Research Article

Gintonin-enriched fraction protects against sarcopenic obesity by promoting energy expenditure and attenuating skeletal muscle atrophy in high-fat diet-fed mice

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ARTICLE INFO

ABSTRACT

Background: Gintonin-enriched fraction (GEF), a non-saponin fraction of ginseng, is a novel glyco-lipoprotein rich in hydrophobic amino acids. GEF has recently been shown to regulate lipid metabolism and browning in adipocytes; however, the mechanisms underlying its effects on energy metabolism and whether it affects sarcopenic obesity are unclear. We aimed to evaluate the effects of GEF on skeletal muscle atrophy in high-fat diet (HFD)-induced obese mice.
Methods: To examine the effect of GEF on sarcopenic obesity, 4-week-old male ICR mice were used. The mice were divided into four groups: chow diet (CD), HFD, HFD supplemented with 50 mg/kg/day GEF, or 150 mg/kg/day GEF for 6 weeks. We analyzed body mass gain and grip strength, histological staining, western blot analysis, and immunofluorescence to quantify changes in sarcopenic obesity-related factors.
Results: GEF inhibited body mass gain while HFD-fed mice gained 22.7 ± 2.0 g, whereas GEF-treated mice gained 14.3 ± 1.2 g for GEF50 and 11.8 ± 1.6 g for GEF150 by downregulating adipogenesis and inducing lipolysis and browning in white adipose tissue (WAT). GEF also enhanced mitochondrial biogenesis threefold in skeletal muscle. Furthermore, GEF-treated skeletal muscle exhibited decreased expression of muscle-specific atrophic genes, and promoted myogenic differentiation and increased muscle mass and strength in a dose-dependent manner ($p < 0.05$).
Conclusion: These findings indicate that GEF may have potential uses in preventing sarcopenic obesity by promoting energy expenditure and attenuating skeletal muscle atrophy.
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1. Introduction

Obesity is a worldwide health problem and is caused by an imbalance between energy intake and energy expenditure [1]. In mammals, there are two different types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT) [2]. WAT is a bioactive endocrine tissue specialized in energy storage, in which triglyceride (TG) accumulates in large unilocular lipid droplets [3]. By contrast, BAT consists of small multilocular lipid droplets and has high levels of mitochondrial biogenesis; dissipates energy and generate heat by thermogenesis [4]. WAT can also turn into BAT-like energy dissipating tissue, when fatty acids produced by TG hydrolysis are oxidized to generate heat [5]. This type of adipocyte has been named a brown-like or beige adipocyte. Given its potential for energy consumption, adipose tissue has become a key anti-obesity drug target [6].

Adipocyte differentiation is related to the onset of obesity [7]. Adipogenesis includes changes in adipocyte morphology, where preadipocytes mature into unilocular adipocytes, as well as changes in gene expression [8]. Adipogenic differentiation is regulated by transcription factors including CCAAT/enhancer-binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor gamma (PPARγ), and fatty acid-binding protein 4 (FABP4); their expression is associated with adipocyte maturation, and, therefore, lipid deposition [9].

According to recent research, one strategy to prevent obesity may include suppressing lipid accumulation and activating energy metabolism through WAT browning [10]. Cold exposure and stimulation of β-adrenergic receptors can increase cyclic AMP and

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activate protein kinase A (PKA)-initiated lipolysis, which in turn induces transcription of lipolytic enzymes such as adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL) [11]. Lipolysis results in the release of free fatty acids (FFAs), which can then be used as fuel for browning [12]. Adipocyte browning requires the phosphorylation of AMP-activated protein kinase (AMPK) and expression of thermogenic proteins, including peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), and PR domain-containing 16 (PRDM16), and ultimately induces the expression of uncoupling protein 1 (UCP1) [13]. UCP1 uncouples the mitochondrial respiratory chain and causes the generation of heat instead of ATP, allowing WAT to consume energy like BAT [14]. Due to the energy expenditure involved in this process, enhancement of BAT-like adipocyte activity may be a potential target for the treatment of obesity [15].

Obesity results in both metabolic impairment and physical dysfunction [16]. Related to this, recent studies have provided new insights that obesity as well as combined low muscle mass and low strength, defined as sarcopenic obesity [17,18]. In addition, the skeletal muscle of obese mice shows reduced mitochondrial biogenesis and oxidative metabolism, which may result in dysfunctional muscle energy homeostasis [19,20]. Mitochondria play an essential role in various biological processes associated with muscle function and energy production [21]. In skeletal muscle, PGC1α is involved in mitochondrial biogenesis and the regulation of energy metabolism: PGC1α activates nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM), which in turn trigger the activation of uncoupling protein 3 (UCP3) [22,23]. UCP3 is a recently discovered uncoupling protein, which is expressed preferentially in the inner mitochondrial membrane of skeletal muscle and increases fatty acid transport into the mitochondria and subsequent mitochondrial oxidation [24]. Therefore, UCP3 in skeletal muscle enhances mitochondrial biogenesis and whole-body energy expenditure [25].

Sarcopenic obesity is one of the most important comorbidities of obesity, and is characterized by low skeletal muscle mass and strength with high fat mass [26]. Moreover, recent studies have shown that HFD-induced obese mice have typical features of skeletal muscle atrophy, such as the loss of muscle mass, decreased muscle fiber size, and impaired exercise capacity [27,28]. Indeed, muscle of obese mice (sarcopenic obesity) was very similar to skeletal muscle from aged mice (sarcopenia) [29,30]. Skeletal muscle atrophy in the obese state occurs when muscle protein degradation exceeds protein synthesis [31]. The ubiquitin proteasome pathway (UPP) is recognized as a primary regulator of this degradation process and is controlled by two muscle-specific ubiquitin E3-ligase F-box proteins, muscle atrophy F-box (MAFbx)/atrogin-1 and muscle ring-finger protein 1 (MuRF1) [32].

Overactivation of the UPP in skeletal muscle atrophy increases the ubiquitination of myogenic regulatory factors (MRFs) [33]. Therefore, to maintain skeletal muscle structure and function, MRFs are required to regulate skeletal muscle differentiation, growth, and regeneration [34]. Myogenesis involves differentiation of skeletal muscle, which induces myoblast fusion and myotube formation [35]. This regeneration process is initiated by myoblast determination protein 1 (MyoD), and then myogenic factor 4 (myogenin) activates muscle-specific proteins, including myocyte enhancer factor-2 (MEF-2) and myosin heavy chain (MYH) [36].

