Korean Red Ginseng (Panax ginseng) Potentiates the Inhibitory Actions of Testosterone on Obesity and Adipogenesis in High Fat Diet-Fed Castrated Mice

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It has been suggested that ginseng is beneficial for ameliorating the aging males' symptoms, such as weight gain, fatigue, erectile dysfunction, and depression, in elderly men with testosterone deficiency. We thus investigated the effects of Korean red ginseng (Panax ginseng C.A. Meyer; Araliaceae) on obesity in a mouse model of testosterone deficiency (castrated C57BL/6J mice). The effects of ginseng extract (GE) and/or testosterone on obesity and adipogenesis in high-fat diet (HFD)-fed castrated C57BL/6J mice and 3T3-L1 adipocytes were examined using in vivo and in vitro approaches. After feeding mice a HFD for 8 weeks, we found that mice also receiving GE and/or testosterone showed decreased body weight, adipose tissue mass, adipocyte size, and hepatic lipid accumulation compared with untreated HFD-fed mice. Expression of adipogenic genes (PPARγ, C/EBPα, and aP2) was decreased by GE and/or testosterone in adipose tissues. Consistent with the in vivo data, lipid accumulation and the mRNA expression of adipogenesis genes in 3T3-L1 adipocytes were decreased by GE, ginsenosides, and testosterone. The inhibitory effects of GE (or ginsenosides) were comparable to those of testosterone, and the effects of co-treatment with GE (or ginsenosides) and testosterone were greater than those of testosterone alone in vivo and in vitro. Our results indicate that ginseng may be able to potentiate the inhibitory effects of testosterone on obesity and adipogenesis in HFD-fed castrated mice, providing possible therapeutic implications in men with testosterone deficiency.

Key Words: 3T3-L1 cell, Adipogenic gene, Ginsenosides, Lipid accumulation, Testosterone deficiency

INTRODUCTION

A growing body of evidence suggests that obesity in the aging men is deeply associated with lowered testosterone levels (Michalakis et al., 2013; Fui et al., 2014; Traish, 2014; Kelly and Jones, 2015). Low testosterone levels induce increased fat mass and testosterone therapy in men with testosterone deficiency results in weight loss and a lower risk of metabolic syndrome (Yassin and Doros, 2013; Francomano et al., 2014; Kelly and Jones, 2015). In mouse studies, knockout of the gene encoding the androgen receptor results in obesity, whereas overexpression of the androgen receptor results in decreased adipose tissue mass (Rana et al., 2011; Semirale et al., 2011; McInnes et al., 2012; Varlamov et al., 2012).

Ginseng has widely been used as a valuable medicine in Korea, China, and Japan for a long period (Yun, 2001; Yin et al., 2008; Park et al., 2012). Pharmacological studies have described the effects of ginseng on the central nervous, endo-

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crine, immune, and cardiovascular systems (Gillis, 1997; Attele et al., 1999; Lu et al., 2009). In addition, ginseng has been suggested to reduce weight gain in animal models of obesity and can effectively regulate genes involved in obesity (Attele et al., 2002; Kim et al., 2005; Karu et al., 2007; Mollah et al., 2009; Lee et al., 2009, 2012). Ginseng also significantly inhibits visceral obesity and adipocyte hypertrophy (Lee et al., 2013, 2014, 2016), which is closely associated with metabolic syndromes (Okuno et al., 1998; Jeong and Yoon, 2009; Lee et al., 2014).

Clinical reports suggested the favorable effects of ginseng on aging males' symptoms (AMS) and male sexual function (Choi et al., 2013; Ernst et al., 2011; Khera and Goldstein, 2011; Moyad and Park, 2012). AMS include testosterone deficiency, erectile dysfunction, depression, fatigue, weight gain, osteoporosis, and type 2 diabetes. Korean red ginseng (Panax ginseng C.A. Meyer; Araliaceae) and Malaysian ginseng increased the serum testosterone concentrations and improved the erectile function compared with placebo when administered to patients with testosterone deficiency (de Andrade et al., 2007; Ham et al., 2009; Tambi et al., 2012; Choi et al., 2013; Jung et al., 2016), indicating the testosterone-enhancing effects of ginseng. Thus, we hypothesized that Korean red ginseng is able to induce weight loss and regulate obesity in castrated male mice, an animal model of men with testosterone deficiency, by regulating the expression of adipogenesis-related genes.

