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The Effect of Aerobic Exercise Training Versus Resveratrol Supplementation on Mitochondrial Biogenesis in Skeletal Muscle of High-fat Diet-induced Obese Mice

Kyung-Il Kim[1], Sang-Min An[2], Hee-Geun Park[1] and Wang-Lok Lee[1]*

[1]*Department of Sports Science, Chungnam National University, Daejeon 34134, Korea*
[2]*Department of Sport and Leisure Studies, Korea University, Sejong 30019, Korea*

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The purpose of this study was to analyze the effects of aerobic exercise and resveratrol supplementation on mitochondrial biogenesis in skeletal muscle of high-fat diet-induced obese mice. In this study, 4-wk-old C57BL/6 male mice were divided into four groups, with 10 animals in each group: a normal diet group (NC), high-fat diet group (HC), high-fat diet group with resveratrol supplementation (HRe), and high-fat diet GROUP with exercise (HE). Aerobic exercise was performed on a treadmill for 40~60 min/d at 10~14 m/min, 0% grade, 4 d/wk for 16 wk. Resveratrol (25 mg/kg bodyweight) was administrated once a day, 4 d/wk for 16 wk. There was a significance difference in COX-IV mRNA expression in the NC group versus that in the HC group ($p<0.05$). The expression of the *SIRT-3, PGC-1a*, and *COX-IV* mRNA genes in the HE group increased significantly as compared with the expression of these genes in the HC and HRe groups ($p<0.05$). These results indicated that high- fat diet-induced obesity did not affect mitochondria biogenesis gene expression in skeletal muscle. In contrast, aerobic exercise training increased the expression of mitochondria biogenesis gene expression in skeletal muscle in high-fat diet-induced obese mice. These findings suggested that aerobic exercise but not resveratrol supplementation had a positive effect on mitochondrial biogenesis in skeletal muscle in high-fat diet-induced obese mice.

Key words : Aerobic exercise, mitochondrial biogenesis, obese, resveratrol, skeletal muscle

Introduction

Obesity is characterized by increased storage of fatty acids in an expanded adipose tissue mass and in peripheral tissues [3]. Chronic obesity from lack of physical activity or high fat diet is a cause for cardiovascular disease, metabolic syndrome, arteriosclerosis, type-2 diabetes [46] and mitochondrial dysfunction [47]. Mitochondrial dysfunction is also the cause of a syndrome of metabolic defects that includes hypomagnesemia, hypertension, and hypercholesterolemia [57]. Defective mitochondrial function in muscle can lead to reduced fatty-acid oxidation and inhibition of glucose transport [38].

Mitochondrial biogenesis is regulated by regulatory factors such as PGC-1α (peroxisome proliferator-activated receptor-ɣ coactivator 1α) and NRF-1 (nuclear respiratory factor 1) [25]. PGC-1α is involved into mitochondrial oxidative metabolism and the maintenance of glucose and lipid [34]. Also, it is highly related to COX-IV (Cytochrome c oxidase subunit IV), ATP and citrate synthase activity [34, 41, 56].

It is known that moderate endurance exercise training improve metabolic and mitochondrial function [21] and regular exercise training improves the function of the mitochondria via the expression of PGC-1α and Tfam (mitochondrial transcription factor A) [22].

SIRT-1 (silent mating type information regulation 2, homolog 1) is activated by the PGC-1α and [7] recent studies have shown that SIRT-3 (silent mating type information regulation 2, homolog 3) exists in the mitochondria and increases PGC-1α that induce mitochondrial biogenesis by regulating mitochondrial number and substrate utilization [52]. In previous studies, Palacios et al. [45] showed that expression level of SIRT-3 was increased in 7-week-old FVB/NJ rats after 6 weeks of wheel-cage exercise. Hokari et al. [20] showed that treadmill running increased the amount of SIRT-3 protein and mitochondria density in the skeletal muscle of the rats. And it has been known that the trained

***Corresponding author**
Tel : +82-42-821-6456, Fax : +82-42-823-0387
E-mail : leewl@cnu.ac.kr

individuals showed a higher number of SIRT-3 proteins and mitochondria compared to sedentary individuals [31]. COX-IV protein plays an important role in aerobic exercise capacity by regulation of mitochondrial oxiative phosphorylation [4, 13, 53]. Also, COX-IV is used as an indicator of aerobic exercise performance because it is controlled by PGC-1α that regulate mitochondrial biogenesis and function [44].