Korean ginseng (*Panax ginseng* Meyer) is an edible medicinal herb, which is commonly used in Korea and other Asian countries for recovering physical balance and stimulating metabolic functions [37]. Korean ginseng is recognized to have various therapeutic effects mediated by highly bioactive components such as saponins,

referred to as ginsenosides, and non-saponin components [38]. Saponins have traditionally been regarded as the bioactive agents of ginseng for scientific investigations, but a newly-discovered active fraction from ginseng is a non-saponin, novel glycolipoprotein called gintonin-enriched fraction (GEF) [39]. Gintonin contains two major components, ginseng major latex-like protein 151 and ginseng ribonuclease-like storage protein. These protein complexes bind to lysophosphatidic acid (LPA) and activate its receptors with high affinity [40]. A recent study showed that GEF regulates lipid metabolism and browning in adipocytes [41]. However, the mechanisms underlying its effects on energy metabolism in WAT and skeletal muscle, and whether it affects sarcopenic obesity, have not been established. Therefore, we aimed to evaluate the effects of GEF on skeletal muscle atrophy in HFD-induced obese mice.

2. Materials and methods

2.1. Preparation of GEF

GEF was prepared from 4-year-old Korean white ginseng (Korea Ginseng Cooperation, Daejon, Korea) [42]. It was ground into small pieces, refluxed with 70 % edible, concentrated, and dissolved in distilled cold water. After centrifugation, the precipitate was lyophilized and this fraction was designated as GEF. Its total protein content was approximately 30.3 %, carbohydrate content was 30 %, and lipid content was 20.2 %.

2.2. Experimental animals and treatments

Animal experiments were approved by the Institutional Animal Care and Use Committee of CHA University (Approval Number 200156). Male ICR mice (4 weeks of age) were purchased from Orient Bio (Seongnam, Korea) and housed in a temperature- and humidity-regulated facility on a 12 h light/dark cycle. After 1 week of adaptation, mice were randomly randomized into four groups ($n = 9$ per group): a chow diet (CD) containing 10 kcal% as fat; a HFD containing 60 kcal% as fat, or a HFD supplemented with GEF at a dose of 50 mg/kg/day or 150 mg/kg/day for 6 weeks (HFD + GEF50 or HFD + GEF150, respectively). During the experimental period, body mass, dietary intake, rectal temperature, and grip strength of the mice were measured weekly. At the end of the experiment, mice were euthanized using CO_2 after fasting for 12 h and tissue samples were collected. The WAT depots and skeletal muscle were immediately weighed.

2.3. Body mass and dietary intake measurements

During the experimental period, mice were weighed weekly at 10:00 a.m. using an analytical balance. Food and water intake were calculated each week as the difference between the quantities supplied and the amount remaining at the end of the week.

2.4. Rectal temperature measurement

The rectal temperature of the mice was measured weekly at 10:00 a.m. using a Testo 925 Type Thermometer (Testo, Lenzkirch, Germany).

2.5. Grip strength measurement

The grip strength of the mice was measured weekly at 10:00 a.m. using a Chatillon Force Measurement System (Columbus Instrument, Columbus, OH, USA).

2.6. Histological analysis

Visceral WAT, *quadriceps* (QUA), and *gastrocnemius* (GAS) muscle samples were fixed in 4 % paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E), mounted on glass slides. Adipocytes and muscle fiber histology was observed using a Nikon E600 microscope (Nikon, Tokyo, Japan). The cross-sectional area (CSA) of the muscle fiber was determined with ImageJ software (Bethesda, MD, USA).

2.7. Immunofluorescence

Tissue samples were deparaffinized and incubated with anti-PKA, anti-UCP1, anti-PGC1α, or anti-UCP3 antibodies. Secondary anti-mouse fluorescein isothiocyanate (FITC)-conjugated and anti-rabbit Alexa Fluor™ 594-conjugated antibodies were then applied. 40,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Waltham, MA, USA) was used to stain the cell nuclei. Fluorescent images were captured using a Zeiss confocal laser scanning microscope (LSM880; Carl Zeiss, Oberkochen, Germany) and Zen 2012 software (Carl Zeiss).

2.8. Western blot analysis

Tissue samples were lysed in lysis buffer (iNtRON Biotechnology, Seoul, Korea), and lysate protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by SDS-PAGE gel electrophoresis and transferred to membranes. Membranes were blocked with 5 % skimmed milk and incubated with the indicated primary antibodies overnight at 4 °C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Antibodies against C/EBPα, PPARγ, FABP4, p-PKA (Ser 114), PGC1α, MyoD, myogenin, and MEF-2 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA); antibodies against ATGL, p-HSL (Ser 563), and p-AMPK (Thr172) were purchased from Cell Signaling Technology (Danvers, MA, USA); antibodies against MGL, PPARα, PRDM16, UCP1, NRF1, TFAM, UCP3, Atrogin-1, MuRF1, and MYH7 were purchased from Abcam (Cambridge, UK); and an antibody against β-actin was purchased from ABM (Richmond, BC, Canada).

2.9. Statistical analysis

Data are expressed as mean ± standard deviation (SD) and were analyzed using one-way ANOVAs, followed by Tukey's post-hoc tests (IBM SPSS Statistics Version 20.0, Armonk, NY, USA). Statistical significance was accepted when $p < 0.05$.

3. Results

3.1. GEF inhibits body mass gain in HFD-fed mice

To determine the anti-obesity effects of GEF *in vivo*, we fed mice a CD, HFD, or a HFD supplemented with 50 mg/kg/day GEF (HFD + GEF50) or 150 mg/kg/day GEF (HFD + GEF150) for 6 weeks. During 6 weeks of GEF treatment, mice were protected from body mass gain (Fig. 1A and B). The body mass of HFD-fed mice was significantly higher than that of the mice fed the CD, while the body masses of GEF-treated mice were significantly and dose-dependently reduced compared with HFD-fed mice. The HFD-fed mice gained 22.7 ± 2.0 g over 6 weeks, whereas the GEF-treated mice gained 14.3 ± 1.2 g on the HFD + GEF50 and 11.8 ± 1.6 g on the HFD + GEF150 (Fig. 1B). This result appeared be a consequence of reduced WAT accumulation in mice fed GEF (Fig. 1C); indeed, the

masses of subcutaneous and visceral WAT depots were significantly lower in GEF-treated mice than HFD-fed mice (Fig. 1D). There were no significant differences in the masses of other organs (Fig. 1E) or in food and water intake among the diet groups (Fig. 1F).

