

Vitamin C and E Supplementation Inhibits Acute Exercise-induced Skeletal Muscle Signaling but does not Alter Marker of Muscle Adaptations

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Authors' contributions

This work was carried out in collaboration between all authors. Author KY designed research, analyzed data (all analyses), performed statistical analyses, and wrote this manuscript. Author TM supported animal experiment and sampling. Author HN supported animal experiment and sampling. Author KS had primary responsibility for final content as the supervisor. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to investigate the effects of vitamin C and E supplementation on acute exercise-induced changes of makers of skeletal muscle adaptation and its signaling pathways in mice.

Methodology: Male C57BL/6 mice were assigned to one of four groups: a control group, exercise group, vitamin C and E supplemented group, and vitamin C and E supplemented exercise group. Mice in vitamin C and E supplemented group were given vitamin C (750 mg/kg weight/day) and vitamin E (150 mg/kg weight/day) for two weeks. One hour after the last supplementation, exercise group mice ran on a treadmill at 25 m/min, 8% grade for 120 min.

Results: Vitamin C and supplementation attenuated exercise-induced oxidative stress ($P < 0.01$). However, vitamin C and E supplementation with vitamins C and E did not alter the acute exercise-induced increase in gene expression of peroxisome proliferator-activated receptor- γ coactivator-1 α

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(PGC-1 α), citrate synthase (CS) and vascular endothelial growth factor (VEGF). On the other hand, vitamin C and E supplementation prevented the phosphorylation of AMP activated kinase (AMPK) and p38 mitogen-activated protein kinase (p38 MAPK) following the treadmill running ($P < 0.05$).

Conclusion: These results suggest that reactive oxygen species (ROS) inhibits exercise-induced skeletal muscle signaling but does not alter mitochondrial biogenesis and angiogenesis in skeletal muscle.

Keywords: Vitamin C; vitamin E; reactive oxygen species; mitochondrial biogenesis; angiogenesis.

1. INTRODUCTION

Endurance exercise training induces adaptations in skeletal muscle such as mitochondrial biogenesis [1], angiogenesis [2] and increase of glucose transporter 4 gene and protein expression [3]. These adaptations are regulated by acute exercise-induced several intracellular signaling pathways, including AMP activated kinase (AMPK) [4], p38 mitogen-activated protein kinase (p38 MAPK) [5], calcium/calmodulin-dependent protein kinase (CaMK) [6] and sirtuin 1 (SIRT1) [7]. Such signaling pathways activate peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) [4-7]. PGC-1 α has been known as a master regulator of mitochondrial biogenesis in skeletal muscle and this cofactor binds to the transcription factors related with skeletal muscle adaptations and enhance these transcriptional activities [8]. Recently, it has been reported that transcription coregulators nuclear corepressor 1 (NCoR1) also regulates oxidative metabolism in skeletal muscle [9]. NCoR1 negatively modulates the skeletal muscle adaptations by suppressing transcription activity of transcription factors related to oxidative adaptation. Thus, exercise-induced skeletal muscle adaptation is regulated by several intracellular signaling pathways [9].

Acute endurance exercise induces the generation of reactive oxygen species (ROS) in muscle [10]. It has been shown that exercise-induced production of ROS also regulates skeletal muscle adaptations. First, Gomez-Cabrera et al. [11] has reported that antioxidant vitamin C supplementation prevents the mitochondrial biogenesis induced by exercise training in rat skeletal muscle. Since then, evidence supporting that antioxidant supplementation attenuates exercise training-induced adaptations in human and rodent skeletal muscle has been published [12-14]. On the other hand, we and another research group have shown that antioxidant supplementation does not alter exercise training-induced adaptations [15-19]. Accordingly, the effects of

antioxidant supplementation on endurance exercise training-induced skeletal muscle adaptations are still not definitive.

