Alteration in cardiac uncoupling proteins and eNOS gene expression following high-intensity interval training in favor of increasing mechanical efficiency

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ARTICLE INFO	ABSTRACT
	Objective(s): High-intensity interval training (HIIT) increases energy expenditure and mechanical energy efficiency. Although both uncoupling proteins (UCPs) and endothelial nitric oxide synthase (eNOS) affect the mechanical efficiency and antioxidant capacity, their effects are inverse. The aim of this study was to determine whether the alterations of cardiac UCP2, UCP3, and eNOS mRNA expression following HIIT are in favor of increased mechanical efficiency or decreased oxidative stress. ***Materials and Methods:*** Wistar rats were divided into five groups: control group (n=12), HIIT for an acute bout (AT1), short term HIIT for 3 and 5 sessions (ST3 and ST5), long-term training for 8 weeks (LT) (6 in each group). The rats of the training groups were made to run on a treadmill for 60 min in three stages: 6 min running for warm-up, 7 intervals of 7 min running on treadmill with a slope of 5° to 20° (4 min with an intensity of 80-110% VO2max and 3 min at 50-60% VO2max), and 5-min running for cool-down. The control group did not participate in any exercise program. Rats were sacrificed and the hearts were extracted to analyze the levels of UCP2, UCP3 and eNOS mRNA by RT-PCR. ***Results:*** UCP3 expression was increased significantly following an acute training bout. Repeated HIIT for 8 weeks resulted in a significant decrease in UCPs mRNA and a significant increase in eNOS expression in cardiac muscle. ***Conclusion:*** This study indicates that Long term HIIT through decreasing UCPs mRNA and increasing eNOS mRNA expression may enhance energy efficiency and physical performance.

Introduction

High-intensity interval training (HIIT) rapidly stimulates metabolic adaptations in skeletal muscle, improves aerobic ability and results in concomitant changes in mitochondrial-associated mRNA and protein levels (1). The increase in exercise-induced energy expenditure is twice the training load (2). The increase in mechanical energy efficiency suggests that the energy needs for daily activities decrease in trained subjects (3). The mechanisms by which exercise could influence energy consumption and mechanical energy efficiency are largely unknown.

Uncoupling proteins (UCPs) are mitochondrial inner membrane proteins, which can disintegrate the proton gradient across the inner mitochondrial membrane and thereby reduce reactive oxygen species (ROS) generation and oxidative stress. Therefore, UCPs may participate in antioxidant defense, but reduced intracellular ATP production due to uncoupling of the mitochondrial oxidative phosphorylation is associated with less efficient metabolism (4). There is very strong evidence that UCP2 and UCP3 are negatively related to mechanical energy efficiency, suggesting that decreased UCPs with training increases mechanical energy efficiency (3). The different expression patterns of UCPs have been reported previously in organs with different metabolic outlines, such as muscle and heart. In contrast to UCP1, which is expressed exclusively in brown adipose tissue, UCP2 is distributed in a variety of tissues including the heart and UCP3 is highly expressed in skeletal muscle and to a lesser extent, the heart. UCP2 and UCP3, due to their close genetic mapping, have a role in metabolic regulation and calcium homeostasis (5). Both of them are involved in the aging heart dysfunction and upregulated under pathophysiological conditions, such as heart failure (6).

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Endothelial nitric oxide synthase (eNOS) is accompanied by a number of physiological processes involved in the regulation of metabolism. eNOS expressed in cardiomyocytes in part mediates the length-dependent increase in cardiac contraction force (7). There is evidence that ablation of eNOS reduces mechanical efficiency and exercise capacity (8). However, the myocardium eNOS uncoupling following exercise inhibits ample NO synthesis and limits the reaction between NO and O_2^- to form peroxynitrite, which is cytotoxic (9).

No data concerning the expression of the UCPs and eNOS mRNA in the cardiac muscle following HIIT has been published so far. Due to the adverse effects of these factors on the mechanical efficiency and antioxidant capacity, the present research was designed to characterize whether changes in the expression of the UCP2, UCP3, and eNOS mRNA following HIIT in the cardiac muscle of rats are in favor of increasing mechanical efficiency or decreasing oxidative stress.