3.2. GEF inhibits lipid accumulation by reducing adipocyte size in WAT

Reduced WAT mass may be a result of smaller adipocyte number or size; therefore, to evaluate the effect of GEF on lipid accumulation, we measured WAT adipocyte size. Representative images of mice suggested that GEF supplementation protected against visceral obesity (Fig. 2A). H&E staining demonstrated that WAT adipocyte size in HFD-fed mice was much larger than in the CD group (Fig. 2B). However, GEF-treated mice had smaller adipocytes than mice fed a HFD without GEF in a dose-dependent manner, such that the size of adipocytes from HFD + GEF150-fed mice were similar in size to those in the CD group. We also performed western blot analysis to determine the molecular mechanisms underlying the inhibitory effect of GEF on increased adipocyte size and lipid accumulation. The expression of key regulators of adipogenesis, C/EBPα, PPARγ, and FABP4, was significantly higher in mice fed a HFD than mice fed a CD (Fig. 2C). However, GEF-treated mice had significantly lower expression of these adipogenic-specific proteins than the HFD-fed mice, implying that adipogenesis was reduced by GEF administration. Taken together, these results suggest that GEF inhibits lipid accumulation by reducing the expression of key adipogenic proteins in WAT.

3.3. GEF increases energy expenditure as heat

Energy metabolism is related with the activation of mitochondrial energy production in WAT. A key fuel in this process are FFAs produced by lipolysis. Hydrolysis of TGs is initiated by p-PKA and lipolytic enzymes including ATGL, p-HSL, and MGL, which release FFAs that can then be oxidized within mitochondria [12]. Therefore, we performed western blot analysis to determine whether GEF stimulates lipolysis. Both groups of GEF-treated mice had significantly higher PKA phosphorylation than GEF-untreated mice in the HFD group (Fig. 3A). GEF treatment also resulted in significantly higher ATGL, p-HSL, and MGL expression in HFD-fed mice (Fig. 3A). AMPK is a key regulator of energy metabolism and thermogenesis, and modulates the expression of several proteins involved in WAT browning, such as PPARα, PGC1α, PRDM16, and UCP1 [13]. Therefore, to investigate whether GEF could induce thermogenic gene expression and promote browning, we performed western blot analysis. We found that GEF-treated mice had significantly higher levels of these proteins than HFD-fed mice, which may contribute to elevated energy expenditure through WAT browning (Fig. 3B). We then measured the rectal temperatures of the mice to determine the effect of GEF on heat generation. Rectal temperatures of GEF-treated mice were significantly higher than the other groups (Fig. 3C). In accordance with the above findings, PKA and UCP1 immunoreactivity were higher in GEF-treated mice than in HFD-fed mice (Fig. 3D). Together, these results suggest that GEF promotes lipolysis and adipocyte browning, resulting in a greater induction of energy expenditure as heat.

3.4. GEF enhances mitochondrial biogenesis in skeletal muscle

Reduced energy expenditure and mitochondrial oxidative capacity in the skeletal muscle is associated with the mitochondrial dysfunction observed in obesity [20]. Various signal transduction systems in skeletal muscle could affect the activation of PGC1α, NRF1, and TFAM. These transcription factors enhance
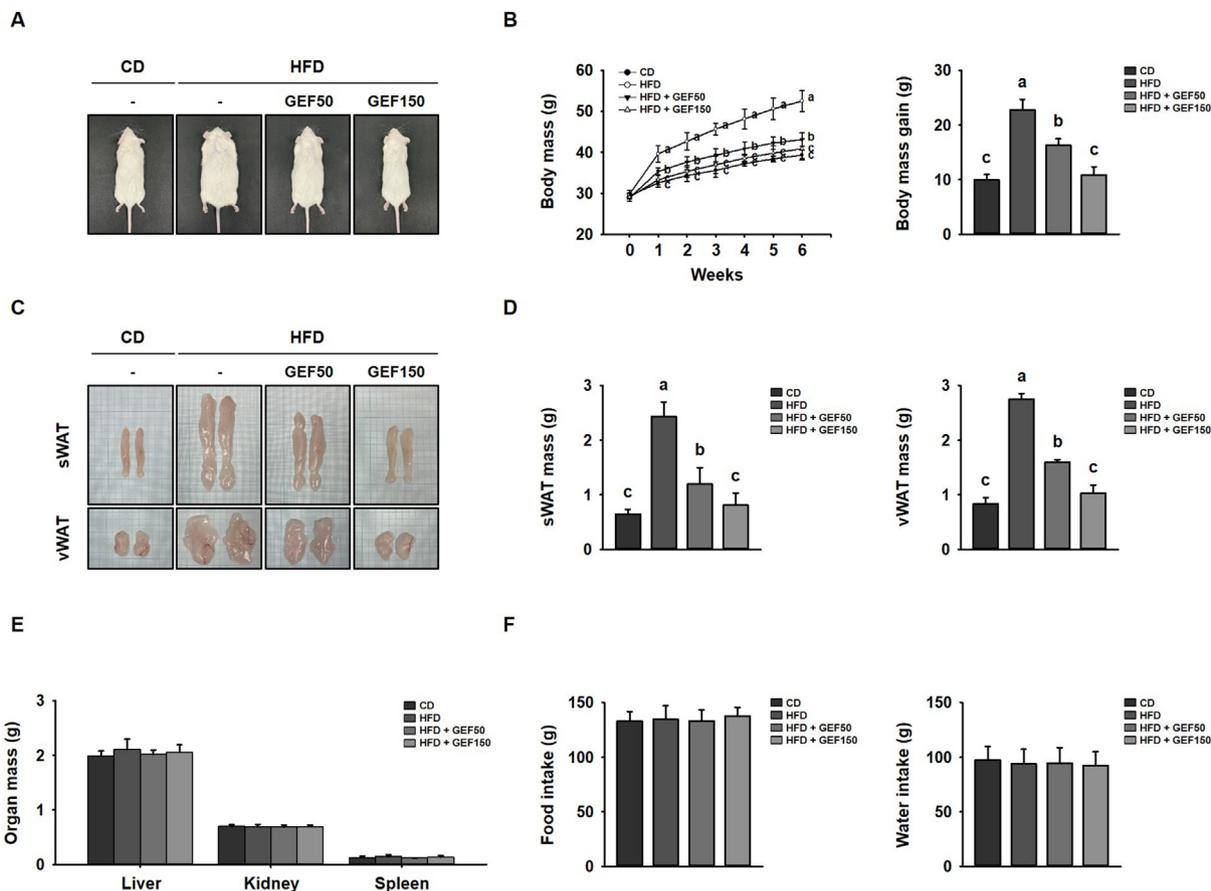
Fig. 1. Effects of GEF on obesity in HFD-fed mice. (A) Representative images of ICR mice. (B) Body mass during the 6 weeks of GEF treatment. (C) Representative images of WAT depots. (D) WAT mass after 6 weeks of GEF treatment. (E) Masses of other organs. (F) Food and water intake during the 6 weeks of GEF treatment. Data were expressed as means ± SDs (n = 8), analyzed using one-way ANOVAs and Tukey's post-hoc tests. Values with different letters are significantly different: $p < 0.05$ (a > b > c).
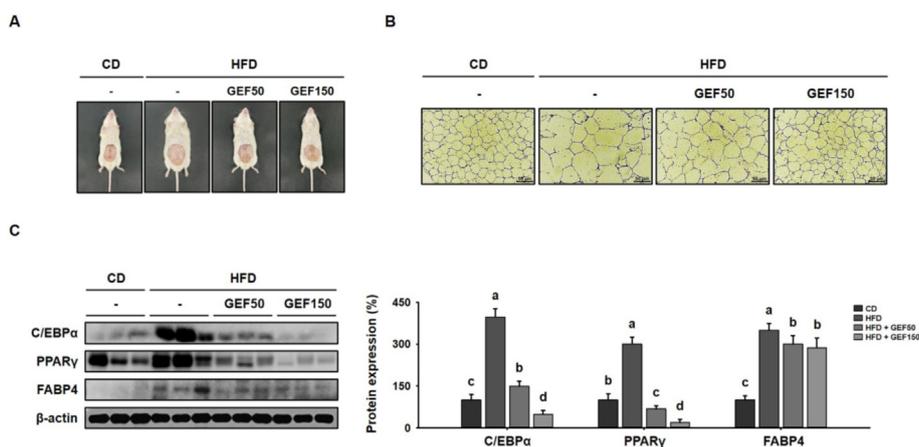