To further understand the role of exercise-induced ROS, it is necessary to investigate the effects of antioxidants on intracellular signaling and markers of skeletal muscle adaptation during acute exercise, because exercise training adaptation reflects an accumulation of acute exercise stimulus. The effects of acute exercise-induced ROS on skeletal muscle signals and adaptations have been evaluated using allopurinol inhibitor of xanthine oxidase (XO) or nonspecific antioxidant vitamin C. It was shown that ROS generated by XO during acute exercise regulates some intracellular signals [20,21]. However, these results only reflect the effects of ROS derived from XO on exercise-induced mitochondrial biogenesis but do not reflect the effects of other sources of ROS such as mitochondria [22] and leukocytes [23]. Wadley and McConell [24] reported that vitamin C does not alter the acute exercise-induced increases in makers of mitochondrial biogenesis. Also, they reported that vitamin C supplementation did not prevent the exercise-induced increase of oxidative stress. Thus, it is likely that their methodology was not suitable to determine the effects of exercise-induced ROS on mitochondrial biogenesis. Vitamin C is water-soluble antioxidant and scavenges radicals [25]. However, it is possible that vitamin C act as a pro-oxidant [26]. On the other hand, vitamin C can react with vitamin E radicals to regenerate vitamin E [27] and a combination of vitamin C and E has high antioxidant capacity and decreases exercise-induced oxidative stress [15, 28]. In addition, to our knowledge, there are no studies investigating the effects of antioxidant supplementation and/or non-exhaustive endurance exercise on skeletal muscle gene expression of NCoR1 that coregulator of oxidative adaptation.

The aim of this study was to investigate the effects of vitamin C and E supplementation on

acute exercise-induced changes of makers of skeletal muscle adaptation and its signaling pathways in mice. We hypothesized that vitamin C and E supplementation would inhibit the activation of AMPK and p38 MAPK during acute exercise and prevent increases in the markers of muscle adaptation after acute exercise.

2. MATERIALS AND METHODS

2.1 Experimental Animals and Protocol

Male C57BL/6 mice (8 weeks old) were purchased from Takasugi experimental animals supply (Kasukabe, Japan). Five animals were housed together in 1 cage (27×17×13 cm) in a controlled environment under a light-dark cycle (lights on at 0900 and off at 2100). The experimental procedures followed the Guiding Principles for the Care and Use of Animals in the Waseda University Institutional Animal Care and Use Committee. The mice were derived into two groups (non-supplemented (NS) and vitamin C and E supplemented (VS), n=40/ groups) and then allocated to either an exercise (n=20) or a sedentary (n=20) group.

Mice in VS group were given vitamin C (750 mg/kg weight/day) and vitamin E (150 mg/kg weight/day) with a feeding needle. Mice in NS group were given vehicle (200 mg of triolein and 20 mg of Tween in 1 ml of saline). Mice were received the antioxidants or vehicle for two weeks. One hour after the last supplementation exercise group mice ran on a treadmill at 25 m/min, 8% grade for 120 min. Immediately or three hours after the treadmill runexercising and sedentary mice were sacrificed under light anesthesia with the inhalant isoflurane (Abbott, Tokyo, Japan). The gastrocnemius muscle was excised, quickly frozen in liquid nitrogen and stored at -80°C until analysis. A portion of gastrocnemius was quickly immersed in RNAlater (Applied Biosystems, Foster City, CA) and stored at -80°C.

Gastrocnemius muscle was homogenized in tissue protein extraction reagent (T-PER; Pierce, Rockford, IL) containing protease inhibitor (Complete mini protease inhibitor cocktail tablets; Roche, Mannheim, Germany) and phosphatase inhibitor (Roche) at 4°C. The homogenate was centrifuged at 10,000 × g for 15 min at 4°C and the protein content of the supernatant was determined by a bicinchoninic acid (BCA) Protein Assay Kit (Thermo, Rockford, IL). This supernatant was used for the measurement of

hydro peroxide (H₂O₂), thiobarbituric acid reactive substances (TBARS) and trolox equivalent antioxidant capacity (TEAC) and immunoblot analysis.

2.2 Measurement of Markers of Oxidative Stress

H₂O₂ level in gastrocnemius muscle was measured with SensoLyte ADHP Hydrogen Peroxide Assay Kit (Ana Spec, San Jose, CA). TBARS, a marker of lipid peroxidation, in gastrocnemius muscle was measured with TBARS Assay Kit (Cayman Chemical Co, Ann Arbor, MI). Non enzymatic antioxidant capacity TEAC in gastrocnemius muscle was measured according to the methods of Re et al. [29].

