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Effects of aged garlic extract and endurance exercise on skeletal muscle FNDC-5 and circulating irisin in high-fat-diet rat models

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BACKGROUND/OBJECTIVES: Irisin, a newly identified hormone, is associated with energy homeostasis. We investigated whether aged garlic extract (AGE) and exercise training intervention could improve body weight, insulin sensitivity, skeletal muscle fibronectin domain containing protein 5 (FNDC-5) levels, and plasma irisin in high-fat diet (HFD).
MATERIALS/METHODS: Male Sprague Dawley rats were fed a ND (normal diet, n = 5) or HFD (n = 28) for 6 weeks. After 6 weeks, all rats were divided into 5 groups for the next 4 weeks: ND, (normal diet, n = 5), HFD (high-fat diet, n = 7), HFDA (high-fat diet + aged garlic extract, n = 7), HFDE (high-fat diet + exercise, n = 7), and HFDEA (high-fat diet + exercise + aged garlic extract, n = 7). Exercise groups performed treadmill exercises for 15-60 min, 5 days/week, and AGE groups received AGE (2.86 g/kg, orally injected) for 4 weeks.
RESULTS: Significant decreases in body weight were observed in the ND, HFDE, and HFDEA groups, as compared with the HFD group. Neither intervention affected the masses of the gastrocnemius muscle or liver. There were no significant differences in glucose levels across the groups. The homeostatic model assessments of insulin resistance were significantly higher in the HFD group, as compared with the ND, HFDA, HFDE, and HFDEA groups. However, skeletal muscle FNDC-5 levels and plasma irisin concentrations were unaffected by AGE or exercise in obese rats. AGE supplementation and exercise training did not affect skeletal muscle FNDC-5 or plasma irisin, which are associated with insulin sensitivity in obese rats.
CONCLUSION: Our results suggest that the protection against HFD-induced increases in body fat/weight and insulin resistance that are provided by AGE supplementation and exercise training may not be mediated by the regulation of FNDC-5 or irisin.

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INTRODUCTION

Obesity, which is caused by energy imbalance and physical inactivity, is strongly associated with increased morbidity and mortality [1]. Rats that receive a high-fat diet (HFD) are suitable animals for obesity-related studies because they are generally sedentary, and the intensity and duration of their exercise can easily be regulated using treadmill machines [2-5].

Many researchers have attempted to inhibit the development of obesity through exercise, diet, and other interventions [6,7]. Preventive strategies that treat obesity through exercise with or without nutritional supplementation can have important effects on is important factor in obesity [8]. Aged garlic extract (AGE) is well-documented alternative source of garlic that is odorless and rich in antioxidants [9]. Moreover, it has been reported that AGE may play a role in the mitigation of cardiovascular disease [10,11], inflammation [12], hypertension [13], and cancer risk [14]. To examine the effects of AGE and exercise as a combined treatment, we recently performed a study of the efficacy of AGE and exercise interventions in HFD induced-obesity. The results of this study confirmed that AGE and exercise were associated with weight loss in obese rats [5].

Recent evidence indicates that numerous hormones regulate energy expenditure, inhibiting weight gain, and are released from adipose tissue and skeletal muscle in rats with HFD-induced obesity [15,16]. In addition, some studies have focused on the protein factors and hormones that are produced in skeletal muscle, and on the regulation of energy expenditure

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factors that are both secreted from skeletal muscle and measurable in the blood [17-19].

One of these hormones, irisin, is secreted into the circulation by contracting skeletal muscle after cleavage from the membrane protein fibronectin type Ⅲ domain containing protein 5 (FNDC-5) as a result of exercise training [20,21]. Proteins that contain this domain are reported to be involved in the change of white adipose tissue into brown adipose tissue, a process that is associated with the expenditure of calories and, accordingly, the generation of heat. Moreover, FNDC-5/irisin is believed to mediate the beneficial effects of exercise on adipose tissues in humans and rodents [18,22]. For example, Elbelt *et al.* [23] have reviewed studies that examined whether FNDC-5/irisin can account for the beneficial effects of exercise in humans. Furthermore, Sanchis *et al.* [24] reported that irisin and the FNDC-5 gene are released from skeletal muscle, indicating that the release of irisin and FNDC-5 promotes energy expenditure during obesity leading to glucose homeostasis. In contrast, circulating levels of irisin, which regulates caloric expenditure, were shown to decrease in exercise-trained humans [25]. The results of other studies have shown that patients with type 2 diabetes have lower irisin levels than subjects with normal glucose tolerance [26,27]. Thus, there is still a paucity of evidence concerning the exact role of irisin in skeletal muscle metabolism.

