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Effects of Downhill Treadmill Running on Uncoupling Protein 3 mRNA Expression

Abstract

Eccentric biased exercise has been reported to elicit more muscle injury than concentric or isometric exercise and potentially generate increased oxidative stress one to two days post exercise. Increased oxidative stress has been shown to up-regulate the expression of UCP3 mRNA. The aim of this study was to investigate the effects of downhill running on skeletal muscle UCP3 mRNA expression. Twenty-four male Sprague Dawley rats were randomly assigned to run continuously for 30 minutes (30-C, n = 6), or run six 5-minute bouts separated by rest periods of 2 minutes (2-R, n = 6), 4 minutes (4-R, n = 6), and 6 minutes (6-R, n = 6) on a 16 degree declined treadmill at a speed of 16 $m \cdot min^{-1}$. Sham control animals (n = 8) were placed in a treadmill chamber during the 30-minute run session. Semi-quantitative RT-PCR was conducted to evaluate UCP3 mRNA levels in the *plantaris*, a muscle used eccentrically during downhill running and *tibialis anterior*, a muscle which undergoes very little eccentric muscle contraction during this exercise. The level of gene expression was normalized to 18 S ribosomal mRNA expression from the same PCR product. Results are reported as mean ± standard error. UCP3 of the *plantaris* muscles from 2-R animals (2.36 ± 0.13) was significantly greater than UCP3 of the *plantaris* from control animals (1.72 ± 0.13), $p < 0.05$. UCP3 of the *tibialis anterior* from the continuous group (1.51 ± 0.17) was significantly less than the UCP3 of the *tibialis anterior* of the control group (2.09 ± 1.4), $p < 0.05$. These data suggest that downhill treadmill running is associated with an increase in UCP3 mRNA expression in the *plantaris* muscle. These results indicate that exercise which is biased toward eccentric exercise may up-regulate UCP3 mRNA during the period post exercise when muscle damage and repair is elevated.

Key words
Eccentric exercise · oxidative metabolism · skeletal muscle · mitochondria

Introduction

UCP3 is a member of the inner mitochondrial membrane protein family that is expressed predominately in skeletal muscle [6]. Although the exact function of UCP3 has yet to be determined, two roles have been proposed. In the first, UCP3 has been shown to facilitate the leaking of protons across the inner mitochondrial membrane, and contribute to the resting respiration rate of skeletal muscle [17, 21]. For example, isolated skeletal muscle mitochondria from UCP3 knockout mice have a decrease in state 4 respiration compared to their wild type littermates [31]. These results suggest that the lack of UCP3 in skeletal muscle results in a greater coupling between the oxidation of fuels and the phosphorylation of ADP to ATP. Furthermore, endogenous levels of UCP3 have oxidative phosphorylation uncoupling capabilities in mammalian mitochondria [31]. However, other investigations

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have proposed an alternate function for UCP3 because UCP3 does not catalyze the basal proton conductance of skeletal muscle in the absence of activators such as superoxide [12] and may, in fact, provide little uncoupling activity at levels normally found *in vivo* [19]. Therefore, the extent to which this uncoupling activity contributes to the overall energy expenditure within skeletal muscle is unknown. UCP3 has also been hypothesized to help lower the mitochondrial production of superoxide and other reactive oxygen species (ROS) and protect against oxidative damage and aging [7]. Brand and others [10] recently reported that mice which underexpressed UCP3 had significantly higher levels of oxidative damage compared with wild-type controls, suggesting that UCP3 functions *in vivo* as part of the antioxidant defenses of the cell.

Eccentric biased exercise has been reported to elicit more muscle injury than concentric or isometric exercise [3]. Recent data collected in our laboratory indicated that a single bout of downhill running resulted in significant damage to those muscles used eccentrically (unpublished observations). In addition, it was observed that six-minute bouts of downhill running interspersed with either 2 or 4 minutes of rest resulted in more damage then continuous downhill running. Lee and colleagues [20] have recently reported that eccentric exercise which resulted in decreased muscle function and delayed onset of muscle soreness, produced signs of increased oxidative stress which reached maximal levels at 24 and 48 h after the exercise. Increased oxidative stress has been postulated to up-regulate UCP3 mRNA levels in the muscle. However, the effects of eccentric muscle contraction on the expression of UCP3 mRNA have not been evaluated.