2.3 Real-time Quantitative PCR

Total RNA was extracted from the gastrocnemius muscle homogenate using Trizol reagent (Invitrogen, Carlsbad, CA) and the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity of total RNA was assessed using the NanoDrop system (NanoDrop Technologies, Wilmington, DE). Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed with the Fast 7500 real-time PCR system (Applied Biosystems) using the Fast SYBR[®] Green PCR Master Mix (Applied Biosystems). The thermal profiles consisted of 10 min at 95°C for denaturation followed by 40 cycles of 95°C for 3 s and annealing at 60°C for 15 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the housekeeping gene, and the $\Delta\Delta CT$ method was used as previously described [30] to quantify target gene expression. All data are represented relative to its expression as fold change based on the values of the sedentary group. Specific PCR primer pairs for each studied gene are shown in Table 1.

2.4 Immunoblot Analysis

Homogenized samples were diluted with homogenizing buffer to 5.5 mg/mL protein. Samples were then mixed with 4 × Laemmli sample buffer (Bio-Rad, Richmond, CA) in 10% 2-mercaptoethanol to 4 mg protein/mL and heated at 60°C for 10 min. Aliquots of samples

Table 1. Primer sequences for real-time RT-PCR analysis

Gene	Forward	Reverse
GAPDH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
CS	GGAGCCAAGAACTCATCCTG	TCTGGCCTGCTCCTTAGGTA
VEGF	CCCCAGAATGAAGGTTACACA	TGTCAAAGGGAGAAGGGTTTT
PGC-1 α	GACTGGAGGAAGACTAAACGGCCA	GCCAGTCACAGGAGGCATCTTT
NCoR1	GACCCGAGGGAAGACTACCATT	ATCCTTGTCGAGGCAATTTG

were separated by electrophoresis on 8% SDS–polyacrylamide gels and then transferred to polyvinyl difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature in tris-buffered saline/0.1% Tween 20 (TBST) with 5% skim milk. Then, membranes were incubated overnight in the TBST in 5% BSA containing anti-phospho-AMPK α Thr¹⁷² (#2535; Cell Signaling Technology, Beverly, MA), anti-AMPK α (#2532; Cell Signaling), anti-phospho-p38MAPK Thr¹⁸⁰/Tyr¹⁸² (#9211; Cell Signaling) and anti-p38 MAPK (#9212; Cell Signaling). The membranes were washed with TBST and incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (1:30000; Bio-Rad), visualized by enhanced chemiluminescence (ECL; GE Healthcare, Arlington Heights, IL) and quantified by densitometry (Las-3000, Fuji photo, Tokyo, Japan).

2.5 Statistical Analysis

Data are presented as mean \pm SE. A two-way analysis of variance (ANOVA) was performed (SPSS V17.0, IBM Japan, Ltd, Tokyo, Japan) to determine the main effects of exercise and/or antioxidant supplementation. If this analysis revealed a significant interaction, Bonferroni's post-hoc test was used to determine the significance among the means. Statistical significance was defined as $P < 0.05$.

3. RESULTS

3.1 Effects of Acute Exercise and Antioxidant Supplementation on Oxidative Stress Marker and Antioxidant Capacity

Single bout of endurance exercise increased in H₂O₂ and TBARS in skeletal muscle ($P < 0.01$, Figs. 1A and B). The acute exercise-induced increase in H₂O₂ and TBARS were prevented in mice given antioxidants ($P < 0.01$, Figs. 1A and B). In addition, 2 weeks of vitamin C and E

supplementation resulted in significantly higher TEAC in skeletal muscle ($P < 0.05$, Fig. 1 C).

3.2 Effects of Acute Exercise and Antioxidant Supplementation on Markers of Skeletal Muscle Adaptation

Gene expression levels of citrate synthase (CS), a marker of mitochondrial biogenesis, and vascular endothelial growth factor (VEGF), a marker of angiogenesis, were significantly increased 3 hour following 120 min treadmill running ($P < 0.01$, Figs. 2A and B). However, antioxidant supplementation did not alter expression levels of CS and VEGF (Fig. 2A and B).

3.3 Effects of Acute Exercise and Antioxidant Supplementation on Phosphorylation of Protein Kinase

Phosphorylation of AMPK and p38 MAPK were significantly increased immediately following endurance exercise ($P < 0.01$, Figs. 3A and B). Antioxidant supplementation prevented the increase in phosphorylation of AMPK and p38 MAPK ($P < 0.05$, Figs. 3A and B).

