Conjugated linoleic acid supplementation enhances insulin sensitivity and peroxisome proliferator-activated receptor gamma and glucose transporter type 4 protein expression in the skeletal muscles of rats during endurance exercise

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ABSTRACT

Objective(s): This study examined whether conjugated linoleic acid (CLA) supplementation affects insulin sensitivity and peroxisome proliferator-activated receptor gamma (PPAR-γ) and glucose transporter type 4 (GLUT-4) protein expressions in the skeletal muscles of rats during endurance exercise.

Materials and Methods: Sprague-Dawley male rats were randomly divided into HS (high-fat diet (HFD) sedentary group, n = 8), CS (1.0% CLA supplemented HFD sedentary group, n = 8), and CE (1.0% CLA supplemented HFD exercise group, n = 8). The rats in the CE swam for 60 min a day, 5 days a week for 8 weeks.

Results: The serum glucose and insulin contents and homeostasis model assessment of insulin resistance (HOMA-IR) value of the CS and CE were significantly decreased compared to those of the HS. The PPAR-γ protein expressions in the soleus muscle (SOM) and extensor digitorum longus muscle (EDL) were significantly higher in the CE than in the HS. In addition, the PPAR-γ protein expression in the SOM of the CS was significantly higher than that in the HS. On the other hand, the GLUT-4 protein expression of the SOM in the CE was significantly higher compared to that in the HS. However, there was no significant difference in GLUT-4 protein expression in the EDL among the groups.

Conclusion: CLA supplementation with/without endurance exercise has role in improvement of insulin sensitivity. Moreover, when CLA supplementation was accompanied by endurance exercise, the PPAR-γ protein expression in SOM and EDL and the GLUT-4 protein expression in SOM were enhanced compared with the control group.

Introduction

Conjugated linoleic acid (CLA) is a fatty acid mixture of positional geometric isomers of octadecadienoic acid (linoleic acid 18:2n-6) with a conjugated double-bond system (1). Commercially, mixed isomer CLA is marketed as a weight-loss supplement. Different isomers of CLA have varied biological functions, such as reducing carcinogenesis, decreasing adipose mass, and modulating immune function and type 2 diabetes (2). CLA also induces hyperinsulinemia and insulin resistance, primarily in mice (3). However, the effects of CLA supplementation on skeletal muscle are still unclear (4).

Obesity is becoming a major public health problem in affluent societies (5). It is well known that diet control and physical exercise are the two main approaches to suppress obesity (6, 7). Previous studies reported that a HFD increases the total energy intake, and that excess dietary fat is greater stored than excess dietary carbohydrate or protein. Thus, an increase in excessive energy intake from fat can reduce physical activity, and this decline of physical activity causes obesity (8, 9).

Physical activity has been considered as a cornerstone in the treatment of obesity (10, 11). Among various types of physical activities, endurance exercise has long been reported to reduce body weight, ameliorate postprandial triglyceride
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Table 1. Composition of experimental diets

<table>
<thead>
<tr>
<th>Variable</th>
<th>HFD(1)</th>
<th>CLA Suppl diet(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>0</td>
<td>200.0</td>
</tr>
<tr>
<td>Starch</td>
<td>200.0</td>
<td>200.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>150.0</td>
<td>150.0</td>
</tr>
<tr>
<td>Lard</td>
<td>350.0</td>
<td>337.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1.0</td>
<td>10.0</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Choline barbiturate</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>DL-a-tocopherol</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>c-9, t-11 CLA</td>
<td>-</td>
<td>6.5</td>
</tr>
<tr>
<td>t-10, c-12 CLA</td>
<td>-</td>
<td>6.5</td>
</tr>
<tr>
<td>Energy (kcal/kg)</td>
<td>5350.0</td>
<td>5350.0</td>
</tr>
<tr>
<td>Protein (% kcal/g)</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>26.0</td>
<td>26.0</td>
</tr>
<tr>
<td>kcal/g</td>
<td>59.0</td>
<td>59.0</td>
</tr>
</tbody>
</table>

