observed unfortunately due to the lack of multiple observation time points. That is, it is true that mitochondria could limit the excessive IMTG storage and mitochondrial damage would lead to lipid hyperaccumulation, whereas the long-term adaptation might allow the coexistence of IMTG accumulation and mitochondria enhancement. This view might provide a novel explanation for exercise-induced IMTG changes in this study from the aspect of mitochondria.

Here, we selected PGC-1α, a transcriptional coactivator recognized as a biomarker of mitochondria biogenesis, and COX IV, the regulatory center of mitochondrial oxidation capacity[47-49], as the indicators of mitochondria adaptation. Our results indicate that the expression levels of PGC1-α and COX IV decreased after 2 and 4 weeks of training, and began to rise when the training duration reached 6 weeks. This indicates that mitochondria might be negatively impacted

within 4 weeks of HIIT, and the positive influence emerged from the sixth week (or earlier). Coincidentally, the changing trend of PGC1-α and COX IV is very similar to that of Carnitine Palmitoyltransferase 1B(CPT-1B), which is responsible for the transfer of free fatty acids into the mitochondria to facilitate muscular lipid oxidation[50, 51]. Therefore, taken together, the same changing trends of CPT1B, PGC1-α, and COX IV and the chaotic lipometabolic proteins changes indicate there is a great possibility that: **1)** the IMTG accumulation observed within 4 weeks of HIIT might be primarily attributed to damaged mitochondria oxidation capacity but not lipid metabolic regulators(since the change of lipid metabolism is unapparent/chaotic), which led to difficulty in IMTG consumption and excessive lipid accumulation. This phase is similar to the *insulin resistance* in type 2 diabetes, and might not be a positive physiological change. **2)** When the training duration reached 6 weeks, mitochondria function and biogenesis began to be improved by training stimulus. Promoted mitochondria oxidation capacity would increase consumption of IMTG, which might explain the disappearance of IMTG increase when the training duration reached 6 weeks. Afterward, with the mitochondria function and biogenesis remaining at high levels, IMTG content gradually increased for more energy substrate storage. FAS might have a prominent contribution to this process because it is the only up-regulated lipogenic enzyme after 6 and 8 weeks of HIIT in this present study. This phase is similar to the *athletes` paradox*, which should be a healthy and positive adaptation induced by exercise training. Additionally, the only unexpected result of mitochondria results is the insignificant change of PGC1-α after 10 weeks of training, which might be explained by the hypothesis that 10-week excessive HIIT caused chronic fatigue and consequently have a negative impact on mitochondria[52, 53]. Currently, studies on exercise-induced fatigue primarily focused on mitochondria function instead of its biogenesis, and the majority of studies are about one bout of exercise instead of excessively chronic training[54-57]. Given this state of the art, further investigation is warranted to examine this hypothesis.

Conclusion

All in all, this study proposes that, for HIIT Rat Modelling, the optimal intervention duration cycle should be longer than 4 weeks if HIIT frame of this study is adopted. Meanwhile, there is a great possibility that mitochondrial changes contribute more to HIIT-induced IMTG accumulation than lipometabolic proteins. Due to the lack of confirmatory mechanism research here, future study is needed to examine this possibility based on our work.

Statements and Declarations

Funding This work was funded by a grant from the Fundamental Research Funds for the Central Universities of China and the National Natural Science Foundation of China (No. 31801002). The authors thank Dr. Jingquan Sun for his help in obtaining the education grant.

Conflict of Interest The authors declare no conflict of interest.

Data availability The supplement WB data used to support the findings of this study has been uploaded and is available from the corresponding author upon request.

Ethical approval The paper is original and has not been published and is not being considered for publication elsewhere.

Ethics Approval and Consent to Participate 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”.