UCP3 mRNA is generally correlated to oxygen uptake corrected for body weight [23] and UCP3 mRNA expression increases in response to aerobic exercise [14,30]. For example, UCP3 is increased for up to 3 h following an acute bout of aerobic exercise [14,30] but it returns to basal levels within 24 h post-exercise [30]. In contrast to the high oxidative cost from running on an incline, in rats downhill running is achieved primarily from eccentric contractions in hind limb muscles [4,24]. Therefore, although the oxidative metabolism is lower for downhill running [28], the level of damage to the muscle is greater [25]. In the current study, we tested the hypothesis that UCP3 expression is increased by eccentric contraction induced through downhill running, where we have shown that significant muscle damage occurs 48 h after exercise. We examined the *plantaris* and the *tibialis anterior* muscles because during downhill running there is a predisposition towards eccentric contractions in the *plantaris* muscle, whereas, the eccentric contractions and muscle damage are minimized in the *tibialis anterior* muscle [3]. In addition we also hypothesized that short rest periods between sets of downhill running would increase the expression of UCP3 mRNA compared to a continuous bout of downhill running. This is based on our recent observation that greater amounts of muscle damage occurred in rats which ran downhill intermittently versus those that ran continuously.

Methods

Animals

Thirty-two male Sprague Dawley rats were studied. All animals were provided food and water *ad libitum* and were maintained at a constant room temperature (72 °F) under controlled lighting conditions (12 : 12-h light-dark cycle) for one week. During this first week, all animals were weighed daily. The mean of these measurements was taken as the *ad libitum* weight. During the second week, the ration of lab chow provided to all animals was reduced so that each rat's weight was gradually reduced to a target weight of 80% of their *ad libitum* weight. The rats' daily rations of standard lab chow were adjusted to maintain their target weights. Each animal was weighed daily to ensure that the proper bodyweight was maintained for a minimum of one week prior to the downhill running protocol. It has been shown that this type of food restriction does not cause stress to the animals but rather enhances health and extends life [13,22]. All methods and procedures were approved by the CDC National Institutes of Occupational Safety and Health (NIOSH) and the Animal Care and Use Committee of West Virginia University.

Downhill treadmill running

Four groups of untrained rats ran on a 16 degree declined motorized treadmill (Columbus Instruments, Columbus, OH, USA) at a speed of 16 $m \cdot min^{-1}$ for 30 minutes. Mild electrical stimulation was applied as needed to maintain the activity of the rats. Rats ran continuously for 30 minutes (30-C, n = 6), or they ran six 5-minute bouts separated by rest periods of 2 minutes (2-R, n = 6), 4 minutes (4-R, n = 6), or 6 minutes (6-R, n = 6). Another group of eight rats served as a sham control group. These control rats were placed in the treadmill chamber with a non-moving belt and active shock grid for 30 minutes. Following the running period, all animals were returned to their housing facilities and allowed access to their food and water.

In order to prevent the influence of the acute bout of endurance running on UCP3 mRNA expression, all animals were sacrificed via decapitation 48 h after the exercise period. This time was also chosen because it has been shown to be the period when muscle damage, DOMS and possibly ROS production may peak. Immediately following sacrifice, *plantaris* and *tibialis anterior* muscles were rapidly excised, weighed, frozen in liquid nitrogen and stored at – 80 °C.

RT-PCR

Total ribonucleic acid (RNA) was extracted from muscles using TriReagent (Molecular Research Center, Cincinnati, OH, USA) and a mechanical homogenizer. RNA was solubilized in 22 µl RNase-free water and measured by absorbance at 260 nm. Two µg of total RNA per muscle were reverse transcribed (RT) according to the directions supplied with Superscript II RNase H^- Reverse Transcriptase (Life Technologies, Bethesda, MD, USA).

UCP3 and 18 S rRNA

Amplification of DNA was the same as reported previously in our laboratory [1,2] with only minor modifications. Briefly, two µg of RNA were reverse transcribed with 1 µl of random decamer primer (Ambion, Austin, TX, USA) so that 18 S rRNA expression could be amplified simultaneously with UCP3 by polymerase

chain reaction (PCR). PCR for the 18 S rRNA and UCP3 was performed using a 1 : 1 solution of 100 ng of 18 S primer pairs and competimer (Ambion, Austin, TX, USA), along with the forward and reverse rat UCP3 primers, 5′-GGAGAACCCAGGAGTGCAGAG-3′ and 5′-TCCGTTCCTTTGGGGGTGTAGA-3′ respectively. The 1 : 1 solution of 18 S primer pairs and competimer (Ambion, Austin, TX, USA) was combined with 100 ng of the UCP3 primers along with 250 µM dNTPs, 10 X PCR Buffer, and 2 units of Taq Polymerase in a final volume of 50 µl. The PCR signal up to 40 cycles (data not shown) for UCP3 remained linear. PCR for 18 S and UCP3 were performed at an annealing temperature of 59.9 °C for 36 cycles.