Recently, many studies have used a variety way to treat obese induced metabolic complication such as caloric restriction and functional nutrition supplementation. Resveratrol (3, 4, 5-trihydroxystilbene, RSV), one of phytochemical, is a polyphenol compound found in berries, grapes, nuts, and several plants source [33, 39]. Resveratrol mimics the metabolic effects of long-term calorie restriction, and many in vitro studies have demonstrated that resveratrol has an anti-obesity potential by inhibiting pre-adipocyte differentiation, decreasing adipocyte proliferation, inducing adipocyte apoptosis, decreasing lipogenesis, and promoting lipolysis and fatty acid β-oxidation [9, 32]. And it has been previously identified as an antioxidation, and anti-inflammation agent. Also, it prevents cardiovascular disease, cancer progression, and metabolic syndrome [12].

Ungvari et al. [55] that resveratrol improves endurance capacity by increasing mitochondria number, reducing reactive oxygen species (ROS) and ameliorating lipid metabolism. This result was supported by the finding that resveratrol mediated activation of AMPK increased intercellular NAD+ level [48, 54] and decreased PGC-1α (peroxisome proliferator activated receptor-ɣ coactivator-1α) acetylation through deacetylation of SIRT-1 (silent mating type information regulation 2 homolog) [5, 35]. In addition, resveratrol contributes to changing the type of muscle twitch fiber and PGC-1α activation by improving the function of mitochondria biogenesis [54]. Surprisingly, the maximal oxygen consumption was significantly increased and average running distance was about 2 times as long in the resveratrol supplementation group compared to the control group [54]. Also, in previous rodent studies, there were positive effects observed with resveratrol dosage (25-30 mg/kg/day or 400 mg/kg/day). These doses for mice would be equivalent to 2,100 or 28,000 mg/kg/day for humans (70 kg). Thus, these doses are unlikely to be obtained from natural or functional foods [48, 54]. Nonetheless, there are only a few studies comparing the effects of exercise training and resveratrol supplementation. In addition, there is little research to our knowledge, comparing the effects of either exercise training or resveratrol supplementation on high fat diet-induced metabolic complication.

Therefore, the purpose of this study was to analyze the effects of either aerobic exercise or resveratrol supplementation on mitochondrial biogenesis (SIRT-1, SIRT-3, PGC-1α, COX-IV mRNA) in skeletal muscle of high fat diet-induced obesity mice.

Material and Methods

Animals and diet

4-week-old male C57BL/6 (Central Experiment Animal, Korea, n=40) mice were housed in cages (5 mice per cage) in a standard experimental laboratory, at a temperature of 22±2℃, with 50±5% humidity. After a one-week acclimatization period, the mice were fed either a high fat diet (45% of energy from fat, Orient Bio Inc., # D12451) or a normal diet (10% of energy from fat, Orient Bio Inc., # D12451) ad libitum for 16 weeks (Table 1).

The classification of groups was classified into total four groups such as normal diet group (NC, n=10), high fat diet group (HC), high fat diet group with resveratrol supplementation (HRe, n=10) and high fat diet with exercise group (HE, n=10) and then resveratrol supplementation and exercise were applied for 16 weeks. All experiments were approved by the Animal Care and Use Committee at the Chungnam National University (CNU-00494).