Figure legends

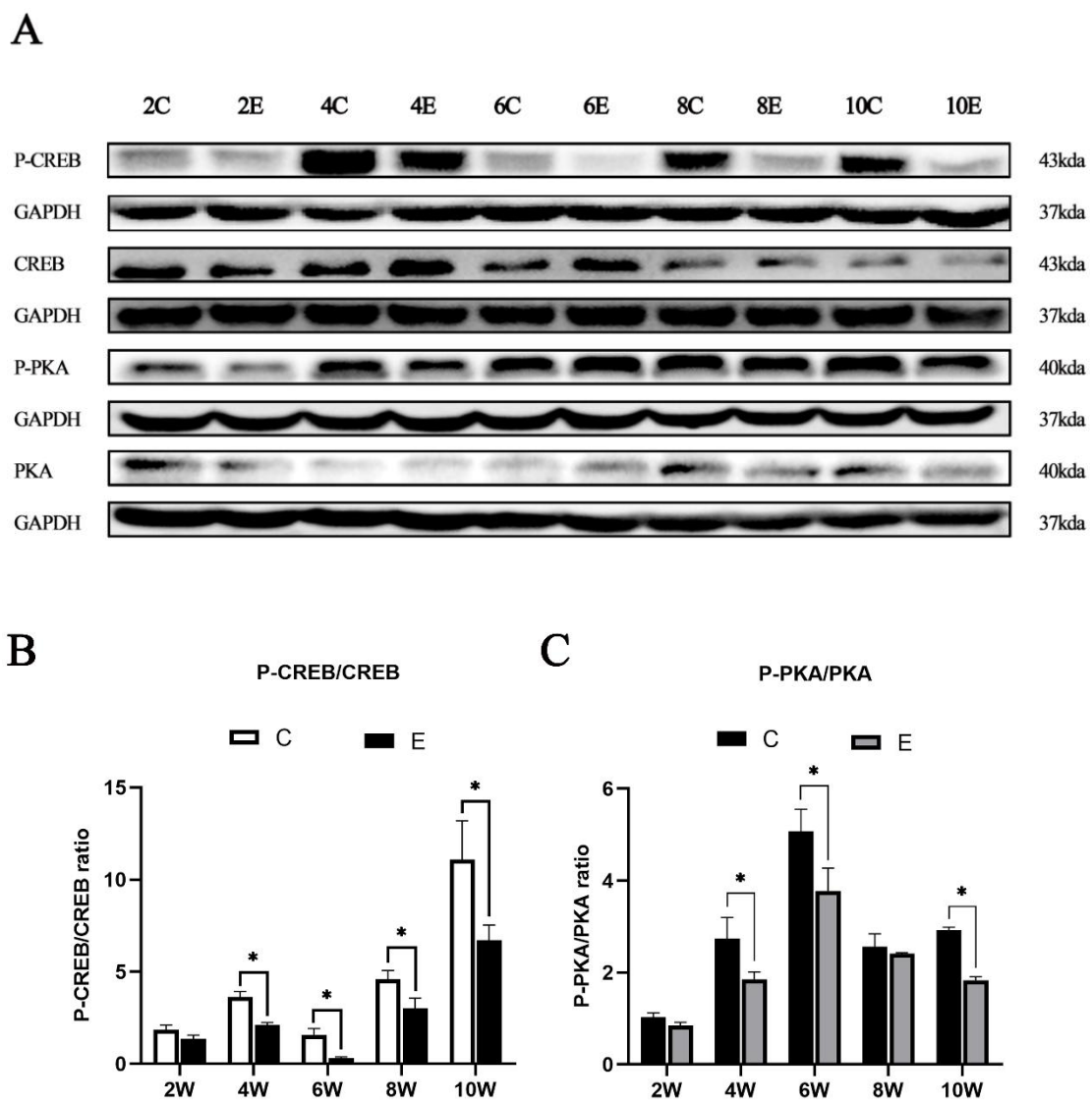

FIGURE 1 | The suppression of the cAMP-PKA pathway when rats were sacrificed. (A) Western blot analysis of intramuscular cAMP-PKA pathway proteins at 72h after the last training session. (B, C) The ratios of P-CREB/CREB and P-PKA/PKA in gastrocnemius. Three bands are used for statistics. The data are presented as the mean ± SD, and significant differences between the two groups were analyzed with the independent-samples t-test. **P*<0.05 vs. corresponding control groups.