Materials and Methods

Animals

A total of 36 male Wistar rats (250±20 g initial weight, 6 rats/group) were acquired from Pasteur Institute of Iran and kept under controlled conditions (12 hr light/12 hr dark cycle; 22±3 °C). The rats had free access to tap water and standard rat food. The ethical codes of treating laboratory animals, set by the Iranian Society for Supporting Laboratory Animals Used for Scientific Purposes, were strictly followed in the present study. The University of Tehran approved the treating and handling method, the training program, and the sampling type of animals conducted in the present study. The animals were randomly divided into control (cont), HIIT for an acute bout (AT1), short term HIIT (ST), and long-term HIIT (LT).

Exercise training program

The training program was developed based on our previous study (10). The rats were habituated to the treadmill (Iranian model, Tehran, Iran) over the first week. Acute HIIT consisted of one exercise session. Animals in the LT group were exercised for 8 weeks, 5 days/wk, and in the ST group were made to run on the treadmill for 3 or 5 sessions (ST3 and ST5). The control group, comprising 6 rats for acute and short-term training and 6 rats for long term training, were placed on a treadmill without running for 60 min/day.

Every session consisted of one hour exercise training performed in three stages:
- Warm-up: running for 6 min at 50-60% VO_{2max}
- Main training: 7 intervals of 7-min running at 5°–20° slope consisting of 4-min running at 80-110% VO_{2max} and 3-min running at 50-60% VO_{2max} Cool-down: running for 5 min at 50-60% VO_{2max}

The intensity of the training program in terms of VO_{2max} was obtained based on the relation of VO_{2max} to speed and treadmill slope (11). Since the best time for rats is during the dark cycle to do exercise training, the training program was conducted in the afternoon during the dark cycle (between 6 pm and 12 am) under red light (12). Using this protocol for 8 weeks was shown to induce mild heart hypertrophy and protect cardiac muscle against ischemia-reperfusion injury in rats (10).

Sampling procedure

The animals in AT1 and other groups were anesthetized with ketamine and pentobarbital (13) immediately or 24 hr after the last exercise session, respectively. The ventricles were excised, dropped into liquid nitrogen, and stored at -80 °C.

RNA extraction and cDNA synthesis

Total RNAs from the heart tissue samples were extracted using the miRCURY™ RNA Isolation Kit (Exiqon, Denmark) according to the manufacturer's instructions. The quality and concentration of total RNA were determined at A $_{260\ nm}$ and impurities were assessed by the A $_{260\ nm}$:A$_{280\ nm}$ ratio using Picodrop P200 system (Alpha Biotech Ltd. UK). RNase-free DNase I (Fermentase, Germany) was used to remove any genomic DNA contamination. First-strand cDNA was synthesized from 1 μg of RNA using Thermo Scientific Maxima First Strand cDNA Synthesis Kit (GmbH, Germany).

Real-time PCR

Real-time PCR was performed using the StepOne™ Real-Time PCR System (Life technologies, USA) and SYBR® Green I PCR Master Mix kit (Life Technologies, USA). As an internal control, HMBS (Hydroxymethylbilane synthase) gene was used for UCP2, UCP3, and eNOS gene expression normalization (14). Primers for the specific genes are listed in Table 1. Each 25 μl reaction volume contained 400–800 nM primers, 12.5 μl SYBR® Green I PCR Master Mix and 2.5 μl of sample cDNA. After a primary denaturation step at 95 °C for 10 min, amplification was performed with 40 cycles of denaturation at 95 °C for 15 sec and annealing at 60 °C for 1 min with collection of fluorescent data. Finally, the specificity of the amplified product was confirmed using a melt curve analysis for each pair of primer at a temperature between 60 °C and 99 °C. The presence and sizes of all PCR products were verified by inspection of the dissociation curve and by gel electrophoresis. The normalized level of expression of genes in each sample was calculated using the Pfaffl method (15). Values were expressed as fold of the control.