3.4 Effects of Acute Exercise and Antioxidant Supplementation on Skeletal Muscle Adaptation Signaling Pathway Gene Expression

Three hours after the endurance exercise, PGC-1 α and NCoR1 mRNA was significantly decreased compared to sedentary mice ($P < 0.01$, Figs. 4A and B). However, antioxidant supplementation did not alter gene expression of PGC-1 α and NCoR1 ($P < 0.01$, Figs. 4A and B).

4. DISCUSSION

The purpose of the present study was to investigate whether antioxidant supplementation affects acute exercise-induced change in makers of skeletal muscle adaptation and its signaling pathways. In contrast to our hypothesis, the

present study showed that combined supplementation with vitamins C and E did not alter the acute exercise-induced increase in markers of mitochondrial biogenesis and angiogenesis. However, interestingly, vitamin C and E supplementation prevented the phosphorylation of AMPK and p38 MAPK following the treadmill running. These results are in accordance with the rodent study of Wadley et al. [21].

It has been reported that a combination of vitamin C and vitamin E reduces oxidative stress in blood and organs [15,28]. In agreement with these studies, we have shown that supplementation with vitamin C (750 mg/kg body weight) and E (150 mg/kg body weight) inhibited exercise-induced oxidative stress (H₂O₂ and TBARS) and increased the basal level of antioxidant capacity (TEAC). Thus, in this study, it seems that combination of vitamin C and E was

appropriate for the evaluation of the effects of inhibiting exercise-induced ROS production on the adaptations in skeletal muscle.

Acute exercise increases markers of skeletal muscle adaptations such as mitochondrial biogenesis [31] and angiogenesis [32]. In this study, we measured CS and VEGF gene expression as markers of skeletal muscle adaptation. Indeed, it has been known that CS protein content and activity reflect the mitochondrial density [33] and VEGF is involved in the regulation of angiogenesis [34]. The results of this study indicate that exercise increased in CS and VEGF gene expression but vitamin C and E did not affect the exercise-induced increase in CS and VEGF gene expression. These results are consistent with the studies suggesting that antioxidant supplementation did not alter exercise training-induced adaptations [15-19].

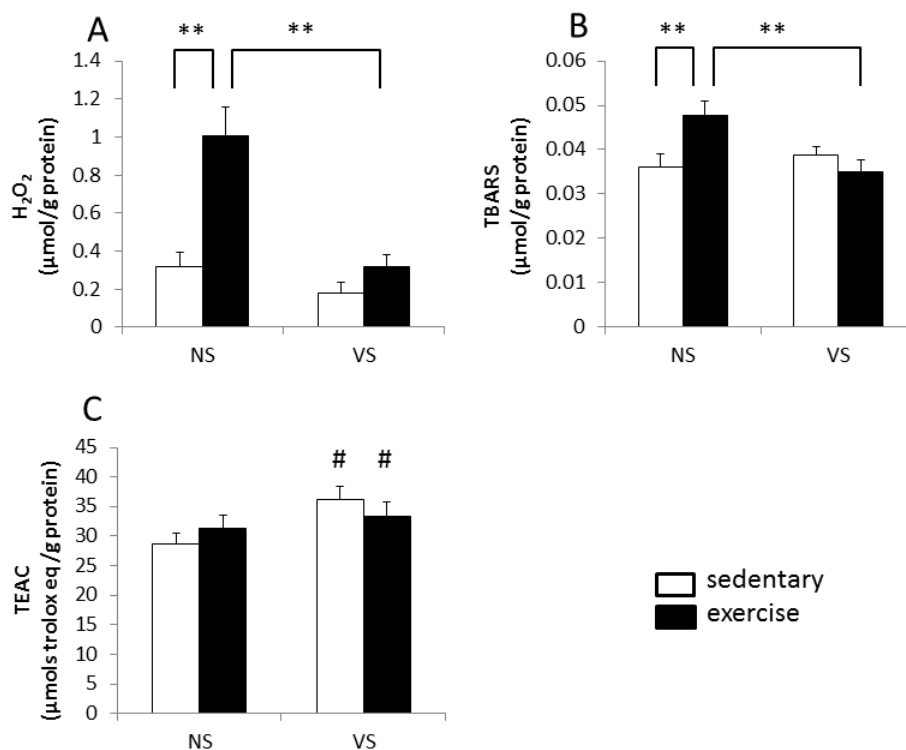


Fig. 1. Hydrogen peroxide (H₂O₂; A), thiobarbituric acid reactive substances (TBARS; B) and trolox equivalent antioxidant capacity (TEAC; C) in the gastrocnemius muscle of mice after 2 weeks of vehicle (NS) or vitamin C and E supplementation (VS) under sedentary condition (sedentary) or immediately after 120 min of treadmill running(exercise). Values are means±SE. #, P<0.05 main effect for vitamin C and E supplemented. **, P<0.01, vs NS exercise group