Following amplification, 10 µl of each PCR product was electrophoresed on 1.5 % agarose gels. Gels were stained with ethidium bromide. Images were captured and signals quantified using Kodak Digital Science 1 D Image Analysis Software version 3.5.4. PCR products for UCP3 were previously verified by DNA sequencing by MWG Biotech Inc. and entering the DNA sequence into BLAST (Basic Local Alignment Search Tool). UCP3 mRNA expression was assessed as a ratio of UCP3/18 S. A one-way ANOVA was used to compare UCP3/18 S mRNA expression. A Tukey post hoc comparison was used to identify which groups were significantly different from one another ($p < 0.05$ was considered significant).

Results

Semi-quantitative UCP3 mRNA expression in the *plantaris* muscle

The contractions of the rat *plantaris* muscle are eccentrically biased during downhill running [3]. The normalized UCP3 mRNA expression in the *plantaris* muscles of the 2-R group of animals was estimated to be 36.7 % greater than that of control animals ($p < 0.001$), 32.9 % greater than that of 6-R animals ($p < 0.05$) and 28.3 % greater than that of continuous animals ($p < 0.05$). 30-C animals did not differ significantly from that of the control animals (Fig. **1**).

Semi-quantitative UCP3 mRNA expression in the *tibialis anterior* muscle

Downhill running induces very few eccentric contractions in the *tibialis anterior* muscle [3]. Normalized UCP3 mRNA expression in the *tibialis anterior* muscles of the 30-Cs animals was estimated to be 27.6 % less than that of the control animals ($p < 0.05$). In addition, the normalized UCP3 mRNA expression from the *tibialis anterior* muscle of the 4-R group of animals was estimated to be 39.9 % less than that of the control animals ($p < 0.001$), 37.7 % less than that of the 2-R animals ($p < 0.05$), and 43.5 % less than that of the 6-R animals ($p < 0.001$) (Fig. **2**).

Discussion

The role of UCP3 in skeletal muscle during exercise is not known. Postulated roles for UCP3 include the regulation of ATP synthesis [8,9] the regulation of free fatty acid oxidation [11,16] and the control of reactive oxygen species (ROS) production [7]. Because oxidation of free-fatty acids may affect UCP3 levels [15] we can-

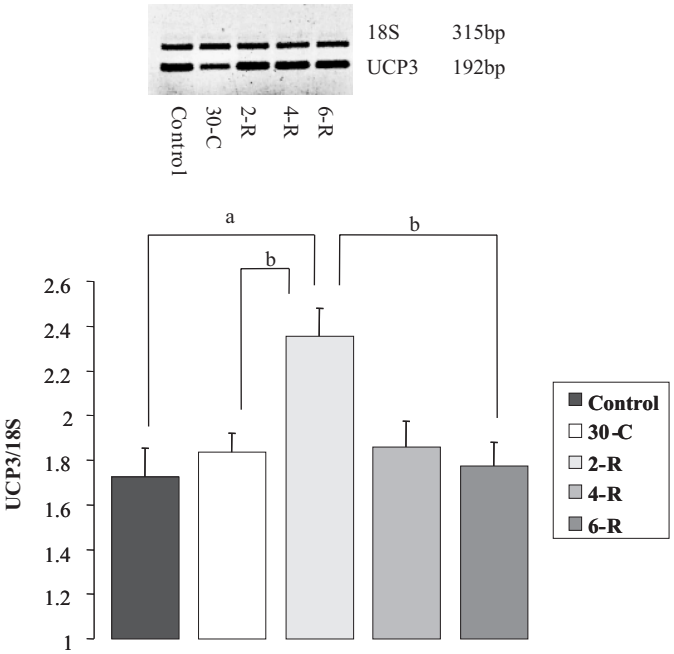

Fig. **1** Effects of downhill treadmill running on UCP3 mRNA expression in the *plantaris* muscle. All experimental animals ran on a 16 degree declined treadmill for 30 minutes at a speed of 16 m/min. Continuous group of animals (30-C) ran continuously for the 30 minutes. 2-R group of animals ran 5-minute bouts interspersed with 2 minute rest periods. 4-R group of animals ran 5 minute bouts interspersed with 4 minute rest periods. 6-R group of animals ran 5 minute bouts interspersed with 6 minute rest periods. UCP3 mRNA expression is reported normalized to 18 S rRNA. Statistical differences are shown as: a = $p < 0.001$, b = $p < 0.05$. Data are presented as mean ± SEM.