Statistical analysis

Data were analyzed using SPSS (version 18.0) and

Table1. Primer sequences used in the Real-time PCR for uncoupling proteins, eNOS, and HMBS genes

Gene	Primers	Sequence
UCP-2	Forward	5′-GGCGGTGGTCGGAGATA-3′
	Reverse	5′-GGCAGAAGTGAAGTGGCAAGGG-3′
UCP-3	Forward	5′-TACAGAACCATCGCCAGGGA-3′
	Reverse	5′-TATCGGGTCTTTACCACATCCA-3′
eNOS	Forward	5′- CGAACAGCAGGAGCTAGAGG-3′
	Reverse	5′- GAGGTGGATCTCTCCTGGGT-3′
HMBS	Forward	5′- TCTAGATGGCTCAGATAGCATGCA-3′
	Reverse	5′- TGTTCAGCAAGAAGATGGTCCA-3′

presented as the mean ± SE. Statistically significant differences between groups were calculated by the one-way ANOVA (Tucky *posthoc* comparison) or the independent t-test. A value of $P<0.05$ was considered significant.

Results

The effects of HIIT on mRNA expression of eNOS, UCP2, and UCP3 in the cardiac muscle were studied. Animals in acute training were killed immediately and the other rats 24 hr after the last exercise bout. There was not a significant exercise effect on HMBS gene expression ($P>0.05$). The effects of HIIT on mRNA expression of eNOS, UCP2, and UCP3 in the cardiac muscle are presented in Figures 1, 2 and 3.

The effect of HIIT on eNOS mRNA expression

As shown in Figure 1A, an acute bout of HIIT induced a small increase in eNOS mRNA level, but there was no significant difference compared to the control group ($P=0.19$). eNOS mRNA were significantly up-regulated, 2.9 and 3.5 fold, in the short term HIIT for 3 and 5 bouts, respectively, compared to the control group (Figure 1B) and by 2.5 fold in the long term HIIT for 8 weeks (Figure 1C).

The effect of HIIT on UCPs mRNA expression

The expression level of UCP2 mRNA did not change following an acute training and short-term training. Cardiac UCP2 mRNA expression was significantly decreased by 44% as compared to that of the control rats after 8 weeks (Figure 2).