Resveratrol supplements and muscle dissection

Resveratrol supplement purchased from Sigma Aldrich Inc. Resveratrol supplement was orally given 25 mg/kg body weight dissolved in a 0.1 ml solution of Dimethyl Sulfoxide (DMSO). The supplements were administered orally using a disposable 1 ml syringe at dose 0.1 ml per mouse 4 times a week. All the mice were sacrificed after fasted for 12 hr under anesthesia using a mixture ketamine (80 mg/kg) and xylazine (10 mg/kg). The gastrocnemius muscle was

Table 1. Formulas of rodent feed

Product	Normal diet		High-fat diet (D12492)	
	g%	Kcal%	g%	Kcal%
Carbohydrate	44.2	58	26.2	20
Protein	18.6	24	26.3	20
Fat	6.2	18	34.9	60
Total		100		100
kcal/gm	3.1		5.24	

Table 2. Exercise protocol

Weeks	Treadmill exercise		
	Speed (m/min)	Time (min)	%VO_2max
1~2	10	40	60
3~5	12	50	70
6~16	14	60	76

dissected, weighed and immediately frozen in liquid nitrogen and stored at -70℃ until analysis.

Exercise protocol

Exercise training was performed on a motor treadmill at moderate intensity for 16 weeks, 4 days/week for 40-60 min/day. The exercise was performed at a speed of 10 m/min for 1-2 weeks, 12 m/min for 3-5 weeks and 14 m/min for 6-16 weeks. This exercise intensity be selected to 60-76% of maximal oxygen uptake [51] because it was known that moderate endurance exercise training affected mitochondrial biogenesis [21]. All groups were exposed to the same noise and handling as the exercise groups (Table 2).

RNA extraction and RT-PCR

In order to extract Total RNA, 40 mg of gastrocnemius muscle tissue was put into Trizol (Qiagen, Germany) and the tissue was grinded for 20 seconds using a homogenizer, and the homogenized solution prepared from the homogenizer was allowed to stand at room temperature for 5 minutes. Chloroform (Sigma, USA) of 200 μl was added into this solution, and then the solution was allowed to stand for 3 minutes at room temperature after mixing for 15 seconds so that the chloroform was wholly mixed well, and was centrifuged (13,000 rpm, 4℃, 15 min.). The only clear supernatant solution was separated from the centrifuged solution into a new tube, and then was allowed to incubation at room temperature for 10 minutes after adding the same amount of isopropanol (Sigma, USA) into this supernatant solution, and it was centrifuged (13,000 rpm, 4℃, 10 min.). 70% ethanol of 1 ml was added on RNA pellet formed on the bottom of tube by centrifugation, and the pellet was washed twice (4,500 rpm, 4℃, 5 min.). If the RNA pellet was completely dried, the RNA pellet was dissolved by adding the 0.01% DEPC-treated distilled water of 30 μl. The array of forward and reverse primer is the same as shown in Table 3. The amplification was performed in a total volume of 20 μl, which included 2 μl of cDNA, 1 μl of each primer (10 pmol/μl), and 16 μl of DEPC (Diethyl pyrocarbonate) water.

Statistical methods

Statistical analysis of the data was performed by SPSS Version 22.0 using one-way ANOVA with LSD post-hoc tests. Statistical significance was defined as α=.05.

Results

Changes of body weight and muscle mass

Fig. 1 shows body and muscle. The body weight of high fat diet groups (HC, HRe, HE) was significantly increased, compared to that of NC group (p<0.05, Fig. 1A). The muscle mass of HE was significantly increased, compared to that of NC, HC and HRe groups (p<0.05, Fig. 1B).

Change of food and calorie intake

Fig. 2 shows food intake and calorie intake. The feed intake of HRe was significantly lower than that of NC, HC and HE groups (p<0.05, Fig. 2A). The calorie intake of NC was lowest, compared to that of HC, HRe and HE groups (p<0.05, Fig. 2B). Notably, the calorie intake of HRe was significantly lower than that of HC and HE groups (p<0.05, Fig. 2B).