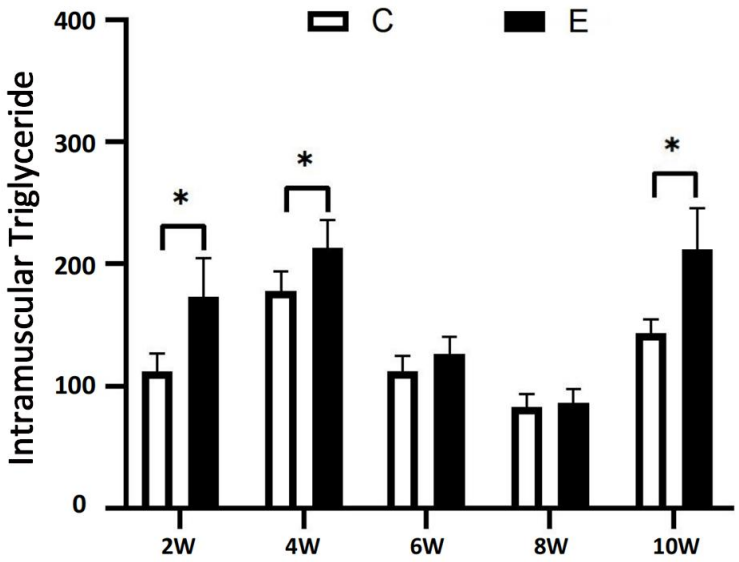

FIGURE 2 | Intramuscular triglyceride accumulation after HIIT.

This figure illustrates the intramuscular triglycerides abundance in gastrocnemius (n=6). The data was presented as the mean ± SD, and significant differences between the two groups were analyzed with the independent-samples t-test. *P<0.05 vs. corresponding control groups.