1) 35% fat of total diet weight were supplied for the HS group 2) 1.0% CLA was added and adjusted to the high-fat diet (HFD) for the CS and CE group 3) 76.81% CLA mixture
CLA: conjugated linoleic acid, CS: CLA supplemented HFD sedentary, CE: CLA supplemented HFD exercise, HS: HFD sedentary

Exercise protocol and sample collection
The exercised rats swam for 60 min a day, 5 days a week for 8 weeks. The water temperature of the swimming pool was maintained at approximately 35±1 °C in a plastic barrel (depth: 50 cm, radius: 25 cm). At the end of the experimental protocol, in an overnight-fasted state, the rats were sacrificed by exsanguination, and blood was drawn from the left ventricle under light diethyl ether anesthesia after the 12-hr fasting period. The skeletal muscles: soleus muscles (SOM) and extensor digitorum longus muscles (EDL) as fast muscle fiber (24) and abdominal fat (AFT) as fat tissue were dissected and immediately snap-frozen in liquid nitrogen. The skeletal muscles and fat tissue were stored at -70 °C until they were analyzed.

Biochemical assays
The serum glucose, triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) levels were analyzed using commercial enzymatic kits (Asan Pharmaceutical Co., Yongin, Korea). The fasting insulin level was measured using standard radio-immunity kits (Linco Research, Inc., St. Louis, MO, USA). The serum low-density lipoprotein cholesterol (LDL-C) level was calculated from TC, TG, and HDL-C concentrations using the following Friedewald formula: \[ (TC - (HDL-C + (TG/5))) \]. Insulin resistance was calculated according to the homeostasis model assessment of insulin resistance (HOMA-IR) using the following formula: \( \frac{\text{fasting glucose level (mmol/l)} \times \text{fasting insulin level (mU/ml)}}{22.5} \).

Western blot analysis
In order to analyses PPAR-γ and GLUT-4 protein expression, muscles (the SOM and EDL) were

Materials and Methods
Experimental animals and diet
Six-week-old male Sprague-Dawley rats weighing 230 to 250 g were obtained (Samtako Co., Osan, Korea) and individually housed in a controlled environment at 23±1 °C with 50±5% relative humidity, under a 12-hr light-dark cycle. All animals were given free access to tap water and food. After an acclimatization period of 1 week, all rats were randomly divided into 3 groups: HS, HFD (35% fat of total diet weight) (22) sedentary group; CS, CLA supplemented HFD sedentary group; and CE, CLA supplemented HFD exercise group. For CLA supplementation, 1.0% CLA (76.82% CLA mixture; 36.8% cis-9,trans-11 CLA, 37.8% trans-10, cis-12 CLA, and 1.2% trans-9,trans-11 CLA) (HK Biotech Co., Gyeongnam, Korea) was substituted for dietary fat in the adjusted HFD (Table 1). During this period, dietary intake was measured daily, and the change in the body weight of each animal was noted weekly. The dietary regimens were based on AIN-76 of the animal diet and were modified from a previous study (23). All of the experimental protocols were approved by the Animal Study Committee of Sunmoon University.

Response, increase the rate of fat oxidation, and improve insulin sensitivity (12-15). However, the physiological and molecular mechanisms for these benefits have not been completely understood (16).

Glucose transporter-4 (GLUT-4) translocation to the plasma membrane through the insulin signaling pathway is well proven (17,18). In addition, thiazolidinediones, which are peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists that decrease insulin resistance, are widely used as a treatment for patients with type 2 diabetes (19). Although PPAR-γ is highly expressed in adipose tissue than in muscle, muscle-specific-PPAR-γ depletion is susceptible to developing insulin resistance in mice (20). Previous studies reported that exercise had improved insulin sensitivity due to an increase in PPAR-γ protein expression (20-21).