In this study, we examined the effects of Korean red ginseng extract (GE) and/or testosterone on obesity and adipogenesis in high-fat diet (HFD)-fed castrated C57BL/6J mice and 3T3-L1 adipocytes. Our findings suggest that ginseng can enhance the actions of testosterone on obesity and adipogenesis in testosterone deficiency.

MATERIALS AND METHODS

GE Preparation

The GE was prepared from 6-year-old Panax ginseng C.A. Meyer (Korea Ginseng Corporation, Seoul, Korea). A voucher specimen was deposited at the laboratory of Korea Ginseng Corporation and the batch number of ginseng used in our study is 6510100112048. Briefly, red ginseng was boiled in distilled water for 24 h at 95°C. The aqueous extracts were filtered and freeze-dried under vacuum to produce GE powder.

For analysis of the quality of GE, GE powder (100 g) was placed into a 1-L flask with a refluxing condenser and extracted twice with 500 mL of water-saturated 1-butanol for 1 h at 80°C. The extracted solution was passed through Whatman filter paper (No. 41) after cooling. The process was repeated twice. The residue and filter paper were washed with 100 mL of water-saturated 1-butanol, and then the filtrate was washed twice with 100 mL of water in a 2-L separating funnel. The butanol layer was then evaporated to dryness. The concentrate was extracted to remove any traces of fat with 100 mL of diethyl ether for 30 min at 36°C in a flask with a refluxing condenser, after which the ether solution was decanted. The quality control of GE was analyzed by the HPLC/ELSD system and the HPLC profile of GE was described previously (Lee et al., 2014).

Animal treatments

For all experiments, 8-week-old male wild-type C57BL/6J mice were housed and bred at Mokwon University with a standard 12-h light/dark cycle. Prior to the administration of a special diet, the mice were given standard rodent chow and water ad libitum. The mice were castrated and then divided into five groups (n = 8/group). The first group received a low-fat diet (LFD, 10% kcal fat, Research Diets, Brunswick, NJ, USA). The second group received an HFD (45% kcal fat, Research Diets). The third group received an HFD supplemented with 0.5% GE (w/w). 50 g GE powder was mixed with 1 kg HFD. The fourth group was fed an HFD and subcutaneously implanted with a testosterone pellet (5 mg testosterone per pellet, Innovative Research of America, Sarasota, FL, USA). The final group received an HFD supplemented with GE and the testosterone implant. After an 8-h fast on the last day of the study, the animals were sacrificed by cervical dislocation. Fat pads were removed, weighed, snap-frozen in liquid nitrogen, and stored at -80°C until use. Portions of the fat pads and liver tissues were prepared for histology. All animal experiments were approved by the Institutional Animal Care and Use Committees of Mokwon University and were carried out in
accordance with the National Research Council Guidelines.

**Histological analysis**

The adipose tissues were fixed in 10% phosphate-buffered formalin for 1 day and processed for paraffin sections. Tissue sections (5 μm) were cut and stained with hematoxylin and eosin for examination by microscopy. To quantify adipocyte size, the stained sections were analyzed using the Image-Pro Plus analysis system (Media Cybernetics, Bethesda, MD, USA).

**3T3-L1 differentiation and analysis of triglyceride content**

Murine 3T3-L1 cells (ATCC, Manassas, VA, USA) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine calf serum (Invitrogen, Carlsbad, CA, USA). The cells were maintained at confluence for 2 days, after which the medium was replaced with DMEM containing 0.5 mM 1-methyl-3-isobutyl-xanthine, 1 μM dexamethasone, and 1 μg/ml insulin, and 10% fetal bovine serum (Invitrogen) (day 0). The cultures were incubated for 2 days to induce adipocyte differentiation, and then the medium was replaced with DMEM containing 10% fetal bovine serum for the remainder of the differentiation process. The cells were treated with 10 μg/ml GE, 10 μg/ml ginsenosides, and/or 100 nM testosterone on days 0~2 only, and the medium was changed every other day (Singh et al., 2006; Oh et al., 2012). On day 8, the cells were fixed in 10% formalin for 1 h and stained with Oil Red O for 2 h. For quantitative analysis, the Oil Red O stain was eluted by adding isopropanol and quantified by measuring absorbance at 520 nm.