Fig. 2. Effects of GEF on lipid accumulation. (A) Representative images of ICR mice. (B) H&E staining of WAT. Scale bar: 50 μm. (C) Western blots of adipogenic proteins (C/EBPα, PPARγ, and FABP4) in WAT. Values with different letters are significantly different: $p < 0.05$ (a > b > c > d).

mitochondrial biogenesis and induce the inner mitochondrial membrane transporter UCP3, which uncouples oxidation and results in energy dissipation [23]. Therefore, we further identified the effect of GEF on mitochondrial biogenesis and mitochondrial energy production in the skeletal muscle of mice. In the HFD group, the expression of PGC1α, TFAM, and UCP3 was significantly lower than the CD group. However, GEF treatment resulted in

significantly higher expression of these proteins in both the QUA and GAS than that in HFD-fed mice without GEF supplementation (Fig. 4A and B). Consistent with this, GEF resulted in stronger immunofluorescence staining intensity of PGC1α and UCP3 in skeletal muscle than that in HFD-fed mice without GEF supplementation (Fig. 4C and D). These results suggest that GEF treatment enhanced mitochondrial oxidative capacity, partially due to
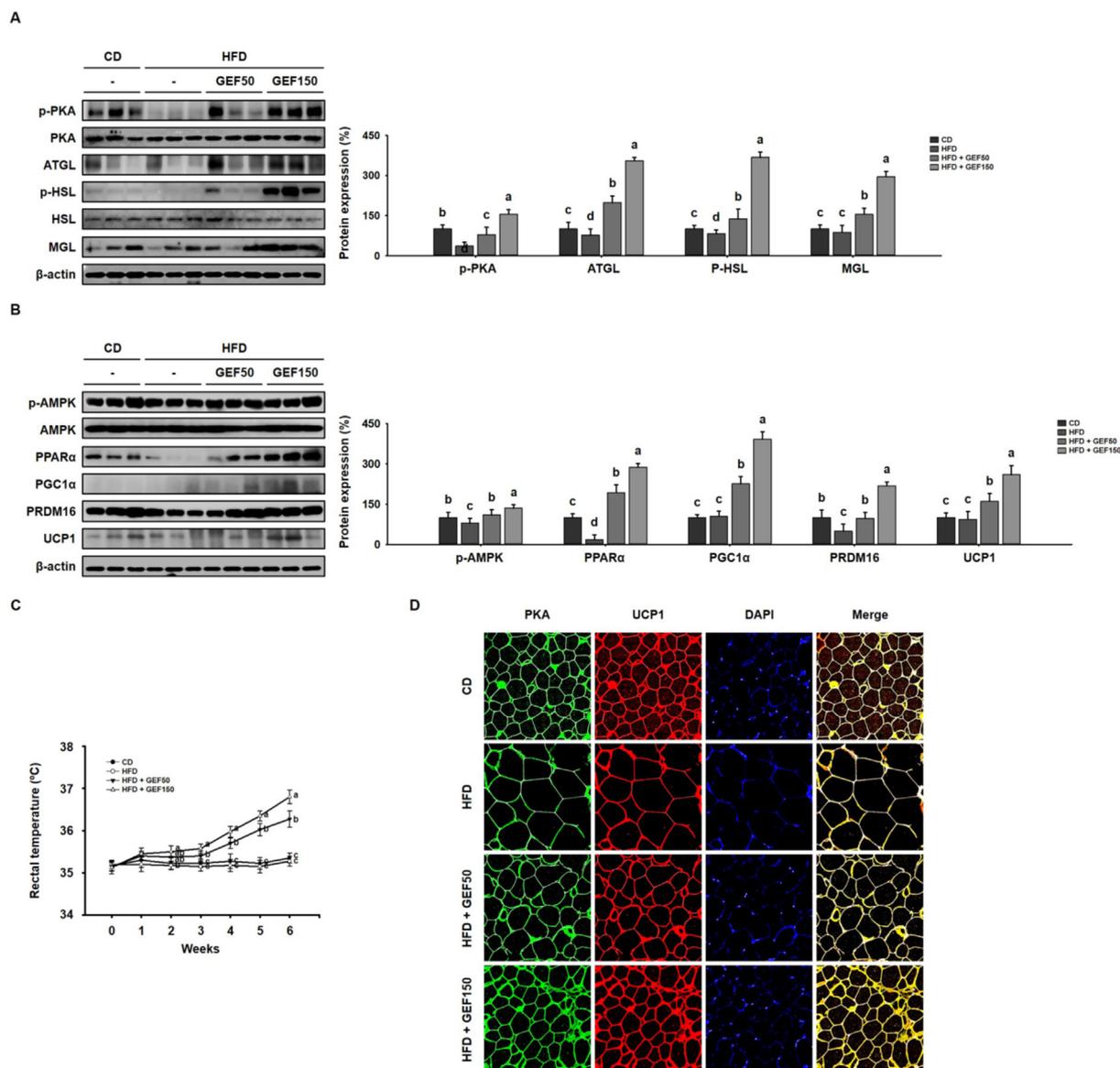
Fig. 3. Effects of GEF on lipolysis and browning. (A) Western blots of lipolytic enzymes (p-PKA, ATGL, p-HSL, and MGL) in WAT. (B) Western blots of proteins involved in browning (p-AMPK, PPARα, PGC1α, PRDM16, and UCP1) in WAT. (C) Rectal temperature during 6 weeks of GEF treatment. (D) Immunofluorescence images of WAT (100X magnification). Values with different letters are significantly different: $p < 0.05$ (a > b > c > d).