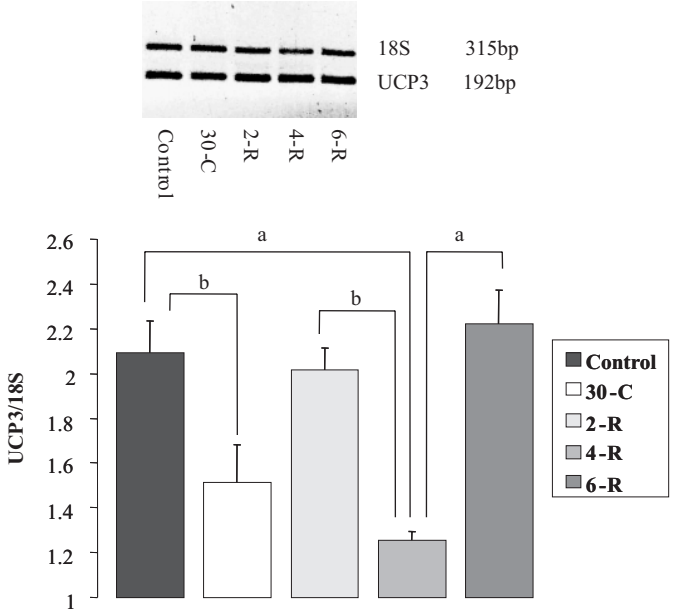

Fig. **2** Effects of downhill treadmill running on UCP3 mRNA expression in the *tibialis anterior* muscle. All experimental animals ran on a 16 degree declined treadmill for 30 minutes at a speed of 16 m/min. Continuous group of animals (30-C) ran continuously for the 30 minutes. 2-R group of animals ran 5 minute bouts interspersed with 2 minute rest periods. 4-R group of animals ran 5 minute bouts interspersed with 4 minute rest periods. 6-R group of animals ran 5 minute bouts interspersed with 6 minute rest periods. UCP3 mRNA expression is reported normalized to 18 S rRNA. Statistical differences are shown as: a = $p < 0.001$, b = $p < 0.05$. Data are presented as mean ± SEM.

not rule out the possibility that free fatty acid oxidation rates were different in some of the groups in this cross-sectional study.

The production of reactive oxygen species (ROS) is greatly increased at times when the proton electrochemical gradient is high [27]. It has been suggested that UCP3 protein may play a role in the production of ROS because the addition of an uncoupling agent, which directly decreases the proton electrochemical gradient, strongly suppresses superoxide anion formation [27]. In addition, mice which lack UCP3 have been shown to have greater ROS production within the mitochondria [31]. Therefore the control of ROS by UCP3 is an attractive hypothesis. Recent data collected in our laboratory indicated that six, 5-minute bouts of downhill running interspersed with either 2 or 4 minutes of rest produced greater signs of muscle damage compared with 30 minutes of continuous downhill running. Data collected by Lee et al. [20] and others showed recently that supra maximal eccentric muscle contractions resulted in blood oxidative stress markers that were significantly elevated at 24 and 48 h post exercise. Although our exercise protocol was different from that used by Lee and coworkers [20] both protocols produced eccentric muscular contractions and muscle damage which became significant 48 h after the exercise. It is possible that there was increased oxidative stress 48 h after the downhill running exercise especially in those animals that ran intermittently. This could explain the significant increase in the expression of UCP3 mRNA in the 2-M group although this is only speculation because no ROS was measured in the present study. The authors are uncertain why the expression of UCP3 mRNA was not increased in the 4-M group. Additional research is required to determine whether downhill running leads to increased oxidative stress and how this may affect the expression of UCP3.