Considering these previous results, it is clear that many studies are necessary to definitively identify the basic mechanisms by which skeletal muscle FNDC-5 and irisin levels are associated with weight loss in obese subjects. The objective of this study was to determine whether AGE supplementation and exercise training result in decreased weight, improved insulin sensitivity, elevated circulating levels of irisin and increased skeletal muscle FNDC-5 levels in obese rats.

MATERIALS AND METHODS

Animals

Thirty-three 3-week-old male Sprague Dawley rats were supplied by Dae Han Biolink Company (Chung Chung Bukdo, Korea), and were maintained (2 animals per cage) on a 12:12-h light-dark cycle at 25℃. The rats received water *ad libitum* with either a normal diet (15.8% kcal from fat) or a HFD (45% kcal from fat) for 10 weeks. All rats were acclimated to their new environment for 7 days. At 4 weeks of age, rats were randomly divided into normal diet (ND; n = 5, D10012G, Research Diets) and HFD (high fat diet group; n = 28, D12451, Research Diets) for 10 weeks. The HFD rats received HFD for 6 weeks, and then were randomly divided into 4 groups for the remaining 4 weeks: the HFD group (high-fat diet, n = 7), the HFDA group (high-fat diet + aged garlic extract, n = 7), the HFDE group (high-fat diet + exercise, n = 7), and the HFDEA group (high-fat diet + exercise + aged garlic extract, n = 7) The experimental study design is shown in Fig. 1. All research procedures were approved by the Institutional Animal Care and Use Committee of Pusan National University (PNU 2008-MY08-01).

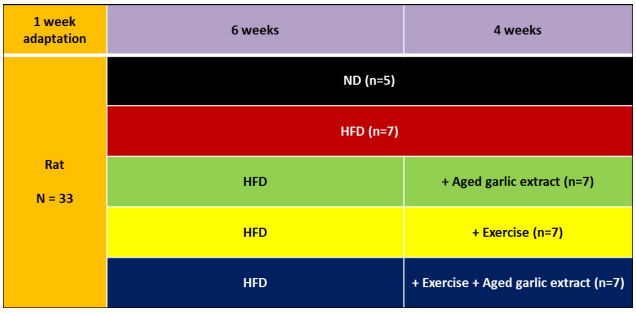

Fig. 1. Flow chart outlining the experimental groups. ND: normal diet, HFD: high-fat diet.

Exercise training protocol

The exercise program consisted of endurance exercise training. The animals in the exercise groups trained on a motor-driven treadmill for 1 week for acclimation. The exercise groups trained at 15 m/min for 45 min during the first week and 60 min during the second week. Further, they trained at 20 m/min for 30 min during the third week and 45 min during the fourth week [5]. The rats were kept in their cages at all times between the exercise periods. The exercise protocols were performed 5 days per week for 4 weeks. These exercise programs are considered to be of moderate intensity [28].

Aged garlic extract supplementation

AGE was obtained from Uiseong Black-Garlic Farming Association of Korea, Co., Ltd. AGE supplementation, and placebo (water) was administered orally via gavage. The supplementation protocol consisted of 1 daily dosage of 2.86 g/kg body weight administered 30 min before exercise for 4 weeks. We have previously shown that this protocol is an effective means of decreasing body weight in Sprague Dawley rats [5,29].

Blood and tissue collection

At the end of the 10 weeks of treatments, the rats were sacrificed by an ether-heparinized syringe via an intraperitoneal injection. Blood from the abdominal aorta was drawn into a heparinized tube. Visceral fat, the gastrocnemius muscle, and the liver were immediately removed and weighted. Subsequently, all tissues were frozen in liquid nitrogen and stored at -80℃ for later analysis.

Plasma measurements

Plasma glucose concentrations were analyzed using an enzymatic kinetic assay (Roche, Germany). Insulin (Millipore, Corp., Billerica, USA) and irisin (Phoenix Pharmaceuticals, Inc., Burlingame, USA) in the blood were assessed using enzyme-linked immunosorbent assays (ELISA). The assay kit was highly sensitive to irisin in animals. Insulin sensitivity was determined using the homeostatic model assessment insulin resistance (HOMA-IR) index. HOMA-IR was calculated as fasting insulin (pmol) × fasting glucose (mmol/L)/ 22.5.