**Reverse transcription-polymerase chain reaction**

Total cellular RNA was extracted from VSC adipose tissues and 3T3-L1 cells using TRIzol reagent (Gibco-BRL, Grand Island, NY, USA). The RNA (2 μg) was denatured for 5 min at 72°C and then immediately placed on ice for 5 min. To generate cDNA, the denatured RNA was mixed with Moloney murine leukemia virus reverse transcriptase, buffer, and a deoxyribonucleotide triphosphate (dNTP) mixture.

| Genes | Gene bank | Primer sequences |
|-------|-----------|------------------|
| **Mouse** | | |
| aP2 | NM_024406.2 | Forward: 5'-CCAAATGTGTGATGCTTTTGTG-3' Reverse: 5'-CTCCTCTTTTGGCTCAATGCC-3' |
| β-actin | NM_007393.5 | Forward: 5'-TGGAAATCCTGTCATCCATGAAA-3' Reverse: 5'-TAAAACGCAGCTCAGTAACAGTCCG-3' |
| C/EBPα | NM_001287514.1 | Forward: 5'-ATTCTGGGCCAACTTCCGGAGC-3' Reverse: 5'-ATCCAGAGGGACTGGAGGTT-3' |
| PPARγ | NM_001308354.1 | Forward: 5'-TGGAAGCCTGATGCTTTATCCCA-3' |
| **Human** | | |
| aP2 | NM_001442.2 | Forward: 5'-TCCAGTAAAAACCTGATTAT-3' Reverse: 5'-ACGCAITCCCACCGATTT-3' |
| β-actin | NM_001101.3 | Forward: 5'-GCAAAGAGGAGCTTCCACC-3' Reverse: 5'-CGTAGATGGGCACAGTGATGG-3' |
| C/EBPα | NM_004364.4 | Forward: 5'-TGGAAGACGCAGGAAGAAG-3' Reverse: 5'-TTCCAAAGCCACAGTACATT-3' |
| PPARγ | NM_138711.3 | Forward: 5'-GCAGGAGAGAGGAAAGGTT-3' Reverse: 5'-AAATATTGCAAAGTGCTGATCATC-3' |
and incubated for 1 h at 42 °C. The cDNA was mixed with PCR primers, Taq DNA polymerase (NanoHelix, Daejeon, Korea), and dNTPs and amplified in an MJ Research thermal cycler (Waltham, MA, USA). The PCR primers are shown in Table 1. The PCR products were analyzed by electrophoresis in a 1% agarose gel and quantified using the GeneGenius bio-imaging system (Syngene, Cambridge, UK). Relative expression levels are presented as the ratio of target gene cDNA to β-actin cDNA.

Statistical analysis

All values are expressed as mean ± standard deviation (SD). Groups were compared by analysis of variance followed by Tukey’s multiple comparison test; \( P < 0.05 \) was considered significant.

RESULTS

Effects of GE and testosterone on body weight and adiposity in HFD-fed castrated mice

Body weight and adipose tissue mass were measured in male castrated C57BL/6J mice on an LFD, HFD, and HFD containing GE with or without testosterone for 8 weeks. The mean body weight of untreated HFD-fed mice was 38.4 ± 1.33 g (Fig. 1A). However, the body weights of HFD-fed mice treated with GE or testosterone were 31.02 ± 1.35 g and 30.08 ± 2.32 g, respectively, representing decreased body weights of 19% and 22% compared with mice fed the HFD (\( P < 0.05 \)). Furthermore, the body weight of HFD-fed mice concomitantly treated with GE and testosterone was 26.96 ± 1.62 g, representing a decreased body weight of 13% by compared with HFD plus testosterone (\( P < 0.05 \)). Treatment with GE and/or testosterone also led to decreased adipose tissue mass in HFD-fed mice. Adipose tissue weights of HFD-fed mice treated with GE or testosterone were 37% or 65% lower than that of untreated HFD-fed mice (\( P < 0.05 \), Fig. 1B). Combined treatment with GE and testosterone further decreased fat weight by 72% compared with HFD-fed mice receiving testosterone only (\( P < 0.05 \)).

Effects of GE and testosterone on adipocyte size in HFD-fed castrated mice

Histological analysis revealed that GE and/or testosterone treatment decreased mean adipocyte size in HFD-fed mice. The adipocyte size decreased by 47% or 69% in HFD-fed mice receiving either GE (6.053 ± 689 μm²) or testosterone.
rone (3,819 ± 379 μm²), compared with that of untreated HFD-fed mice (11,517 ± 731 μm²; *P*<0.05; Fig. 2). Co-treatment with GE and testosterone (1,695 ± 78 μm²) further decreased the mean adipocyte size by 53% compared with that of HFD-fed mice receiving testosterone only (*P*<0.05).