Change of SIRT-1 and SIRT-3 mRNA

Fig. 3 shows the gene expression of SIRT-1 and SIRT-3 mRNA. There was no significance among all the groups in SIRT-1. However, the gene expression of SIRT-3 mRNA in HE was significantly increased, compared to that in HC and HRe groups (p<0.05, Fig. 3B). Also, the gene expression of SIRT-3 in NC was a little increased but not significantly

Table 3. Primer sequences used for Real-time PCR

Gene	Forward	Reverse primer
GAPDH	GAGAGTGTTTCCTCGTCC	AATGAAGGGGTCGTTGATGG
SIRT-1	GATGACGATGACAGAACGTC	GAATTGTTCGAGGATCGGTG
SIRT-3	AGACTTGGGTCCTCTGAAAC	CTCCCACACAGAGGGATATG
PGC-1α	CTGTGTGTCAGAGTGGATTG	GCAGCACACTCTATGTCACT
COX-IV	TCTTGGTCTTCCGGTTGC	CTCTGGAAGCCAACATTCTG

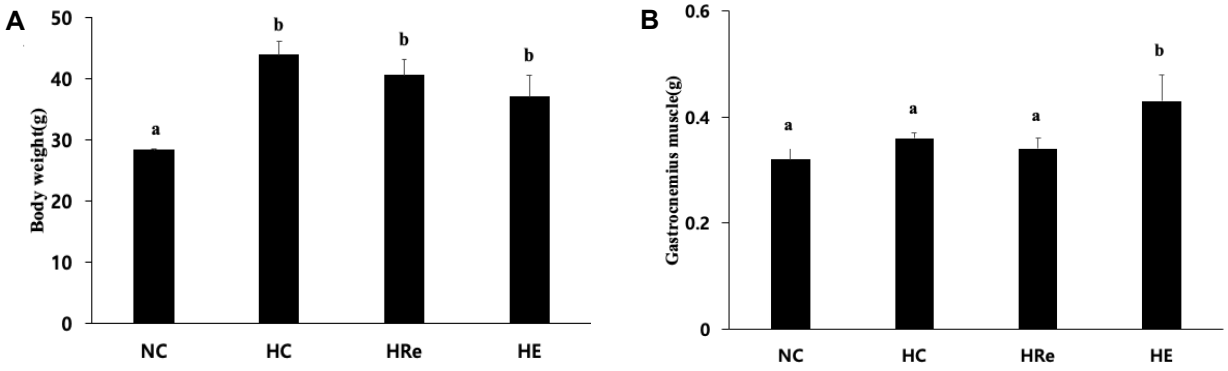

Fig. 1. The comparison of body and muscle weight among groups. (A) body weight; (B) gastrocnemius muscle weight. NC, normal diet control; HC, high fat diet control: HRe, high fat diet with resveratrol; HE, high fat diet with exercise. Values represent Mean ± SD. Different alphabet shows significantly different (p<0.05).

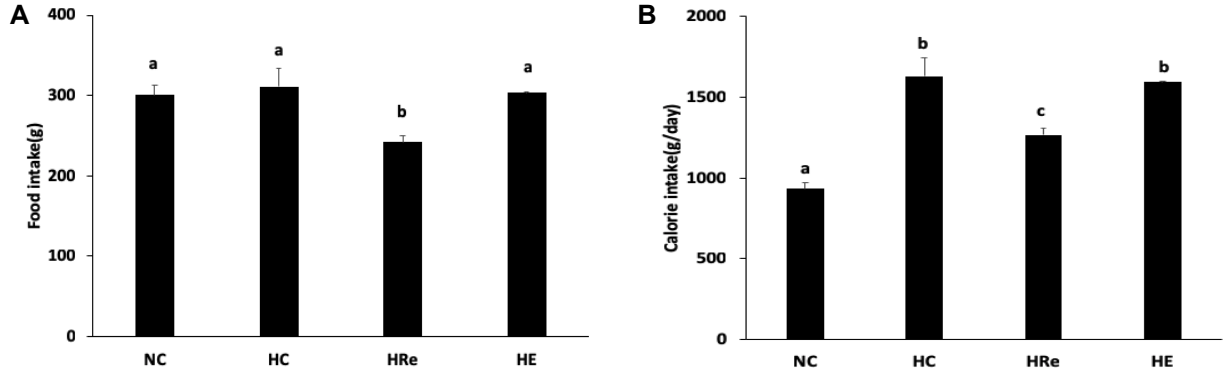

Fig. 2. The comparison of food and calorie intake among groups. (A) food intake; (B) calorie intake. NC, normal diet control; HC, high fat diet control: HRe, high fat diet with resveratrol; HE, high fat diet with exercise. Values represent Mean ± SD. Different alphabet shows significantly different (p<0.05).