Immunoblotting

The levels of FNDC-5 were determined using the cytosolic protein fraction via a western immunoblot analysis. Gastrocnemius muscles were homogenized in an ice-cold buffer containing the following: 50 mM HEPES, 10mM EDTA, 100 mM NaF, 50 mM Na pyrophosphate, and 10 mM Na orthovanadate supple-

mented with phosphatase and protease inhibitor cocktails (Sigma-Aldrich, Co., St. Louis, USA).

Muscles were homogenized 3 times and centrifuged for 15 min at 14,000 rpm (4℃). The protein concentration in the homogenates was determined using a bicinchoninic acid assay kit (Pierce Biotechnology, Co., Rockford, USA). Proteins were subjected to SDS/PAGE, transferred onto polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA, USA), blocked in 5% fat-free milk for 1 h at room temperature, and incubated with primary antibodies in 5% milk overnight at 4℃. The FNDC-5 antibody was from Abcam, and the GAPDH antibody was from the Cell Signaling Company. Following 3 washes in TBS-T, membranes were incubated at room temperature for 60 min in blocking buffer with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, USA). Following 3 washes in TBS-T, an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, USA) was used for visualization. Densitometry was performed using an Eastman Kodak, Co. (Rochester, USA) film cartridge and film, a scanner interfaced with a microcomputer, and the Image Analysis 1.62 software program (National Institutes of Health, Bethesda, USA).

Statistical analysis

All data are presented as mean ± standard errors of the mean (SEM). Statistical analyses were performed using SPSS version 19.0 (IBM SPSS, Inc., Chicago, USA). The significance of differences among groups was assessed using one-way analysis of variance with an appropriate post hoc test (*Tukey*). Values of $P < 0.05$ were considered significant.

RESULTS

AGE and exercise interventions mediate weight loss in obese rats.

Thirty-three Sprague Dawley rats were fed ND (n = 5), or HFD (n = 28) for 6 weeks. This design is consistent with other studies that used the model of rats with HFD-induced obesity, which have previously shown that HFD promotes increases in body weight, food intake, and adipose tissue after 6 weeks [5]. During the AGE and exercise interventions, body weight decreased significantly in the ND, HFDE, and HFDEA groups, as compared with the HFD only group. In particular, reductions in the HFDEA group were significantly greater than other groups (Fig. 2A). These data on body weight distribution also revealed a strong inhibition of weight gain in the groups that received 4 weeks of exercise. Visceral fat increased significantly more in the HFD group than in the other groups (Fig. 2B). We also found that exercise training played a role in controlling body fat. Despite dramatic reduction in body weight and visceral fat, neither intervention affected the mass of the gastrocnemius muscle (Fig. 2C) or liver (Fig. 2D).

AGE and exercise interventions improve insulin sensitivity in obese rats.

To test the hypothesis that AGE supplementation and exercise training induce insulin sensitivity in obese rats, we measured glucose, insulin, and HOMA-IR after overnight fasting. HFD-fed rats are known to cause obesity in rats, and increase the levels

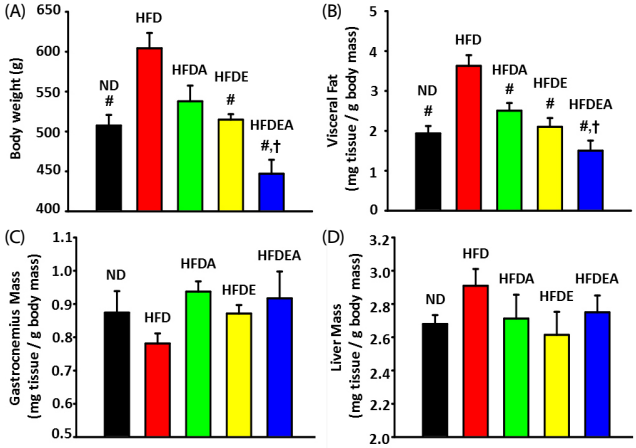

Fig. 2. Effects of AGE supplementation and exercise intervention on body weight (A), visceral fat (B), gastrocnemius mass (C), and liver mass (D) in high fat diet rats. After 4 weeks of AGE supplementation and exercise intervention, body weight, visceral fat, gastrocnemius muscle and liver mass were measured following fasting overnight. All values are expressed as mean ± SE. $^{\#}P<0.05$ vs. HFD, $^{\dagger}P<0.05$ vs. HFDA, ND: normal diet (n = 5), HFD: high-fat diet (n = 7), HFDE: high-fat diet + exercise (n = 7), HFDA: high-fat diet + aged garlic extract (n = 7), HFDEA: high-fat diet + exercise + aged garlic extract (n = 7).