The aim of this study was to investigate the effects of CLA supplementation on the insulin resistance and PPAR-γ and GLUT-4 protein expression in the skeletal muscles of rats on a HFD during endurance exercise.

homogenized on ice with a polytron homogenizer in 20 mmol/l Tris-HCl buffer (pH 7.5) containing 5 mmol/l ethylenediaminetetraacetic acid, 2 mmol/l phenylmethylsulfonyl fluoride, and 1:200 protease inhibitor cocktail (Sigma, St Louis, MO, USA). The protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. An aliquot of tissue extract containing 20 µg of protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) in a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA). After treating with blocking buffer (phosphate-buffered saline (PBS) containing 10% skim milk) for 90 min, the membrane was incubated with primary polyclonal antibodies for 2 hr, followed by five 10-min washes with PBS (5% Tween 20). The membranes were washed and then incubated with horseradish peroxidase (HRP)-conjugated anti-goat immunoglobulin G (IgG) or anti-rabbit IgG (Santa Cruz, CA, USA) for 1 hr, followed by five 10-min washes with PBS (5% Tween 20). The target proteins were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscatawy, NJ, USA). The films were photographed and the protein bands of interest were quantified with band analyzer software (Bio-Rad, Hercules, CA, USA).

Statistical analysis
The data were expressed as the means ± standard error of the mean (SEM) using the SPSS/PC program. Statistical analyses were conducted using one-way ANOVA followed by LSD’s post hoc test to verify significant difference among groups. Statistical significance was set at P<0.05.

Results
The weight-related variables
The changes in the weight-related variables are summarized in Table 2. The body weight, weight gain, and AFT in the CS and CE were significantly lower than those in the HS (P<0.001). Additionally, the body weight (P<0.05), weight gain (P<0.01), and AFT mass (P<0.05) in the CE were significantly reduced compared to those in the CS. In the weight of the skeletal muscles, the SOM in the CE was significantly increased compared to those in the HS (P<0.001), whereas the EDL did not show any significant differences among the groups.

Changes in serum parameters
The changes in the serum parameters are presented in Table 3. The serum TG, TC, HDLC, and LDLC levels in the CS and CE were significantly lower than those in the HS (P<0.001). Furthermore, the serum TC concentration was found to have significantly reduced in the CE compared to that in the CS (P<0.01).

Changes in serum glucose and insulin contents and HOMA-IR level
As shown in Figure 1, the serum glucose and insulin contents and HOMA-IR value of the CS (glucose: P<0.001, insulin: P=0.006, HOMA-IR: P=0.004) and CE (glucose: P=0.001, Insulin: P=0.001, HOMA-IR: P=0.001) were significantly decreased

### Table 2. The level of body weight, weight gain, fat weight, and skeletal muscles weight among groups

<table>
<thead>
<tr>
<th>Variables (g)</th>
<th>HS (n = 8)</th>
<th>CS (n = 8)</th>
<th>CE (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>468.1 ± 3.3</td>
<td>433.2 ± 5.9***</td>
<td>414.2 ± 6.5***</td>
</tr>
<tr>
<td>Weight gain</td>
<td>193.7 ± 3.2</td>
<td>167.7 ± 3.1***</td>
<td>149.7 ± 5.5***</td>
</tr>
<tr>
<td>AFT</td>
<td>17.5 ± 0.6</td>
<td>12.6 ± 0.5**</td>
<td>10.3 ± 0.8**</td>
</tr>
<tr>
<td>SOM</td>
<td>0.125 ± 0.003</td>
<td>0.159 ± 0.004***</td>
<td>0.162 ± 0.007***</td>
</tr>
<tr>
<td>EDL</td>
<td>0.167 ± 0.004</td>
<td>0.172 ± 0.006</td>
<td>0.178 ± 0.002</td>
</tr>
</tbody>
</table>

### Table 3. The level of serum total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, and triglycerides among groups