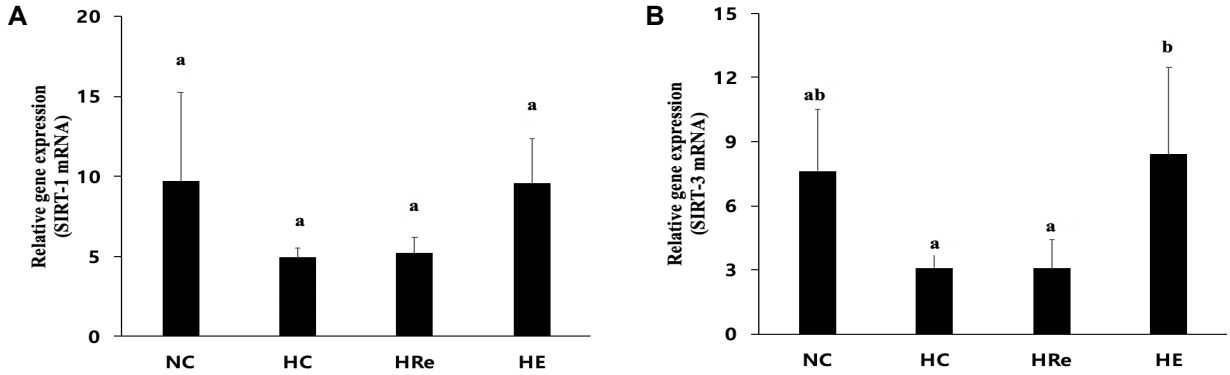

Fig. 3. The comparison of SIRT-1 and SIRT-3 mRNA among groups. (A) SIRT-1 mRNA; (B) SIRT-3 mRNA. NC, normal diet control; HC, high fat diet control: HRe, high fat diet with resveratrol; HE, high fat diet with exercise. Values represent Mean±SD. Different alphabet shows significantly different (p<0.05).

changed, compared to HC and HRe groups.

Change of PGC-1α mRNA and COX-IV mRNA

Fig. 4 shows the gene expression of PGC-1α and COX-IV mRNA. The gene expression of PGC-1α mRNA in HE was significantly increased compared to that in the other groups. Also, the gene expression of COX-IV mRNA in HE was significantly increased, compared to the other groups (p<0.05, Fig. 4B). Moreover, the COX-IV mRNA of NC was significantly increased, compared to that of HC (p<0.05, Fig. 4B).