UCP3 mRNA was elevated by ∼ 4-fold following an acute bout of training ($P<0.01$). UCP3 mRNA did not change after short-term training while it was decreased by 48% in long term training compared to that of the control group (Figure 3).
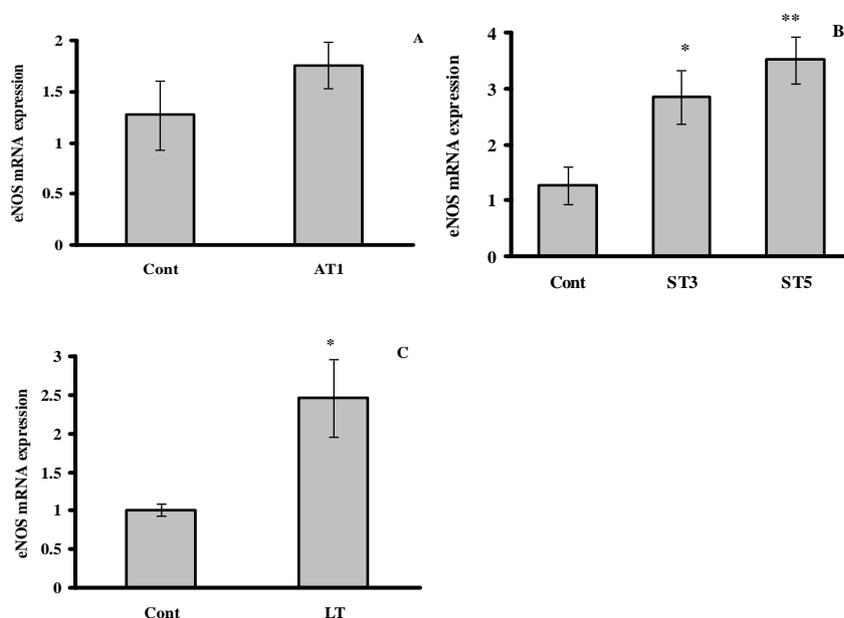
Figure 1. Relative abundance of endothelial nitric oxide (eNOS) mRNA in rat cardiac muscle in response to an acute bout of high-intensity interval training (HIIT, AT1), short term HIIT for 3 and 5 bouts (ST3 and ST5), and long term HIIT for 8 weeks (LT). It normalized with HMBS (using the corresponding 18S rRNA values). The results are expressed as mean±SEM, * $P<0.05$, **$P<0.01$ vs. control rats
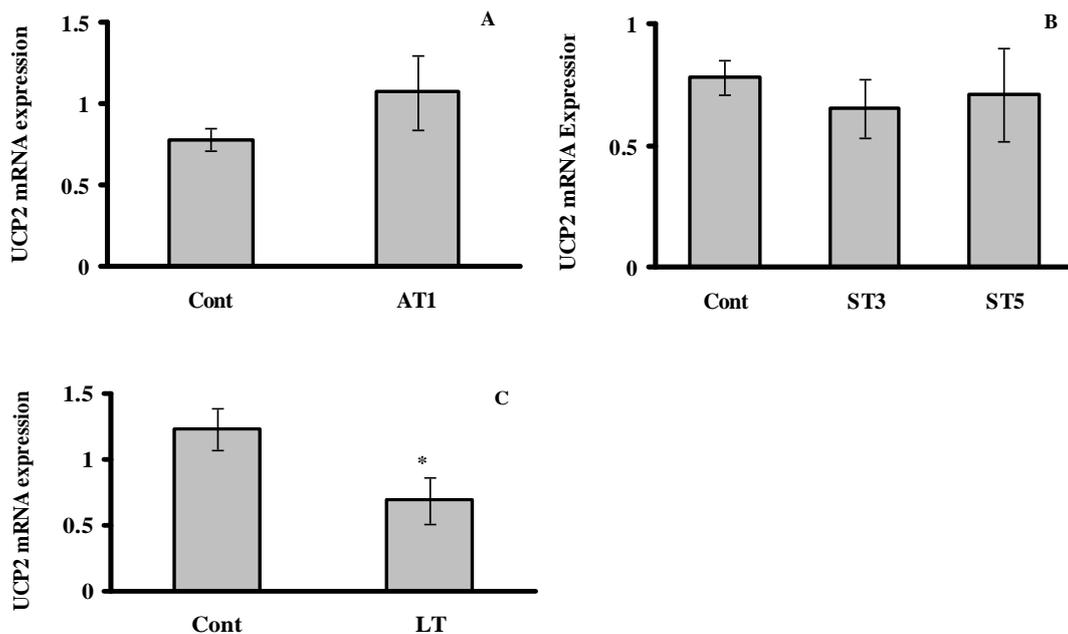
Figure 2. Relative abundance of uncoupling protein 2 (UCP2) mRNA in rat cardiac muscle in response to an acute bout of high-intensity interval training (HIIT, AT1), short term HIIT for 3 and 5 bouts (ST3 and ST5), and long term HIIT for 8 weeks (LT). It normalized with HMBS (using the corresponding 18S rRNA values). The results are expressed as mean±SEM. *$P<0.05$ vs. control rats
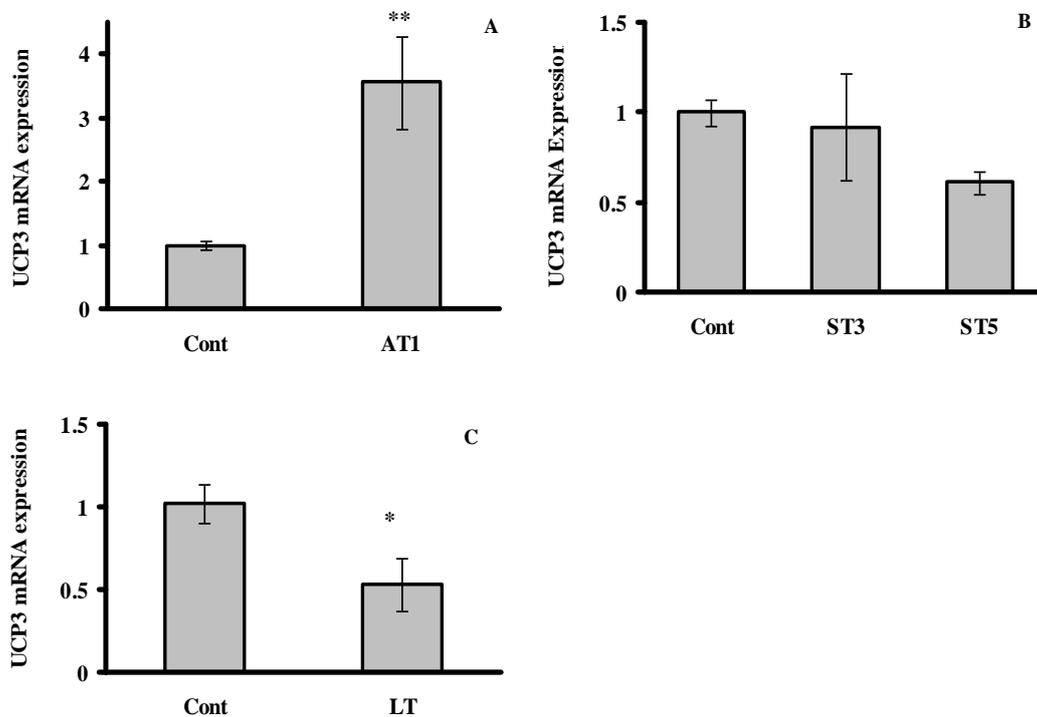
Figure 3. Relative abundance of uncoupling protein 3 (UCP3) mRNA in rat cardiac muscle in response to an acute bout of high-intensity interval training (HIIT, AT1), short term HIIT for 3 and 5 bouts (ST3 and ST5), and long term HIIT for 8 weeks (LT). It normalized with HMBS (using the corresponding 18S rRNA values). The results are expressed as mean±SEM. *$P<0.05$ vs. control rats