**Effects of GE and testosterone on expression of adipogenesis-associated genes in adipose tissues of HFD-fed castrated mice**

Expression patterns of genes involved in adipogenesis were investigated in adipose tissues of the castrated mice. GE administration decreased mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), and adipocyte fatty acid-binding protein 2 (aP2) in the adipose tissue of untreated HFD-fed mice, and testosterone decreased PPARγ and aP2 mRNA levels (*P*<0.05; Fig. 3). Co-administration of GE and testosterone decreased C/EBPα and aP2 mRNA levels compared with HFD-fed mice treated with testosterone alone (*P*<0.05).

**Inhibition of lipid accumulation by GE, ginsenosides, and testosterone in 3T3-L1 adipocytes**

We then examined the ability of GE (10 μg/ml), ginsenosides (10 μg/ml), and testosterone (100 nM) to prevent lipid accumulation in 3T3-L1 cells. After incubation in differentiation medium, the untreated 3T3-L1 cells (control) showed a marked accumulation of lipid droplets, as shown by the increase in Oil Red O staining (Fig. 4A). However, treatment with GE, ginsenosides, or testosterone decreased lipid accumulation by 34%, 52%, and 48%, respectively, compared with the control (Fig. 4B). Combination treatment with ginsenosides and testosterone further decreased triglyceride.
content compared with testosterone alone.

Effects of GE, ginsenosides, and testosterone on adipogenic gene expression in 3T3-L1 adipocytes

Treatment with GE, ginsenosides, or testosterone also decreased the expression of adipogenic genes compared with the control (Fig. 5). Treatment with GE decreased mRNA levels of C/EBPα and aP2, and ginsenosides decreased mRNA levels of PPARγ, C/EBPα, and aP2 (P<0.05). The inhibitory effects of ginsenosides were greater than those of GE. Testosterone treatment also decreased mRNA levels of all three genes. Co-treatment with ginsenosides and testosterone decreased mRNA levels of PPARγ, C/EBPα, and aP2 compared with testosterone alone (P<0.05).

DISCUSSION

Based on reports showing that low testosterone leads to obesity and its related metabolic diseases (Fui et al., 2014; Kelly and Jones, 2015) and that GE enhances testosterone effects in men with testosterone deficiency (de Andrade et al., 2007; Ham et al., 2009; Tambi et al., 2012; Jung et al., 2016), we examined the effects of ginseng on obesity and adipogenesis in testosterone-deficient castrated mice. Our results indicate that both GE and testosterone can prevent adipogenesis, adiposity, and obesity in HFD-fed castrated mice, and these effects are mediated in part through reducing the expression of adipogenic genes. Our findings also suggest that GE may be able to potentiate the inhibitory effects of testosterone on obesity and adipogenesis in obese castrated mice.
We found that 8 weeks of HFD feeding resulted in increased body weight in castrated mice (38.4 ± 1.33 g) compared with sham-operated mice (33.44 ± 2.24 g), as well as increased adipose tissue mass (data not shown), consistent
with previous studies reporting that testosterone deficiency leads to obesity. Administration of GE significantly decreased body weight and adipose tissue mass in the HFD-fed castrated mice by 19% and 37%, respectively, compared with untreated HFD-fed castrated mice. Our results are supported by previous reports showing that GE induced weight loss in several animal models of genetically and diet-induced obesity (Mollah et al., 2008; Lee et al., 2009, 2012, 2013). Weight loss was also observed with testosterone treatment, which decreased body weight and adipose tissue mass by 22% and 65%, respectively, in HFD-fed castrated mice. Testosterone plays a key role in the pathology of metabolic diseases such as obesity, and low testosterone levels are associated with increased fat mass and reduced lean mass in adult males (Kelly and Jones, 2015). In men with testosterone deficiency, testosterone therapy can produce significant and sustained weight loss, lower BMI, and decrease waist circumference (Yassin and Doros, 2013; Francomano et al., 2014), with the increase in testosterone proportional to the amount of weight loss. Moreover, body and adipose tissue weights in mice treated with the combination of GE and testosterone were lower than that of mice treated with testosterone alone.