elevated UCP3 activity, leading to higher mitochondrial energy production in HFD-fed mice.

3.5. GEF inhibits muscle mass loss and skeletal muscle atrophy

Skeletal muscle atrophy results in loss of muscle mass and reduced exercise capacity [26]. Therefore, during experimental periods, we evaluated the effect of GEF on exercise performance using grip strength. Although HFD-fed mice had significantly lower grip strength than CD-fed mice, GEF-supplemented mice had significantly higher grip strength than CD-fed mice (Fig. 5A). In addition, GEF treatment appeared to increase skeletal muscle size (Fig. 5B). Although the HFD resulted in a significantly lower muscle mass and a lower ratio of skeletal muscle mass to whole-body mass than CD-fed mice, this reduction was prevented by GEF treatment (Fig. 5C). Ubiquitin-mediated protein degradation is demonstrated by high levels of atrophic factors, which increase under most skeletal muscle atrophy conditions [32]. HFD-fed mice had significantly higher protein levels of two muscle-specific ubiquitin

ligases, Atrogin-1 and MuRF1, than the CD-fed group. However, GEF supplementation resulted in significantly lower expression of Atrogin-1 and MuRF1 than that in HFD-fed mice without GEF supplementation (Fig. 5D and E). These results suggest that GEF prevents HFD-induced skeletal muscle atrophy in obese mice.

3.6. GEF increases muscle fiber size and myogenic transcription factors

Myogenesis, the process of skeletal muscle hypertrophy and regeneration, is necessary for the development of muscle fiber after muscle atrophy [34]. According to histological analysis of skeletal muscle cross sections, HFD feeding resulted in smaller muscle fiber size than that in the CD group (Fig. 6A). However, GEF-treated mice had a significantly and dose-dependently higher CSA of skeletal muscle than HFD-fed mice (Fig. 6A). Changes in skeletal muscle morphology are associated with myogenic regulatory mechanisms during differentiation [36]. Therefore, we investigated whether GEF treatment increased the expression of MRFs, including MyoD,
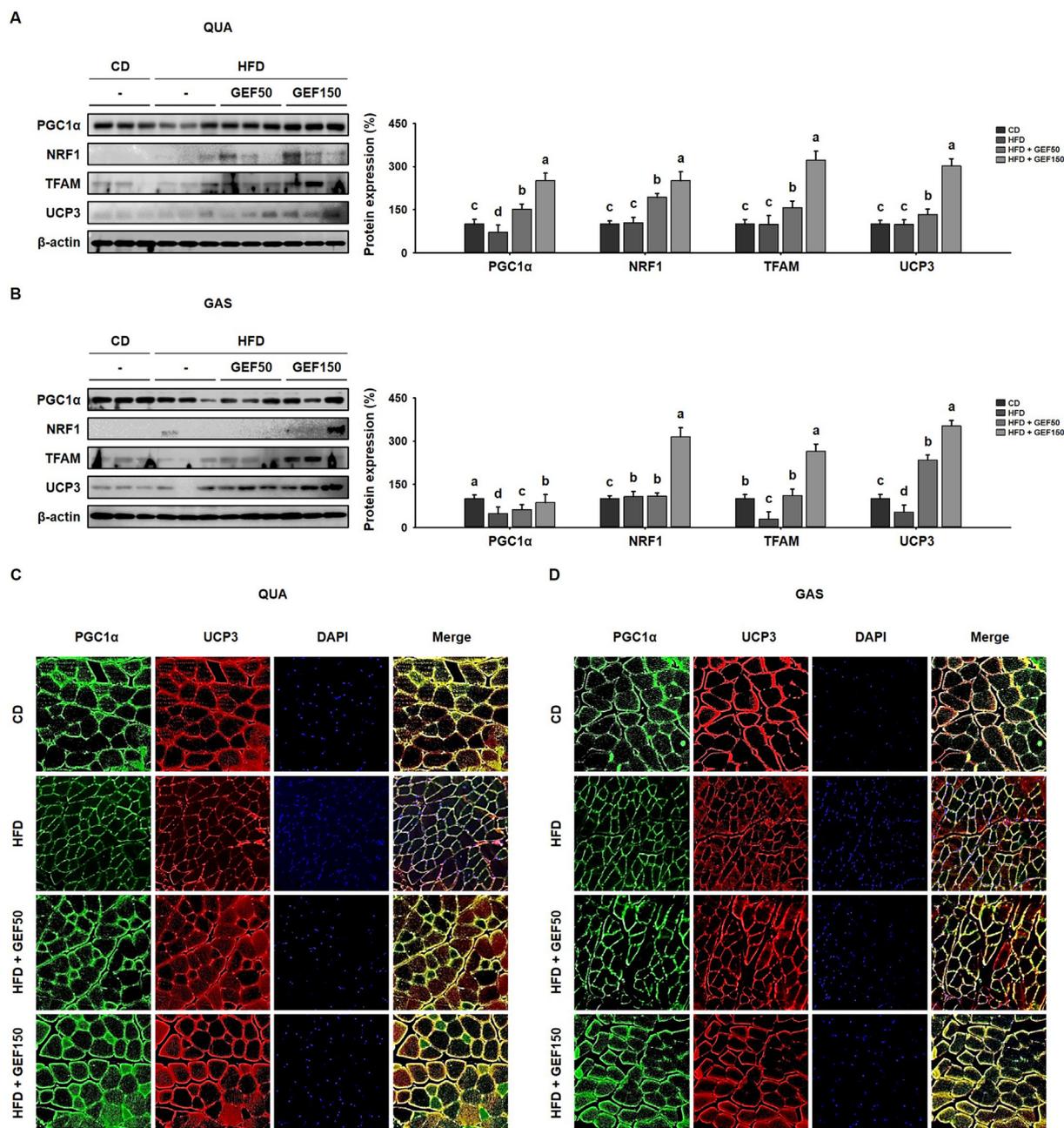
Fig. 4. Effects of GEF on mitochondrial biogenesis in skeletal muscle. (A) Western blots of proteins involved in mitochondrial biogenesis (PGC1α, NRF1, TFAM, and UCP3) in QUA. (B) Western blots of proteins involved in mitochondrial biogenesis (PGC1α, NRF1, TFAM, and UCP3) in GAS. (C) Immunofluorescence images of QUA (100X magnification). (D) Immunofluorescence images of GAS (100X magnification). Values with different letters are significantly different: $p < 0.05$ (a > b > c > d).