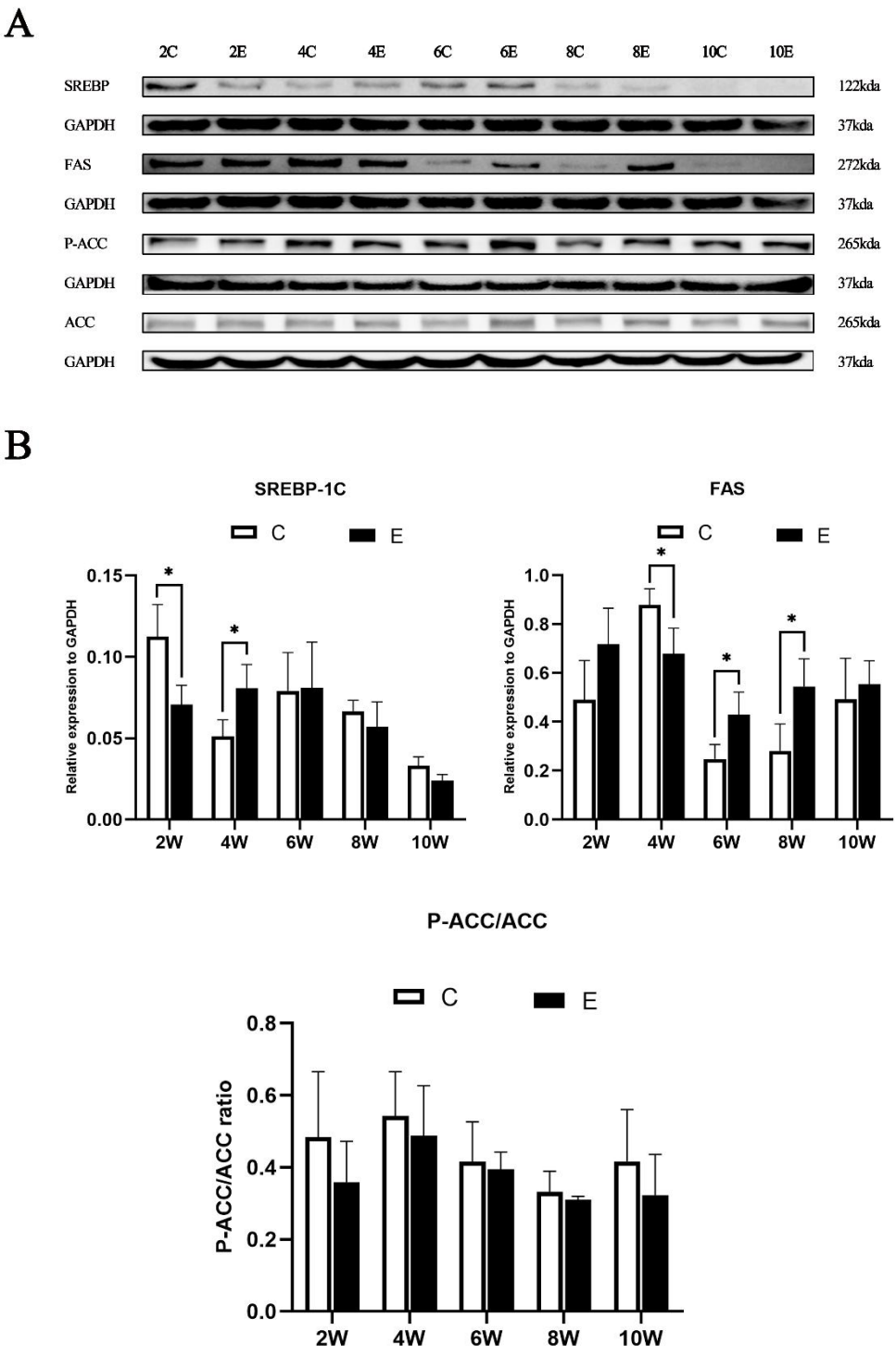

FIGURE 3 | The expression levels of lipogenesis-related proteins after HIIT.
(A) Western blot analysis of lipogenesis-related proteins. (B) Fold protein expression of SREBP-1C, FAS, and the ratio of P-ACC/ACC. Three bands are used for statistics. The data are presented as the mean ± SD, and significant differences between the two groups were analyzed with the independent-samples t-test. *P<0.05 vs. corresponding control groups.