In addition, we evaluated the effects of antioxidant supplementation on exercise-induced changes in intracellular signaling i.e., AMPK, p38 MAPK, PGC-1 α and NCoR1. We observed that vitamin C and E supplementation attenuated the exercise-induced phosphorylation of AMPK and p38 MAPK. These proteins regulate activation and/or expression of PGC-1 α [4,5]. However, despite these effects of supplementation, in the present study, gene expression of PGC-1 α was not affected by antioxidant supplementation. PGC-1 α is the key factor of skeletal muscle adaptations and modulates CS [35] and VEGF [36] expression. Thus, it is likely that significantly increased CS and VEGF gene expression levels observed in both exercise groups (with or without

vitamin C and E) arose from the augmentation of the PGC-1 α by exercise, irrespective of antioxidant supplementation. These observations agree with findings of the study of Wadley et al. [21], who reported that XO inhibition by allopurinol prevented the exercise-induced phosphorylation of p38 MAPK and ERK but did not alter the increases in acute exercise-induced signal gene expression levels such as PGC-1 α and the training adaptations in rat skeletal muscle. In the present study, it is unclear why PGC-1 α and makers of skeletal muscle adaptation were not affected by antioxidant supplementation, even though phosphorylation of AMPK and p38 MAPK were prevented by the antioxidant supplement. It has been reported that

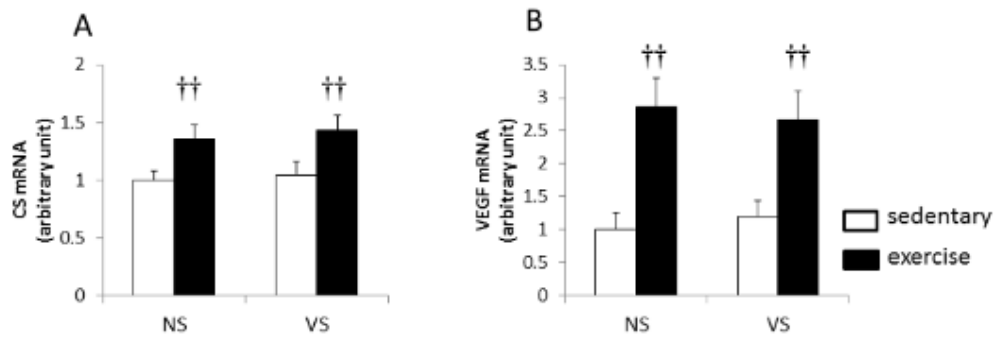


Fig. 2. Citrate synthase (CS; A) and vascular endothelial growth factor (VEGF; B) mRNA in the gastrocnemius muscle of mice after 2 weeks of vehicle (NS) or vitamin C and E supplementation (VS) under sedentary condition (sedentary) or 3 hour after 120 min of treadmill running(exercise). Values are means \pm SE. ††, P<0.01, main effect for exercise