myogenin, MEF-2, and MYH7. The expression of these myogenic proteins was significantly higher in GEF-treated mice than that in HFD-fed mice without GEF treatment (Fig. 6B and C). This is consistent with the observed increase in CSA of skeletal muscle induced by GEF. These results indicate that GEF protects against obesity-induced skeletal muscle atrophy through regeneration of skeletal muscle by increasing the expression of MRFs.

4. Discussion

Sarcopenic obesity is a combined accumulation of body fat and abnormal skeletal muscle loss, which is associated with muscle dysfunction and further metabolic disorders [43]. Recent studies of

sarcopenic obesity have resulted in increased interest in dietary components as a strategy to increase mitochondrial energy expenditure in both WAT and skeletal muscle, and prevent obesity-induced skeletal muscle loss to treat both obesity and skeletal muscle atrophy [44]. In this study, we aimed to establish whether GEF prevented sarcopenic obesity by reducing lipid accumulation, stimulating energy expenditure, and attenuating skeletal muscle dysfunction in HFD-fed mice. GEF, a non-saponin fraction of ginseng, is a novel bioactive G protein-coupled LPA receptor ligand that is rich in hydrophobic amino acids. GEF-mediated LPA receptor activation is a key regulator of various GEF effects [40]. Recent studies have shown that GEF has anti-inflammatory and anti-metastatic actions [39,45]. It has also been reported that GEF
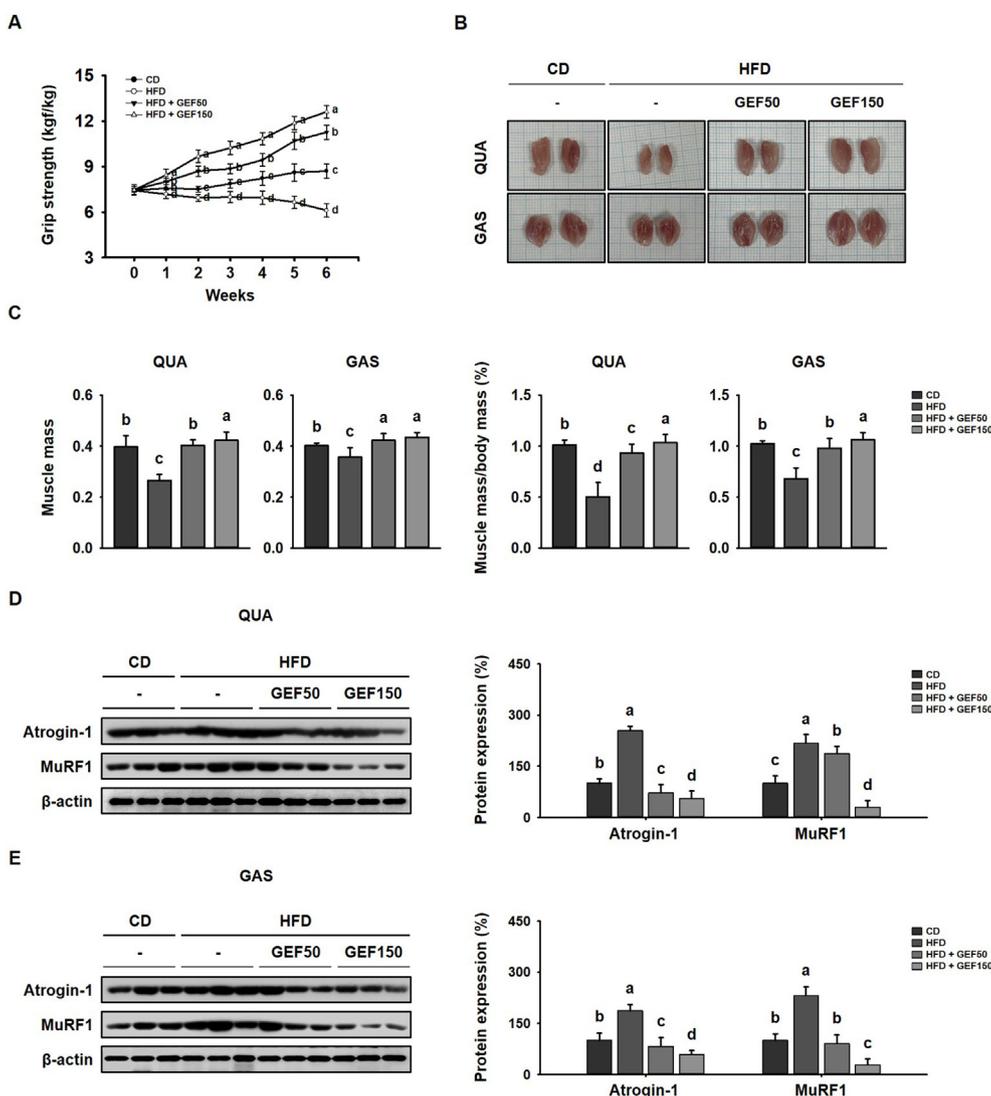
Fig. 5. Effects of GEF on skeletal muscle atrophy in HFD-fed mice. (A) Grip strength during 6 weeks of GEF treatment. (B) Representative images of QUA and GAS. (C) Skeletal muscle mass after 6 weeks of GEF treatment. (D) Western blots of atrophic proteins (Atrogin-1 and MuRF1) in QUA. (E) Western blots of atrophic proteins (Atrogin-1 and MuRF1) in GAS. Values with different letters are significantly different: $p < 0.05$ (a > b > c > d).