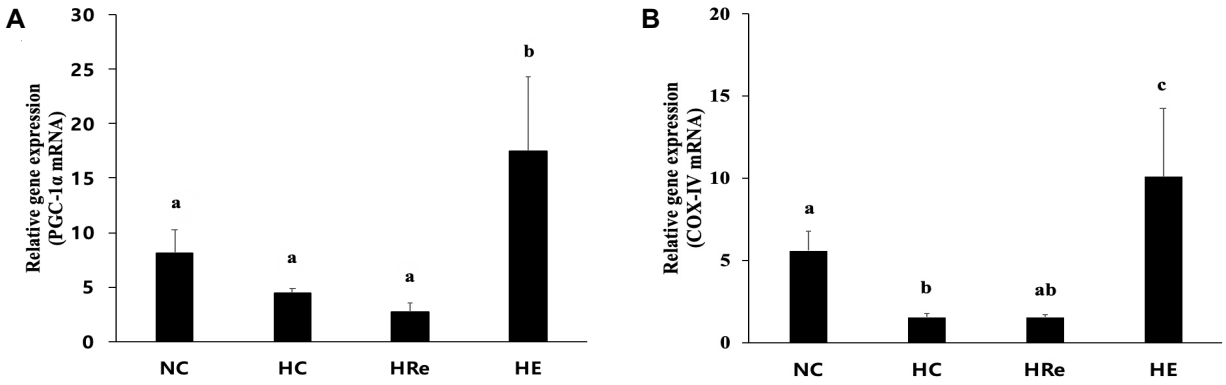

Fig. 4. The comparison of PGC-1α and COX-IV mRNA among groups. (A) PGC-1α mRNA; (B) COX-IV mRNA. NC, normal diet control; HC, high fat diet control: HRe, high fat diet with resveratrol; HE, high fat diet with exercise. Values represent Mean±SD. Different alphabet shows significantly different (p<0.05)

Discussion

A high fat diet is known to increase body weight and fat accumulation so that to make obese. Moderate exercise increases energy expenditure and has been recommended for the treatment of obesity [26]. Also, it is known that resveratrol treatments reduce body weight compared to non-treatment in animals [1]. In this study, we found that body weight was not significantly reduced by resveratrol supplementation for 16 weeks in high fat-induced obese mice. This result is opposed to the fact that there was significant weight reduction with resveratrol supplementation by both 10 or 30 mg/kg/day in obese mice [9]. However, our previous results with 10mg/kg had showed no significant effect on body weight [24]. Even if the dosage of resveratrol supplementation was changed to 25 mg/kg/day in this study, the body weight was not significantly changed. It seems that the effect of resveratrol supplementation to reduce the body weight is not clear, depending on the level of obese, duration and doses in obese mice [9, 46]. Also, the gastrocnemius muscle mass of HE group was significantly increased compared to that of other groups in this study. It seems that exercise training not resveratrol supplementation has a positive effect to increase skeletal muscle weight. Thus, further study is needed to understand better with a various dosage and duration of resveratrol supplementation on body weight and muscle mass.

In previous studies of obese animals, resveratrol supplementation appears to mimic effect of caloric restriction. Also, it was reported that the effect of caloric restriction is caused by a protein called sirtuin [28]. Sirtuin is activated by NAD+-dependent deacetylases during the fasting condition in the brain, liver and kidney [31]. Further, it has been shown that sirtuin regulates metabolism and gene expression by stimulating the activation of SIRT-1 deacetylation. It even recovers damaged genes by activating PGC-1α, inhibiting weight gain and aging [2, 3, 29, 35]. In previous studies, the expression level of the SIRT-1 molecule was significantly reduced in the obese group compared to non-obese group [37]. Also, high fat diet-induced obese mice inhibited the activation of the SIRT-1 mRNA by the influence of white adipose tissue and metabolic dysfunction of the muscle tissue [8].

In this study, the SIRT-1 mRNA was not significantly changed in all the groups. However, there was a tendency that the expression of SIRT-1 mRNA of NC and HE group were higher by 51% than that of HC and HRe group. These results are consistent with the results of previous study [8, 37]. Therefore, it is considered that the high-fat diet has a negative effect on the SIRT-1 activation of skeletal muscle in high fat diet-induced obese mice but when considering the dose of resveratrol, the negative effect size might be different.

SIRT-3 is expressed in the mitochondria of the brain, heart, liver, adipose and skeletal muscle [36] and may be increased in AMP/ATP ratio, AMPK activation and regulate oxidative phosphorylation of mitochondria [16, 43]. However, Lanza et al. [31] found that SIRT-3 protein and number of mitochondria are less expressed in sedentary individuals, and chronic high-fat diet inhibits SIRT-3 protein expression and induces hyperacetylation of mitochondrial protein [19]. Several previous studies have reported that aerobic exercise increases the expression of SIRT-3 in skeletal muscle [20, 27, 45]. Gurd et al. [15] reported that the expression of SIRT-3, PGC-1α by treadmill running and SIRT-3

protein in the skeletal muscle were increased by exercise and dietary restriction [45]. In human studies, SIRT-3 expression in muscle was also increased by aerobic exercise [31].