<table>
<thead>
<tr>
<th>Variables (g)</th>
<th>HS (n = 8)</th>
<th>CS (n = 8)</th>
<th>CE (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>74.6 ± 2.5</td>
<td>67.6 ± 2.9***</td>
<td>62.4 ± 1.6***##</td>
</tr>
<tr>
<td>HDLC</td>
<td>20.1 ± 1.3</td>
<td>33.0 ± 2.5***</td>
<td>37.2 ± 3.3***</td>
</tr>
<tr>
<td>LDLC</td>
<td>26.0 ± 1.2</td>
<td>14.4 ± 2.8***</td>
<td>7.7 ± 3.3***</td>
</tr>
<tr>
<td>TG</td>
<td>125.9 ± 3.8</td>
<td>104.6 ± 2.6***</td>
<td>99.6 ± 1.1***</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SEM. ***P<0.001 compared with the HS group; ##P<0.01 compared with the CS group. TG; triglycerides, TC; total cholesterol, HDLC; high density lipoprotein cholesterol, LDLC; low density lipoprotein cholesterol. CLA; conjugated linoleic acid, CLA; conjugated linoleic acid, CS; CLA supplemented HFD sedentary, CE; CLA supplemented HFD exercise, HS; HFD sedentary
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Figure 1. Changes of serum glucose and insulin contents and homeostasis model assessment of insulin resistance (HOMA-IR) level among groups compared to those of the HS. Furthermore, the glucose content of the CE was significantly reduced compared to that of the CS (P=0.005)

PPAR-γ and GLUT-4 protein expression level according to skeletal muscle fiber type

As shown in Figure 2, PPAR-γ protein expressions in the SOM (P=0.005) and EDL (P=0.043) were higher in the CE than those in the HS. In addition, the PPAR-γ protein expression in the SOM of the CS (P=0.035) was significantly higher than that in the HS. On the other hand, the GLUT-4 protein expression of the SOM in the CE was significantly higher compared to that in the HS (P=0.034). However, there was no significant difference in the GLUT-4 expression of the EDL among the groups (Figure 3).

Discussion

To the best of our knowledge, this is the first study to investigate the effects of CLA supplementation with/without endurance exercise on insulin resistance and PPAR-γ and GLUT-4 protein expression by analyzing the skeletal muscle fiber types of rats on a HFD. Our results suggest that CLA supplementation and endurance exercise independently affected the insulin sensitivity and PPAR-γ and GLUT-4 protein expression according to the skeletal muscle fiber type.

Previous studies have reported that CLA decreases body mass and fat mass, while improving serum lipids profiles (25-28). Our results show that not only weight-related parameters such as body weight, weight gain, and fat tissue weight, but also serum parameters such as TG, TC, HDLC, and LDLC in the CLA-supplemented groups with/without endurance training were significantly reduced compared to the control group. Some studies reported that endurance exercise results in the significant decrease of fat mass and body mass as well as an improved serum lipids profiles (29, 30). However, in our study, in the case of CLA supplementation, the endurance exercise group have tended lower in body weight, weight gain, AFT mass, and glucose and TC concentrations compared to the CS. These results suggest that endurance exercise during CLA supplementation might result in the utilization of fat as a substrate during exercise, and that endurance exercise reduces body mass and body fat mass. Previous studies reported that the lipid profile values did not increase above the normal range in six-week-old male Sprague-Dawley rats fed with...
HFD for 8 weeks (20, 23). However, in our study, statistically significant differences were shown in the serum lipid profile concentrations after 8 weeks of treatment. Those levels may ameliorate serum lipid profiles when one engages in at least 8 weeks of endurance exercise with/without CLA supplementation. These results suggest that the combination of CLA supplementation and endurance exercise may be recommended for utilizing fat as a substrate, reducing body mass and body fat mass, and ameliorating serum lipid profiles compared to CLA supplementation or endurance exercise alone rather than in conjunction with each other.

Many animal studies have tried to induce a model of obesity using a dietary composition of approximately 35 to 40% lard and also shown that HFDs induce hyperglycemia, hyperinsulinemia and insulin resistance (31-34). It is also reported that endurance exercise ameliorates the serum insulin concentration and HOMA-IR level in rats fed a HFD (35). In this study, CLA supplementation with/without endurance exercise decreased the insulin content and HOMA-IR value. These results suggested that CLA supplementation could potentially results in lower insulin resistance regardless of participation in endurance exercise.