suppresses adipocyte differentiation and lipid accumulation *in vitro* [41]. However, it remains unclear whether GEF suppresses body mass gain and promotes energy expenditure *in vivo* or protects against sarcopenic obesity. Therefore, this study examined the effects of GEF on the metabolism of HFD-fed mice, with emphasis on mitochondrial energy expenditure and obesity-induced skeletal muscle atrophy.

We used a HFD-fed mouse model to examine the effects of GEF on the development of sarcopenic obesity. Feeding a HFD to mice without GEF for 6 weeks efficiently induced obesity, along with a significant increase in body mass, especially WAT mass. The changes in body mass were accompanied by changes in overall metabolic status, as indicated by adipocyte size in WAT [46]. As expected, GEF administration to HFD-fed mice dramatically inhibited body mass gain and growth of WAT adipocytes. Lipid accumulation in WAT plays a critical role in the development of obesity and is regulated by various adipogenic-specific proteins, including C/EBPα, PPARγ, and FABP4. Therefore, reducing adipocyte size in WAT through the regulation of these adipogenic proteins may be a possible strategy in preventing obesity. Indeed, a recent

study reported that GEF reduces lipid accumulation by reducing the expression of adipogenic proteins in 3T3-L1 cells [41]. Building on this *in vitro* work, in this study, we showed that expression of C/EBPα, PPARγ, and FABP4 was significantly lower in the WAT of GEF-treated mice than in that of HFD-fed mice, implying that GEF may reduce lipid synthesis by inhibiting adipogenesis in WAT.

The current study focused not only on inhibiting adipocyte differentiation, but also on stimulating lipolysis of accumulated fat by energy expenditure [47]. Energy expenditure can be stimulated by inducing lipolysis and promoting thermogenic capacity [48], thus attaining BAT-like characteristics in WAT, which is a potential strategy for combating obesity. Lipolysis involves the hydrolysis of TG, stored in WAT, into glycerol and FFAs, and is activated by the phosphorylation of PKA. As a downstream lipolytic target of p-PKA, ATGL stimulates p-HSL and MGL successively [49]. In our previous study, a PKA activator (forskolin) or an inhibitor (H89) were used to determine whether GEF increases lipolysis by activating PKA and we showed that forskolin upregulated p-PKA expression and GEF had an additive effect to that of forskolin *in vitro* [41]. Moreover, in this *in vivo* study, our data demonstrated that GEF induced lipolytic
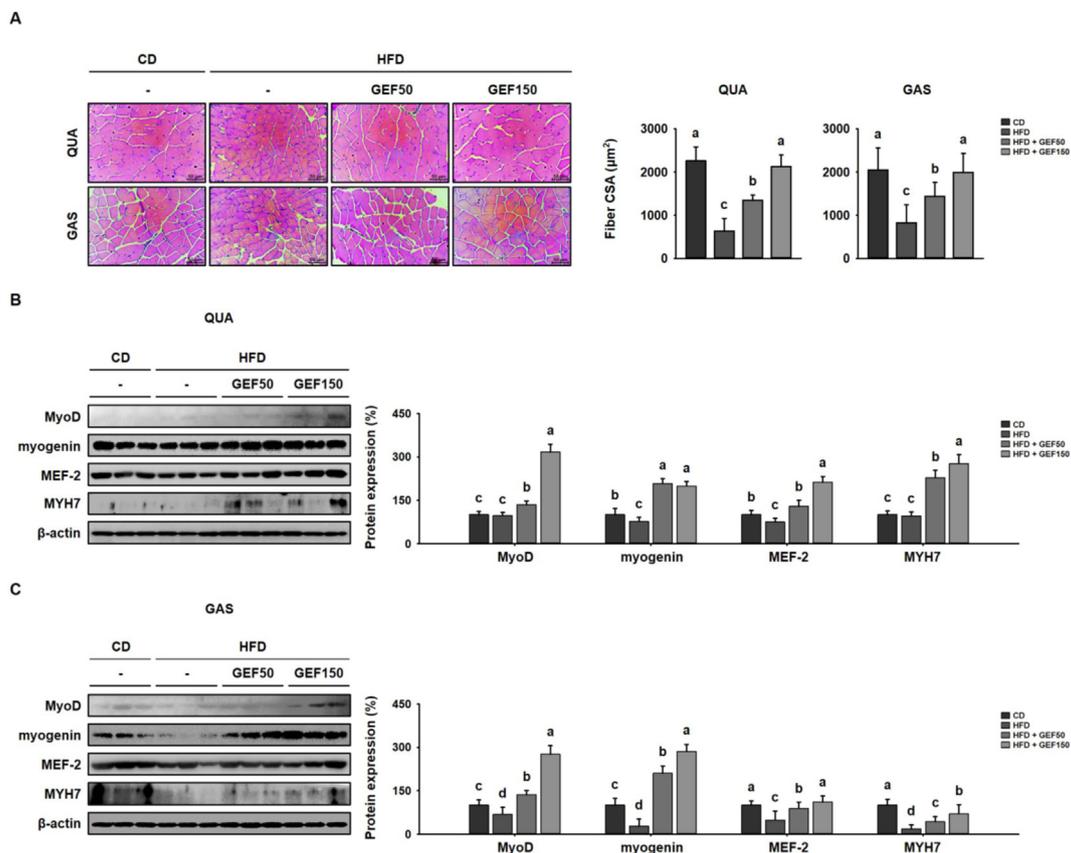
Fig. 6. Effects of GEF on skeletal muscle regeneration in HFD-fed mice. (A) H&E staining of skeletal muscle. Scale bar: 50 μm. Quantification of the mean CSA was measured from QUA and GAS. (B) Western blots of myogenic proteins (MyoD, myogenin, MEF-2, and MYH7) in QUA. (C) Western blots of myogenic proteins (MyoD, myogenin, MEF-2, and MYH7) in GAS. Values with different letters are significantly different: $p < 0.05$ (a > b > c > d).

enzyme expression in WAT of HFD-fed mice, a process required for WAT browning.