Discussion

This study describes the changes in the cardiac UCP2, UCP3, and eNOS gene expression following different periods of HIIT. The current data provides evidence that an acute bout of HIIT induced an increase in UCP3 and eNOS mRNA expression, while long-term HIIT for 8 weeks decreased UCPs mRNA expression and increased eNOS mRNA expression.

HIIT represents a time efficient strategy alternative to classic endurance training (ET) that enhances rapid adaptations in skeletal muscle and exercise performance. Gibala *et al* demonstrated 2.5 hr of HIIT produced molecular and cellular adaptations in human skeletal muscles similar to 10.5 hr of endurance training (16). However, Holloway *et al* reported that ET and HIIT induce divergent signals in the heart. So that ET for 4 weeks increased eNOS content, while HIIT did not. They found that in the presence of hypertension, HIIT and ET have opposing effects on cardiac remodeling (17). Swimming training for 10 weeks led to increased expression of eNOS of skeletal muscle paralleled with decreases in blood pressure and heart rate in spontaneously hypertensive rats (18). These results suggest that different types of exercise training in different situations have diverse effects on biomarkers expression. In addition, the alterations in eNOS are tissue-specific. Our results showed that eNOS mRNA was increased after HIIT in the cardiac muscle. Previous studies demonstrated that downregulation of eNOS exerts following effects on ATP levels and oxidative phosphorylation complexes in skeletal muscles of rodents (8, 19). On the other hand, nitric oxide production through eNOS plays an important role in the regulation of cardiac hypertrophy (20) and protection against myocardial ischemia-reperfusion injury (21). Taken together, eNOS upregulation following HIIT has a role in increased energy efficiency and physical performance and enhances cardioprotection.