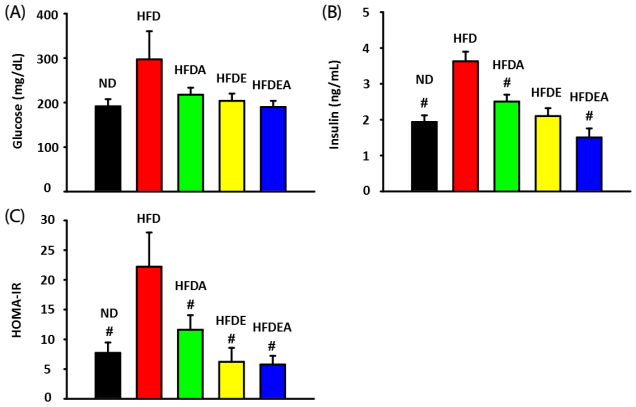

Fig. 3. Effects of AGE supplementation and exercise intervention on glucose (A), insulin (B), and HOMA-IR (C) in high fat diet rats. After 4 weeks of AGE supplementation and exercise intervention, glucose, insulin and HOMA-IR were measured, following fasting overnight. All values are expressed as mean ± SE. $^{\#}P<0.05$ vs. HFD, ND: normal diet (n = 5), HFD: high-fat diet (n = 7), HFDE: high-fat diet + exercise (n = 7), HFDA: high-fat diet + aged garlic extract (n = 7), HFDEA: high-fat diet + exercise + aged garlic extract (n = 7).

of glucose and insulin in their blood [30]. In the present study, glucose levels did not significantly differ across the groups (Fig. 3A). Although we observed no significant differences in glucose, we found that insulin (Fig. 3B) and HOMA-IR (Fig. 3C) were significantly higher in the HFD group, as compared with the ND, HFDA, HFDE, and HFDEA groups.

Neither AGE nor exercise improves skeletal muscle FNDC-5 protein and plasma irisin levels.

Recent studies have shown that FNDC-5 in skeletal muscle and serum irisin are markedly elevated in obesity and type 2 diabetes with exercise training [31]. To investigate whether the effects of the AGE and exercise interventions were mediated through a reduced body weight in rats with HFD-induced obesity, we examined the effects of the AGE and exercise interventions

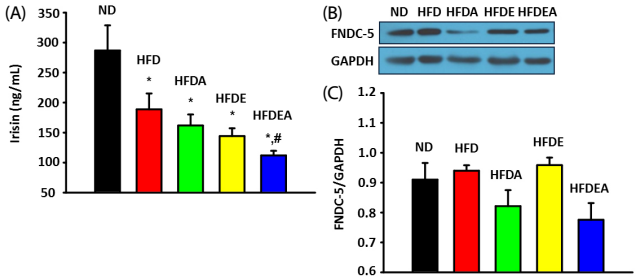

Fig. 4. Effects of AGE supplementation and exercise intervention on irisin (A) and FNDC-5 (B,C) in high fat diet rats. After 4 weeks of AGE supplementation and exercise intervention, plasma irisin was measured following fasting overnight and skeletal muscle FNDC-5 was used by westernblot on the gascronemius. All values are expressed as mean ± SE. * P< 0.05 vs. ND, # P< 0.05 vs. HFD, ND: normal diet (n = 5), HFD: high-fat diet (n = 7), HFDE: high-fat diet + exercise (n = 7), HFDA: high-fat diet + aged garlic extract (n = 7), HFDEA: high-fat diet + exercise + aged garlic extract (n = 7).

on muscle FNDC-5 and serum irisin concentration. As presented in Fig. 4A, 4B, and 4C, there was no improvement in skeletal muscle FNDC-5 levels or plasma irisin concentration. Although the HFD only group had a significantly lower blood irisin concentration than the ND group (as expected), the irisin concentrations were not attenuated in the HFDA, HFDE, or HFDEA groups. Furthermore, protein levels of FNDC-5 in skeletal muscle did not differ in the HFD, HFDA, HFDE, or HFDEA groups.