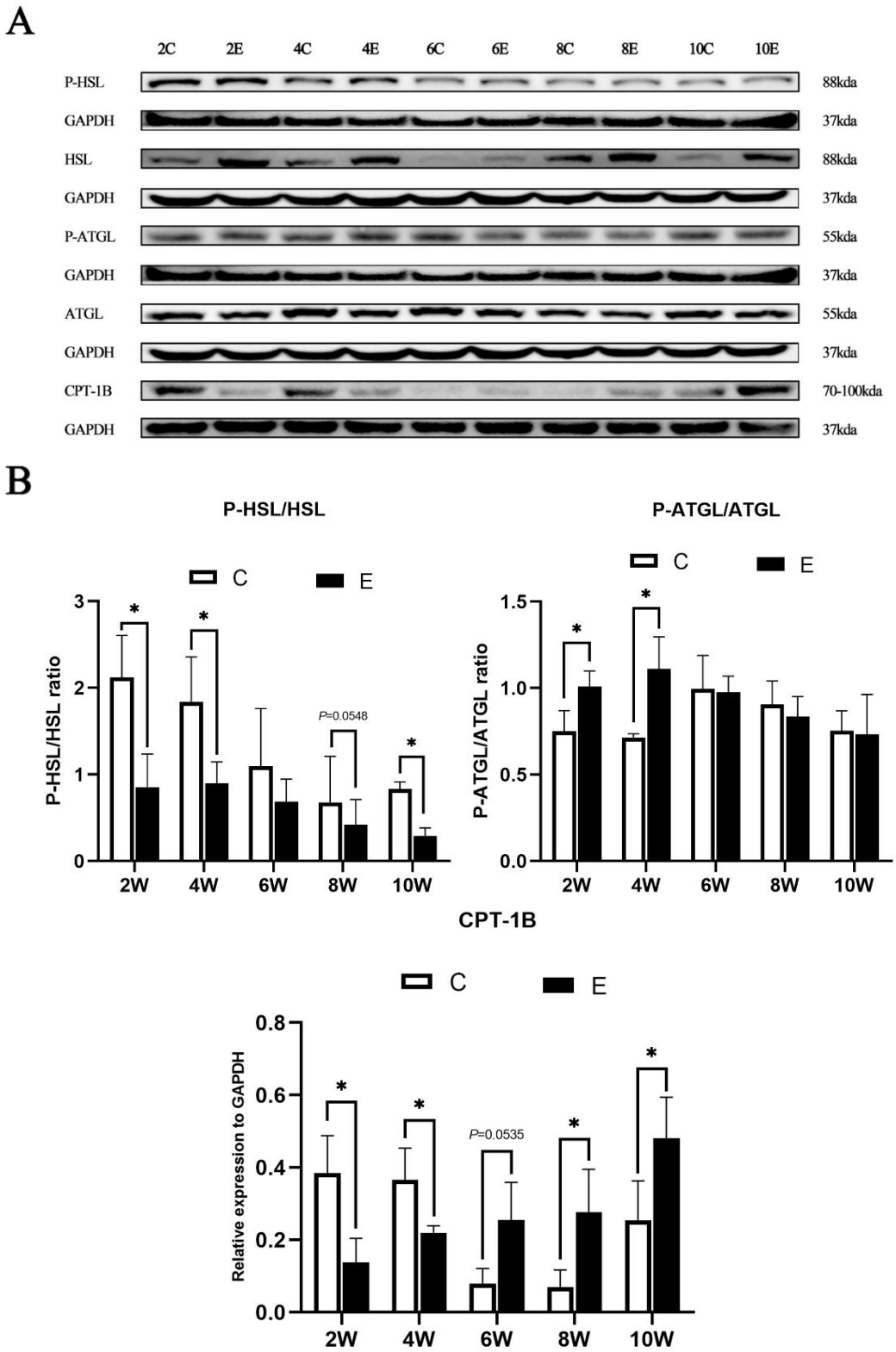

FIGURE 4 | The expression levels of lipolysis-related proteins after HIIT.

(A) Western blot analysis of lipolysis-related proteins. (B) Fold protein expression of P-HSL/HSL, P-ATGL/ATGL and CPT-1B. Three bands are used for statistics. The data are presented as the mean ± SD, and significant differences between the two groups were analyzed with the independent-samples t-test. *P<0.05 vs. corresponding control groups.

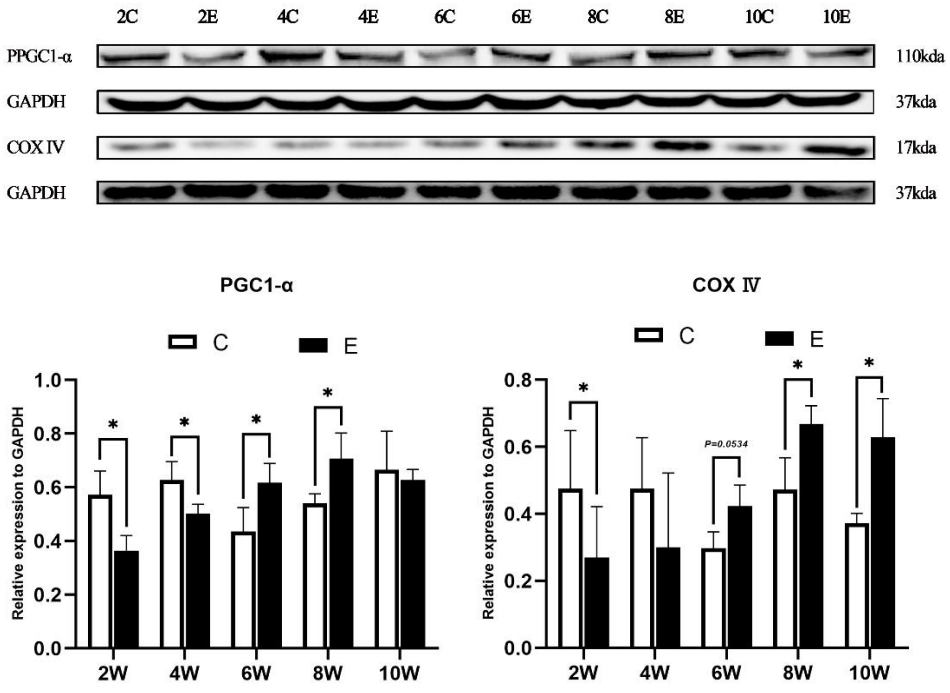

FIGURE 5 | The expression levels of mitochondrial biomarkers after HIIT. Western blot analysis of mitochondrial biomarkers (PGC1-α and COX IV) and corresponding fold protein expressions. Three bands are used for statistics. The data are presented as the mean ± SD, and significant differences between the two groups were analyzed with the independent-samples t-test. **P*<0.05 vs. corresponding control groups.

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