Alternatively, there is evidence to suggest that this protein facilitates a proton leak across the inner mitochondrial membrane, and this may be a contributor to the resting respiration rate of skeletal muscle [17,21]. Recent observations in L6 cells show that overexpression of UCP3 increases oxidative metabolism [18]. Furthermore, data from McPherron and Lee [23] show that myostatin null mice have both lower UCP3 mRNA levels and oxygen uptake when corrected for body weight. Together, these observations suggest that UCP3 may play a role in oxidative metabolism. The most important finding from the present study is that UCP3 expression increased 48 h post-exercise, after bouts of eccentric running exercise coalesced with short rest intervals, because estimates of UCP3 mRNA increased in the *plantaris* muscles of 2-R animals. Longer rest intervals or continuous exercise did not increase UCP3 expression over control levels. This correlates, in part, with recent data collected in our laboratory which showed that more muscle damage was produced when rats underwent a single bout of 30-minute intermittent downhill running compared with continuous downhill running (unpublished data). The increase in UCP3 in the 2-R group was not merely from acute exercise responses, because skeletal muscle UCP3 expression is increased for up to 3 h following an acute bout of aerobic exercise [14,30] and returns to basal levels within 24 h after exercise [5,26,30]. The second finding is that UCP3 expression is decreased in the *tibialis anterior* muscles of animals that ran downhill for 30 continuous minutes and in animals that ran 5-minute downhill bouts interspersed with 4-minute rest periods. These findings suggest that both activity and rest intervals may have effects on UCP3 expression, which persist 48 h post exercise, and that non-eccentrically active muscles have different UCP3 responses to downhill running.

Estimated levels of *tibialis anterior* UCP3 mRNA expression decreased 48 h after 30 minutes of continuous downhill running and after six, 5-minute bouts of downhill running interspersed with 4 minutes of rest. These findings suggest that the rest intervals between sets of eccentric running may result in a biphasic UCP3 adaptation in non-eccentric antagonist muscles. Our data imply that in non-eccentrically active muscles, acute rest periods are too short or too long to affect UCP3 levels, but there is a point where optimal rest periods in non-eccentrically active muscles reduces UCP3 (4-R animals). Further work is required to determine if the changes in UCP3 measured 48 h after exercise is from altered oxidative metabolism and substrates during this recovery period, which is regulated in part by the length of rest between periods of exercise. Furthermore, additional experiments are needed to determine if rest intervals also affect UCP3 levels after concentric-uphill running exercise. Because oxygen uptake per unit of body weight is lower in muscles expressing low levels of UCP3 [23] it is possible that the increases in UCP3 in the *plantaris* after eccentric contractions with short (i.e., 2 min) rest intervals, represent altered oxygen consumption after eccentric exercise. Likewise, the reduced UCP3 levels in the *tibialis anterior* in 30-C and 4-R animals may represent reduced oxidative metabolism in these non-eccentrically active muscles. It is also possible that limited or no muscle damage resulted in the non-eccentrically active muscles and that this may have affected the expression of UCP3 mRNA. However, identifying the mechanism leading to these changes is beyond the scope of the current study. All animals were exposed to a small food restriction that resulted in a target weight for each animal of 80% of their *ad libitum* weight. This amount of caloric deficit does not cause stress to the animals and actually improves the overall health of the animal [13,22]. These animals are also more willing to exercise and do so with less stress. Because our control animals were exposed to the same caloric restricted conditions as the exercised animals, it is unlikely that the differences in UCP3 mRNA expression observed in the *plantaris* of 2-R animals or the *tibialis anterior* of 30-C and 4-R animals is the effect of this small caloric restriction diet. It has been reported that forced exercise may decrease food consumption in *ad libitum* fed rats [29]. However, this suppression of feeding was reported with compulsive exercise and not with a single submaximal bout of forced exercise, and therefore we conclude that it is unlikely that the feeding behavior of the animals within this study was suppressed following the single submaximal bout of exercise.

We have shown that UCP3 mRNA expression in the *plantaris* muscle is increased 48 h after a downhill running protocol (i.e., intensive eccentric exercise) consisting of six, 5-minute bouts of running separated with 2 minutes of rest. In addition, we have shown that UCP3 mRNA expression in the *tibialis anterior* muscle is decreased 48 h after 30 minutes of continuous downhill running (i.e., no eccentric exercise) as well as after six, 5-minute bouts of downhill running interspersed with 4 minutes of rest. Additional research is required to determine the factors associated with eccentric muscle contractions that regulate the expres-

sion of UCP3 mRNA and to determine if ROS has a role in regulating UCP3 expression during eccentric exercise.

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