Our data revealed that UCP3 gene expression is up-regulated in response to an acute bout of HIIT in rat cardiac tissue. An increase in UCP3 gene expression is thermodynamically unfavorable because it decreases the efficiency of oxidative phosphorylation (22). Acute exercise can result in oxidative stress more than trained exercise. In rat heart, an increase in antioxidant enzyme activities was more in acute exercise as compared to those in chronic exercise, possibly the heart copes with the enhanced production of ROS during exhaustive exercise (23). Previous studies have shown that UCP3 expression can be rapidly upregulated in response to an acute bout of exercise or contractile activity in mammalian skeletal muscles, possibly owing to increased ROS generation (22). UCPs overexpression appears to be a physiological response to elevated temperature and oxidative stress of exercise in the cardiac muscle (24). Thus, increased UCP3 serves as an early response to antioxidant protection. It is suggested that this adaptation precedes MnSOD upregulation temporally and it could be an important molecular mechanism to maintain the integrity of mitochondria at the expense of ATP production and oxidative phosphorylation efficiency under potential oxidative stress (22). On the other hand, increased UCP3 mRNA after an acute exercise seems to be related to changes in fatty acid metabolism. It was postulated that the primary function of UCP3 would be in the handling of those fatty acids that cannot be oxidized (25). After acute exercise in the fasted state more fatty acids are released from the adipose tissue than can be oxidized, thus explaining the upregulation of UCP3 (3).

Although during an acute bout of exercise, the first strategy of mitochondria to reduce oxidative stress is decreasing the production of O_2^- by overexpressing UCP2/UCP3, endurance training reduces acute stress-induced UCP2/UCP3 expression and activity in rats. By the way, endurance training significantly elevated MnSOD activity and protein level (24, 26). It was reported that acute exercise increases skeletal muscle UCP3 expression in untrained but not trained humans (27). Generally, exercise adaptation of MnSOD may augment mitochondrial tolerance to ROS production and hence a lower UCP2/UCP3 activation during intensive exercise maintains the efficiency of oxidative phosphorylation (26). Our results also showed that UCP2 and UCP3 mRNA levels, 24 hr after the last exercise bout of short and long term HIIT, decreased considerably in the cardiac muscle. Downregulation of UCPs after chronic HIIT is in accordance with the findings obtained by other researchers who reported 8–10 weeks of endurance training program in rats, lowers considerably UCP2 and UCP3 mRNA levels in the skeletal muscles and in the heart (28, 29). Study showed that decreased UCP2 expression in the high-intensity exercise group compared to the low-intensity exercise group is due to an increase in ATP production as a part of the improvement in exercise efficiency through inhibiting unnecessary energy utilization following a decrease in glucose storage level (30). UCPs are negatively related to mechanical energy efficiency. The lower levels of UCPs mRNA in the skeletal muscle of trained subjects suggest that exercise training induces an adaptive physiological response improving mechanical efficiency (3, 31). Therefore, a decreased expression of UCPs in the cardiac muscle increases cardiac metabolic efficiency, which in turn would allow a higher capacity to perform mechanical work during subsequent exercise (28). Nevertheless, several studies have produced

somewhat conflicting results. In obese mice, swim training for 6 weeks increased the levels of UCPs mRNA and proteins in adipose tissue and skeletal muscles (32). Treadmill exercise at low and moderate intensities for 8 weeks increased UCP2 protein expression of brown adipose tissue in Zucker rats compared to the control group (30). These discrepancies between the reports may be due to variations in the type and duration of training. Additionally, the findings suggest that acute exercise and repeated training with different intensities might have opposite effects on UCPs mRNA expression.

Conclusion

The present data suggested that an acute bout of HIIT induced a significant increase in eNOS and UCP3 mRNA level, whereas long-term HIIT resulted in a significant decrease in UCPs and a significant increase in eNOS mRNA levels compared to the control group. After considering all the results, long term HIIT may induce an adaptive physiological response improving metabolic efficiency and physical performance and enhance cardioprotection by downregulation of UCPs expression and upregulation of eNOS in the cardiac muscle. Future studies are needed to compare the alterations of antioxidant enzymes between acute and chronic HIIT.

Acknowledgment

Conflict of interest

No conflict of interest to disclose.

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