In this study, there was no significance among NC, HCh and HRe groups, it means that high fat diet-induced obese did not affect to the gene expression of SIRT-3. However, there was a tendency that high fat diet-induced obese made the gene expression of SIRT-3 reduced. Because the gene expression of SIRT-3 mRNA in HC was the lowest compared to that in the other groups. These results are a little similar to the results of previous study [19]. Therefore, high-fat diet may have a negative effect on SIRT-3 activation of skeletal muscle in high fat diet-induced obese mice. In this study, the expression of skeletal muscle SIRT-3 mRNA of high-fat diet and exercise group (HE) was a significantly increased compared to that of high-fat diet group (HC). These results are consistent with the results of previous studies [15, 20, 27, 45]. Therefore, it is considered that the aerobic exercise has a positive effect on the SIRT-3 activation of skeletal muscle in high fat diet-induced obesity mice.

Das et al. [11] was reported that resveratrol induces activation of SIRT-3 protein and has been shown to have a beneficial effect on health by activating the SIRT-3. In this study, there was no significant difference of SIRT-3 mRNA expression level in the normal diet group (NC), the high-fat diet group (HC) and high-fat diet group with resveratrol treatment (HRe) of the skeletal muscle. Those result is contrast to the study by Das et al. [11]. It seems that even if the scientific evidence to supports the ability to regulate the SIRT-3 by resveratrol has been reported, the effect of resveratrol supplementation would be different depending on the way of doses and treatment [11, 42]. Further, as the effect of resveratrol was compared to the exercise intervention, the effect of resveratrol might be reduced in statistics. Therefore, further research will be needed.

Excessive fat accumulation or obesity is a major cause of mitochondrial dysfunction and reducing proteins such as mitochondrial biogenesis and PGC-1α acting on oxidative phosphorylation causes skeletal muscle mitochondrial dysfunction [23]. Several previous studies have reported decreased PGC-1α expression and mitochondrial function in skeletal muscle of high-fat diet mice [30, 40, 49]. Also, the expression level of PGC-1α was significantly lower in obese subjects than in normal subjects [37]. Decreased expression of PGC-1α reduces exercise adaptability and be caused acute inflammation and mitochondrial muscle disease [17, 18].

In this study, there was no significant difference of expression of skeletal muscle PGC-1α mRNA among normal diet group (NC), high-fat diet group (HC) and high-fat diet with resveratrol (HRe). However, the expression level of skeletal muscle PGC-1α mRNA in HE group was significantly higher than that in other groups. Further, there was not a significance but tendency that the expression of PGC-1α mRNA of HE group was higher than that of NC group by 83%. This is consistent with the results of previous studies [23, 30, 49]. It seems that high-fat diet is considered to have a negative effect on the PGC-1α activation of skeletal muscle in induced obesity mice. However, the study of the effect of resveratrol on PGC-1α mRNA is needed further to understand better.

COX-IV protein plays an important role in aerobic exercise capacity by involved in the regulation of mitochondrial oxiative phosphorylation [4, 13, 53] and COX-IV is used as an indicator of aerobic exercise performance because it is controlled by PGC-1α that regulate mitochondrial biogenesis and function [44]. In this study, the expression level of the COX-IV mRNA of the skeletal muscle in the high-fat diet group (HC) was the lowest compared to that in the other groups. Also, the COX-IV mRNA of the skeletal muscle was most affected by the aerobic exercise training. This result is consistent with the previous study [44]. Therefore, high-fat diet group (HC) is considered to have a negative effect on COX-IV activation of skeletal muscle in induced obesity mice.