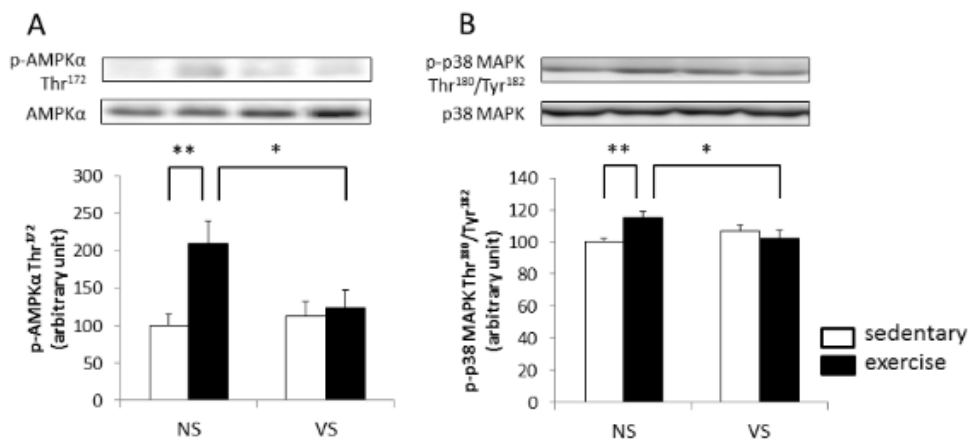


Fig. 3. Phosphorylation of AMP activated kinase (AMPK)- α (A) and p38 MAPK (B), in the gastrocnemius muscle of rats after 2 weeks of vehicle (NS) or vitamin C and E supplementation (VS) under sedentary condition (sedentary) or immediately after 120 min of treadmill running (exercise). Values are means \pm SE. *, P<0.05, vs NS exercise group. **, P<0.01, vs NS exercise group

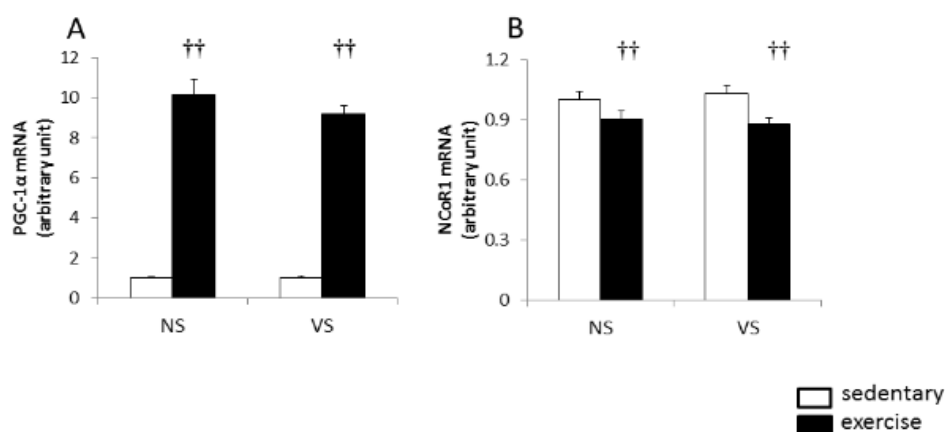


Fig. 4. peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α ; A) and nuclear corepressor 1 (NCoR1; B) mRNA in the gastrocnemius muscle of mice after 2 weeks of vehicle (NS) or vitamin C and E supplementation (VS) under sedentary condition (sedentary) or 3 hour after 120 min of treadmill running(exercise). Values are means \pm SE. ††, P<0.01, main effect for exercise

PGC-1 α is regulated by several intracellular signals such as AMPK [4], p38 MAPK [5], CaMK [6] and SIRT1 [7]. The limitation of the present study is that we were unable to examine the effect of vitamin supplementation on intracellular signals except for AMPK and p38 MAPK. Hence, it remains unclear what kind of signal activated PGC-1 α in present study. However, it is possible that the increase in PGC-1 α gene expression was induced by intracellular signals except for AMPK and p38 MAPK.

NCoR1 is transcription coregulator that negatively regulate skeletal muscle oxidative metabolism [9]. However, it is unclear whether expression level of this gene is changed by exercise and/or antioxidants. Especially, to our knowledge, there are no studies investigating the effects of antioxidant supplementation and/or non-exhaustive acute exercise on skeletal muscle NCoR1. Our results show that NCoR1 mRNA was decreased 3 hours after the non-exhaustive treadmill exercise but was not altered by antioxidant supplementation. Thus, it is likely that exercise-induced gene expression of CS in this study does not only result from the increase in PGC-1 α but also the decrease in NCoR1 with exercise.

5. CONCLUSION

In conclusion, the combined supplementation with vitamin C and E prevented the acute exercise-induced phosphorylation of AMPK and p38 MAPK. However, despite these effects on signaling, supplementation with vitamin C and E

did not alter the exercise-induced change in gene expression of coregulator PGC-1 α and NCoR1, and makers of adaptation, CS and VEGF. These results suggest that acute exercise-induced ROS does not alter mitochondrial biogenesis and angiogenesis in skeletal muscle.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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