Adipocyte browning is a UCP1-mediated pathway that can increase energy expenditure by initiating thermogenesis in WAT. Several studies provide the concept that this process occurs through transdifferentiation of white adipocytes into adipocytes with a BAT-like phenotype [50]. Our data suggest that GEF might increase energy expenditure by initiating the browning of WAT. In GEF-treated mice, it appeared that FFAs released from lipolysis were transported into mitochondria, where the electrochemical gradient was dissipated as heat through UCP1-mediated uncoupling. In support of this theory, GEF appeared to stimulate the UCP1 pathway in the WAT of HFD-fed mice. AMPK is a key regulator in energy metabolism and induces the expression of many genes associated with WAT browning [13]. Our results demonstrated that GEF supplementation resulted in activation of AMPK and increased expression of PPARα, PGC1α, PRDM16, and UCP1, which is associated with the transformation from a WAT-to BAT-like phenotype. Moreover, during the experimental period, HFD-fed mice lost their thermogenic capacity, indicated by a reduced body temperature. However, GEF-treated mice had a much higher body temperature, which means generate more energy as heat. Following the above findings, we demonstrated that energy expenditure by browning requires the activation of PKA and ultimately induces UCP1. Indeed, we showed that PKA and UCP1 immunoreactivity were higher in GEF-treated mice than in HFD-fed mice. These results suggest that GEF could enhance energy metabolism through lipolysis and UCP1-mediated browning pathway.

As obesity has reached epidemic proportions globally, the adverse effects of obesity on many health-related diseases have

become a focus of recent research. The strong relationship between obesity and sarcopenia affecting skeletal muscle atrophy by the two major components of body composition: fat and muscle. The relative increase in body fatness with muscle loss may increase the risk of physical disability. Thus, worsening of muscle function associated with high fat has been considered the factor for sarcopenic obesity, such as mitochondrial dysfunction and skeletal muscle atrophy. Mitochondria are involved in skeletal muscle function, being the primary site of ATP production by oxidative phosphorylation [51]. Skeletal muscle of obese mice displays lower mitochondrial function and decreased citrate synthase activity, which is consistent with the initiation of skeletal muscle atrophy [52]. PGC1α is a necessary mediator of mitochondrial biogenesis in skeletal muscle and leads to the stimulation of regulatory transcription factors involved in energy metabolism, including NRF1 and TFAM. NRF1 binds to the TFAM promoter before TFAM binds to the promoter of mitochondrial DNA and initiates its transcription and replication [53]. Moreover, UCP3, a mitochondrial inner membrane transporter, has a vital ability in mitochondrial biogenesis and energy metabolism in skeletal muscle [54]. Therefore, we investigated whether a reduction in skeletal muscle mitochondrial dysfunction resulting from GEF supplementation prevented obesity-induced skeletal muscle atrophy. We found that GEF-treated mice exhibited elevated expression of mitochondrial biogenesis-related genes, including PGC1α, NRF1, TFAM, and UCP3, in both QUA and GAS. These results suggest that GEF stimulates mitochondrial biogenesis, resulting in browning of WAT and the promotion of energy expenditure as heat.

In the current study, HFD-induced obese mice showed skeletal muscle atrophy, as indicated by the reduction of skeletal muscle

mass and exercise capacity, and induction of the UPP pathway [27,55]. The UPP pathway is the E3-ligase F-box protein system that modulates skeletal muscle degradation, and these changes are mediated by the upregulation of the muscle-specific atrophic genes, Atrogin-1 and MuRF1 [56]. In this study, we demonstrated that GEF administration prevented obesity-induced reduces in skeletal muscle mass and grip strength in the HFD-fed mice. In line with this, HFD-induced obese mice exhibited high levels of muscle atrophic factors Atrogin-1 and MuRF1. However, GEF treatment of these mice resulted in a reversal of obesity-induced skeletal muscle atrophy, by inhibiting the degradation of muscle-specific proteins. This is consistent with the roles of MRFs, and the observation that increases in MRF levels lead to differentiation of skeletal muscle. Myogenesis is the differentiation process of myoblasts to myotubes that leads to the development of muscle fibers [57]. Because myogenic differentiation processes physically interacts with muscle fiber, myogenesis is necessary for skeletal muscle hypertrophy and regeneration after muscle atrophy [58]. During differentiation, skeletal muscle increases the expression of MRFs such as MyoD, myogenin, MEF-2, and MYH by activation of myogenic regulatory mechanisms. MyoD is a key differentiation marker of skeletal muscle and is responsible for muscle development, which induces downstream myogenic factors, including myogenin, MEF-2, and MYH [59]. Myogenin upregulates myoblast fusion into myotubes, which form the structure of differentiated muscle cells [60]. Based on the results from H&E staining, GEF-treated mice displayed larger skeletal muscle fiber size than HFD-fed mice. Consistent with this, the expression of MyoD, myogenin, MEF-2, and MYH7 was higher, implying that GEF protects against obesity-induced skeletal muscle atrophy through promotion of muscle regeneration.

The present study demonstrated for the first time that GEF may prevent sarcopenic obesity by suppressing body mass gain, fat accumulation, and obesity-induced skeletal muscle atrophy. We revealed the molecular mechanism by which GEF downregulates adipogenesis and significantly increases the transcription of UCP1 in WAT through AMPK activation. These results establish an essential role for GEF in modulating WAT browning and energy expenditure. In addition, we have provided evidence that GEF enhances mitochondrial function and biogenesis in skeletal muscle. Consistent with enhanced mitochondrial function, the protective effect of GEF on obesity-induced skeletal muscle atrophy may be due to synergistic effects of inhibiting the UPP pathway and activating MRFs. We also found that GEF protected from skeletal muscle atrophy, increasing skeletal muscle mass, muscle fiber size, and exercise capacity; these results were accompanied by prevention of fat gain in the GEF-treated mice. In conclusion, we demonstrate that GEF may have potential uses for preventing sarcopenic obesity by promoting energy expenditure and attenuating skeletal muscle atrophy.

Declaration of competing interest

All authors have no conflict of interest to declare.

Acknowledgments

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