Several previous studies have reported acute high-intensity cycle exercise was significantly increased the protein of skeletal muscle PGC-1α and COX-IV [6, 10]. Short et al. [53] reported that levels of PGC-1α and Cox-IV mRNA were increased after 16 weeks of aerobic exercise and Greene et al. [14] reported that the protein level of AMPK, PGC-1α and COX-IV which are closely related to mitochondrial biogenesis were significantly increased 12 weeks of moderate intensity exercise. In this study, high-fat diet with aerobic exercise group (HE) significantly increased expression of skeletal muscle COX-IV mRNA comprared to the high-fat diet (HC). Therefore, aerobic exercise is considered to have a positive effect on the activation of skeletal muscle COX-IV in induced obesity mice.

Resveratrol supplementation has been reported to induce transcription of nuclear-encoded mitochondrial sub-signaling substances such as COX-IV and to improve mitochondrial function as well as mitochondrial respiration [50]. In

this study, high-fat diet group with resveratrol treatment (HRe) showed no significant difference in the expression level of skeletal muscle COX-IV mRNA compared to the high-fat diet group (HC). Although these results are in contrast to the results of Scarpulla et al. [50], it seems that the expression of COX-IV was not affected because the expression of PGC-1α had not been activated. Therefore, resvertrol supplementation is considered to have no effect on the activation of skeletal muscle COX-IV in the skeletal muscle of high fat diet-induced obese mice.

Taken together, in this study, there were no significant effect of resveratrol supplementation on the body weight and mitochondria biogenesis markers compared to aerobic exercise. It means that the effect of resveratrol supplementation was not enough to reduce body weight and to activate mitochondrial biogenesis whereas aerobic exercise training had a positive effect to reduce body weight and improve mitochondria biogenesis gene activation. In conclusion, aerobic exercise training was more effective than resveratrol supplementation to increase mitochondrial biogenesis markers in high fat diet induced obese mice. Furthermore, aerobic exercise has a kind of medicine to ameliorate high fat diet-induced metabolic complication. However, the effect of resveratrol supplementation should be researched further with a variety of doses and duration.

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초록 : 고지방식이로 유도된 비만 쥐의 골격근에서 유산소 운동 훈련 또는 레스베라트롤 투여가 미토콘드리아 생합성에 미치는 영향

김경일[1] · 안상민[2] · 박희근[1] · 이왕록[1]*
([1]충남대학교 스포츠과학과, [2]고려대학교 사회체육학과)

본 연구에서는 고지방식이로 유발된 비만 쥐의 골격근에서 유산소 운동과 레스베라트롤 투여가 미토콘드리아 생합성에 미치는 영향을 조사하였다. 4주령 C57BL/6의 수컷 쥐를 이용하여, 일반 식이 그룹(NC, n=10), 고지방식이 그룹(HR, n=10), 레스베라트롤 투여와 고지방식이 그룹(HRe, n=10), 유산소 운동 그룹(HE, n=10)으로 분류하였다. 유산소 운동은 16주 동안 40~60 min/day 동안 10-14m/min, 0% grade의 강도로 주당 4회 트레드밀 운동을 실시하였고, 레스베라트롤은 16주 동안 1일 1회, 주당 4회 체중 당 25 mg/kg을 투여하였다. COX-IV mRNA 발현은 NC와 HC 그룹 간에 유의한 차이가 있었으며(p<0.05), HE 그룹의 SIRT-3, PGC-1α 및 COX-IV mRNA 발현은 HC 및 HRe 그룹에 비해 유의하게 증가하였다(p<0.05). 또한, 오직 HE 그룹의 PGC-1α 및 COX-IV mRNA의 발현만이 HC 그룹에 비해 유의하게 증가하였다(p<0.05). 이상의 결과를 종합해보면, 고지방식이로 유발된 비만 쥐는 골격근에서 미토콘드리아 생합성 유전자 발현에 영향을 나타내지 않는 것으로 보인다. 하지만, 유산소 운동 훈련은 고지방식이로 유발된 비만 쥐의 골격근에서 미토콘드리아 생합성 유전자 발현을 증가시키는 것으로 나타났다. 이러한 연구 결과는 레스베라트롤 투여가 아닌 유산소 운동이 고지방식이로 유도된 쥐의 골격근에서 미토콘드리아 생합성에 긍정적인 영향을 미친다는 것을 시사한다.