DISCUSSION

This is the first study to determine whether the plasma irisin level was involved in the anti-obesity effect mediated by AGE supplementation or exercise in HFD-induced obese rats. Three major findings were evident: First, AGE supplementation and exercise training provided protection against HFD-induced increases in body weight and visceral fat in rats. Second, AGE supplementation and exercise training ameliorated the HFD-induced insulin resistance in obese rats. Third, skeletal muscle FNDC-5 protein levels and circulating irisin levels were not affected, although AGE supplementation and exercise significantly improved the obesity status of HFD rats. These results provide the first indication that AGE and exercise interventions have a protective effects against HFD-induced insulin resistance that are independent of skeletal muscle FNDC-5 and circulating plasma irisin in HFD-induced obese rats.

Obesity has been strongly associated with HFD and physical inactivity, which increase tissue resistance to insulin [32]. The majority of people who are obese develop impaired glucose tolerance and insulin resistance. Therefore, improvements to impaired glucose tolerance and insulin resistance can be achieved by decreasing body fat mass [33]. Additionally, recent studies have suggested that exercise training and/or interventions involving phytochemical compounds can improve impaired glucose tolerance and insulin resistance, and thereby inhibit the accumulation of fat in adipose tissue in obese rats [34,35]. In our previous study of obese rats, HFD increased body weight and the weights of various organs and tissues, but AGE supplementation and exercise training reduced body weight [5]. In accordance with previous studies, we also have recently observed that AGE supplementation and exercise training can inhibit the development of obesity and improve insulin levels, as observed in the HFDEA group. Our results do not suggest any specific mechanism by which the inhibition of weight gain may be explained. Based on our results, however, one could postulate that the modulatory effects of AGE supplementation and exercise training appear to improve insulin sensitivity, which is an attainable goal for anti-obesity interventions.

Recently, it has been established that irisin plays a significant role in energy metabolism and glucose tolerance and, further, that irisin can change the browning of adipose tissue in exercise subjects [19,36,37]. In the original study on this topic, Bostom *et al.* [20] and Swick *et al.* [38] reported that exercise training induces the expression of FNDC-5 gene in the skeletal muscle of mice. Moreover, Kraemer *et al.* [39] have observed that prolonged aerobic exercise results in increased irisin concentrations in young men and women. Previous studies also determined that decreased body weight and improved insulin sensitivity were strongly associated with increased skeletal muscle FNDC-5 and serum irisin [26,27,31].

Given the improvements in body weight and insulin sensitivity that have been observed following AGE supplementation and exercise interventions, we further investigated whether these interventions may increase skeletal muscle FNDC-5 and serum irisin. However, we could not observed any training-induced increases in skeletal muscle FNDC-5 or plasma irisin levels, despite decreases in body weight, decreases in tissue weights, and improved insulin sensitivity in rats with HFD-induced obesity. In accordance with previous studies, Huh *et al.* [40] observed that obese individuals with surgical weight loss have decreased the irisin levels. In addition, Timmons *et al.* [41] showed that skeletal muscle FNDC-5 mRNA expression is not associated with insulin sensitivity in endurance-trained subjects. Therefore, our findings may imply that blood irisin and skeletal muscle FNDC-5 levels are not the only determinants of induced obesity; another underlying mechanism of HFD-induced obesity must be present, which blood irisin and skeletal muscle FNDC-5 levels both have roles.

Although we observed improvements in body weight and insulin resistance as a result of AGE administration and exercise, there were no changes in plasma irisin or skeletal muscle FNDC-5. However, further studies are necessary to investigate the roles of plasma irisin and skeletal muscle FNDC-5 during phytochemical supplementations and exercise interventions for other physiological and pathological conditions.

In conclusion, the results of this study have demonstrated that AGE supplementation and exercise training provided protection against HFD-induced increased body fat/weight and insulin resistance in obese rats. The results have also indicated that these anti-obesity effects may not be mediated by improved plasma irisin or skeletal muscle FNDC-5, which are associated with insulin sensitivity in obese rats. However, the underlying molecular mechanism by which and exercise treatments have anti-obesity effects for HFD-induced obesity should be investigated more extensively in further studies.

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