The effects of CLA on skeletal muscle are still unclear. The most pronounced effect of CLA isomer feeding was a stimulation of protein synthesis, which enhanced lean body mass. This stimulatory effect was mainly observed in the muscles of sedentary rats (36-39). Regular physical activity leads to a number of adaptations in skeletal muscle (40). Skeletal muscle fiber types have been traditionally classified as “slow twitch” and “fast twitch” according to their expression of myosin heavy chain isomers. The slow twitch fibers mainly depend on oxidative pathways, and the fast twitch fibers mainly depend on glycolytic pathways for ATP production (41). In this study, the SOM and EDL, which are hind-limb muscles, were selected as typical slow and fast twitch fibers, respectively. We found a considerably increased SOM mass in the case of CLA supplementation regardless of participation in endurance exercise, while there was no significant difference in EDL mass. This result seems to indicate that the increase in SOM mass is a result of swimming exercise.

It is well established that the mechanism for elevating glucose uptake is mainly by the translocation of GLUT-4 vesicles from the intracellular pool to the plasma membrane through insulin binding to its receptor (42-44). Although the exact correlation between PPARs and GLUT-4 protein expression is not clearly understood, PPAR-γ protein is reported to play an important role for regulation of GLUT-4 gene expression in skeletal muscle tissues (45). Our study also showed that the PPAR-γ protein expression in the SOM and EDL of the CE was significantly higher compared to that in the HS, and the PPAR-γ protein expression in the SOM of the CS tended to be significantly higher than that in the HS. Previous studies have shown that PPAR-γ protein expression increases with exercise in plasma and adipose tissue (46, 47), and is upregulated by endurance or a moderate level of intense exercise (48, 49). Our finding is in accordance with previous studies, which show that the PPAR-γ protein expression of the SOM in the CE rats was significantly higher than that of the HS rats. However, the PPAR-γ protein expression of the CLA-supplemented non-exercised group in the EDL did not show significant improvement compared to the HS group. As mentioned above, the soleus muscle, as slow twitch fibers, were mainly activated during swimming, and it might be assumed that changes in protein expression are affected by external factors such as obtained muscle type during exercise and the type and duration of the exercise. Thus, further studies considering the time and type of exercise including prolonged exercise and muscular exercise are needed to better elucidate the relationship between exercise and protein expression.

Our study showed that the muscle GLUT-4 protein expression of the SOM in the CE was significantly higher than that in the HS, while the GLUT-4 protein expression of the SOM in the CS was not significantly different compared to that in the HS.
It is reported that the slow twitch fibers are more responsive to insulin than the fast twitch fibers (50). These results are likely due to the fact that swimming exercise mainly mobilizes the SOM, which increases in response to endurance training, thus facilitating glucose uptake into the trained muscle. In our study, the GLUT-4 protein expression in the EDL did not show significant improvement. Taken together, our results indicate that the combined case of CLA supplementation and endurance exercise improve not only the GLUT-4 expression level, but also the PPAR-γ expression level in the SOM. Skeletal muscle comprises a relatively large mass in the body and is an important target tissue for glucose metabolism by insulin (51, 52). Therefore, we can assume that the significant changes in the GLUT-4 activity of the CE in the SOM may have significant physiological effects because the soleus muscle is the major target tissue of insulin for glucose uptake during swimming.

Taken together, even when accompanied by a HFD, CLA supplementation with/without endurance exercise plays a role in the suppression of obesity and the improvement of insulin sensitivity. When CLA supplementation was accompanied by endurance exercise, the PPAR-γ protein expression in the SOM and EDL and the GLUT-4 protein expression in the SOM were enhanced compared to the control group. In addition, the PPAR-γ protein expression in the SOM of the CS was enhanced by only CLA supplementation.

Conclusion

In conclusion, CLA supplementation under a non-endurance exercising condition in rats may play a role in enhancing the PPAR-γ protein expression in red muscles. In addition, CLA supplementation under an endurance exercise condition in rats may enhance the PPAR-γ protein expression in the SOM and EDL and the GLUT-4 protein expression in the SOM. However, there was no synergic effect of CLA supplementation and endurance exercise on insulin resistance in the serum and protein expression in skeletal muscles of rats under the condition of CLA supplementation with/without endurance exercise.

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Conflict of interests

Authors have no conflict of interests.

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