In our study, we found that adipocytes were smaller in GE- or testosterone-treated HFD-fed mice than in untreated HFD-fed mice, and co-treatment with GE and testosterone further decreased adipocyte size compared with testosterone treatment alone. These results indicate that GE and testosterone effectively inhibit adipocyte hypertrophy in HFD-fed castrated mice and that GE potentiates the ability of testosterone to inhibit adipocyte hypertrophy. Adipocyte hypertrophy is closely related to metabolic syndromes, such as insulin resistance, type 2 diabetes, hypertension, atherosclerosis, dyslipidemia, and nonalcoholic fatty liver disease. The hypertrophied adipocytes secrete large amounts of inflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1), which stimulates macrophage infiltration in mice and humans (Xu et al., 2003; Curat et al., 2004). This inflammatory response ultimately leads to the deposition of ectopic fat in liver, muscle, and pancreas (Bluher, 2009). Visceral adipocytes in obese individuals are insulin resistant, possibly as a consequence of adipose cell expansion. Large adipocytes increase the levels of circulating free fatty acids, tumor necrosis factor α, and leptin, which are associated with insulin resistance (Okuno et al., 1998; Jeong and Yoon, 2009; Oh et al., 2015). Therefore, GE and testosterone may alleviate metabolic disease by inhibiting adipocyte hypertrophy.

Adipogenesis involves excess fat accumulation and lipogenic gene expression during differentiation of preadipocytes into mature adipocytes (Rosen and Spiegelman, 2000). PPARγ and C/EBPα are major transcription factors of early-stage adipocyte differentiation. Their expression activates the target gene aP2, which plays a role in lipogenesis (Rosen et al., 1999). Based on their ability to decrease body weight and adipocyte size, we hypothesized that GE and testosterone regulate the expression of adipogenesis-associated genes. Our results showed that GE and testosterone negatively regulate the expression of PPARγ, C/EBPα and aP2 in the adipose tissue of castrated mice, and co-administration of GE and testosterone further decreased adipogenic gene expression compared with testosterone alone. Our findings are supported by previous studies describing that red ginseng downregulates PPARγ and aP2 expression in adipose tissue of rats with HFD-induced obesity (Jung et al., 2015) and androgens such as testosterone and dihydrotestosterone downregulate PPARγ and C/EBPα expression in human adipose stem cells (Chazenbalk et al., 2013). These results support our hypothesis that decreased body weight gain, adipose tissue mass, and adipocyte size following GE and testosterone treatments are due to the downregulation of adipogenesis genes.

We showed that in vitro adipogenesis is inhibited by GE, ginsenosides, and testosterone by staining 3T3-L1 adipocytes with Oil Red O. Ginsenosides, which comprise approximately 3~6% of ginseng, were more effective than GE in preventing the accumulation of intracellular triglycerides, likely because ginsenosides exert most of the pharmacological activity of ginseng (Attele et al., 1999; Huang, 1999). Co-treatment with ginsenosides and testosterone further decreased lipid content in 3T3-L1 cells compared with testosterone alone. In parallel with the reduced lipid accumulation, treatment with ginsenosides or testosterone decreased PPARγ, C/EBPα, and aP2 expression in 3T3-L1 cells, and
co-treatment with ginsenosides and testosterone further decreased expression of these genes compared with testosterone alone. Our data are consistent with several reports demonstrating that individual ginsenosides (Rb1, Rc, and Rf) inhibit adipogenic activities by downregulating PPARγ, C/EBPα, and aP2 in 3T3-L1 cells (Oh et al., 2012; Siraj et al., 2015; Yang and Kim, 2015), and a study reporting that testosterone inhibits adipogenic differentiation and decreases C/EBPα and PPARγ2 protein and mRNA levels in 3T3-L1 cells (Singh et al., 2006). Taken together, our in vitro data support the in vivo results showing that GE and testosterone inhibit adipogenesis and obesity by downregulating adipogenesis-related genes.

In conclusion, the results of our study show that ginseng and testosterone can prevent obesity and adipogenesis in HFD-fed male castrated mice and suggest that these processes are mediated in part by the inhibition of adipogenesis gene expression. The inhibitory effects of GE (and ginsenosides) on obesity were comparable to those of testosterone. In addition, combination treatment provided effects greater than those of testosterone alone, indicating that ginseng may be able to replace or potentiate the inhibitory actions of testosterone on obesity and adipogenesis. Our findings suggest that ginseng may act as an anti-obesity drug in men with testosterone deficiency.

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CONFLICT OF INTEREST
The author declares no conflict of interest.

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